MOLECULAR AND CELLULAR BIOLOGY, July 1993, p. 4203–4213
0270-7306/93/134203-11$02.00/0
Copyright © 1993, American Society for Microbiology
Vol. 175, No. 13

In Vivo Analysis of Sequences Necessary for CBP1-Dependent Accumulation of Cytochrome *b* Transcripts in Yeast Mitochondria

TELSA M. MITTELMEIER[1] AND CAROL L. DIECKMANN[1,2]*

Departments of Molecular and Cellular Biology[1] and Biochemistry,[2] Life Sciences South, 426, University of Arizona, Tucson, Arizona 85721

Received 2 March 1993/Returned for modification 29 March 1993/Accepted 19 April 1993

In *Saccharomyces cerevisiae*, cytochrome *b*, an essential component of the respiratory chain, is encoded by the mitochondrial gene *cob*. The *cob* transcription unit includes the $tRNA^{Glu}$ gene from positions −1170 to −1099 relative to the *cob* ATG at +1. The initial *$tRNA^{Glu}$-cob* transcript undergoes several processing events, including removal of $tRNA^{Glu}$ and production of the mature 5′ end of *cob* mRNA at nucleotide −954. The nuclear gene product CBP1 is specifically required for the accumulation of *cob* mRNA. In *cbp1* mutant strains, *cob* transcripts are not detectable by Northern (RNA) blot analysis, but the steady-state level of $tRNA^{Glu}$ is similar to that of wild type. The results of a previous study led to the conclusion that a 400-nucleotide region just downstream of $tRNA^{Glu}$ is sufficient for CBP1 function. In the present study, the microprojectile bombardment method of mitochondrial transformation was used to introduce deletions within this region of *cob*. The analysis of *cob* transcripts in strains carrying the mitochondrial deletion genomes indicates that a 63-nucleotide sequence that encompasses the cleavage site at −954 is sufficient both for CBP1 function and for correct positioning of the cleavage. Furthermore, the data indicate that CBP1 prevents the degradation of unprocessed *cob* transcripts produced by endonucleolytic cleavage at the 3′ end of $tRNA^{Glu}$.

In the yeast *Saccharomyces cerevisiae*, mitochondrial biogenesis is a complex process involving both nuclear and mitochondrial genes (for reviews, see references 21 and 46). Nuclear genes required for the maintenance of respiratory-competent mitochondria, termed *PET* genes, fall into several phenotypic classes (for a review, see reference 44). One class of *PET* genes consists of those that have pleiotropic effects on the expression of mitochondrial genes, while a second class includes those that are required for the expression of individual mitochondrial genes. The nuclear *PET* gene *CBP1* is of the latter class and is required specifically for expression of the mitochondrial gene *cob*, encoding cytochrome *b* (14, 15).

As illustrated in Fig. 1, mitochondrial *$tRNA^{Glu}$* and *cob* are cotranscribed from a promoter upstream of the tRNA gene (10, 11). Processing of the primary transcript involves endonucleolytic cleavages at the 5′ and 3′ ends of $tRNA^{Glu}$ (8, 24) and cleavage at nucleotide (nt) −954 (relative to the *cob* AUG at +1) to produce the mature 5′ end of *cob* mRNA (5). In wild-type cells, the steady-state level of $tRNA^{Glu}$ is approximately 15-fold greater than that of mature *cob* mRNA (32), presumably reflecting a difference in the degradation rate of $tRNA^{Glu}$ compared with that of *cob* transcripts. In *cbp1* mutant strains, $tRNA^{Glu}$ accumulates to near wild-type levels, but *cob* mRNA is undetectable by Northern (RNA) blot analysis and the cells are unable to respire (15). Therefore, CBP1 is required posttranscriptionally for the accumulation of *cob* mRNA and may play a role in regulating the steady-state level of *cob* mRNA compared with that of $tRNA^{Glu}$.

CBP1 could act directly or indirectly to allow the accumulation of *cob* mRNA by any one or a combination of the following mechanisms: (i) CBP1 could prevent the degradation of unprocessed *cob* transcripts produced by endonucleolytic cleavage at the 3′ end of $tRNA^{Glu}$ (Fig. 1), (ii) CBP1 could prevent the degradation of mature *cob* mRNA, or (iii) CBP1 could cleave precursor transcripts to produce mature *cob* mRNA that is resistant to degradation. An understanding of the interaction between CBP1 and *cob* transcripts will provide insight into the factors which determine the rate of RNA degradation in yeast mitochondria and the mechanisms by which nuclear gene products affect mitochondrial gene expression.

The goal of this study was to define *cob* sequences that are sufficient for CBP1 function. Previously, we analyzed transcripts from a strain with a spontaneous deletion in the mitochondrial genome which fused *cob* sequence 5′ of −696 to the untranslated leader of the *oli1* gene (30). Accumulation of the hybrid *cob-oli1* mRNA was dependent on CBP1, leading to the conclusion that *cob* sequence between the 3′ end of $tRNA^{Glu}$ at −1099 and nt −696 is sufficient for CBP1 function. Here we describe the analysis of *cob* transcripts in strains with deletions within the −1099 to −696 interval of *cob*. The deletions were introduced into the mitochondrial genome by the microprojectile bombardment method of mitochondrial transformation (25, 38), which has been used previously to investigate the requirements for translation of mitochondrial *cox3* mRNA in *S. cerevisiae* (19). Using this approach, we have defined a 63-nt region of *cob* RNA that is sufficient both for CBP1 function and for correct positioning of the cleavage that produces the mature 5′ end of *cob* mRNA at −954. Furthermore, the data indicate that CBP1 prevents the degradation of unprocessed *cob* transcripts produced by endonucleolytic cleavage at the 3′ end of $tRNA^{Glu}$.

MATERIALS AND METHODS

Strains and media. The *S. cerevisiae* strains used in this study are listed in Table 1. Strains without mitochondrial DNA (mtDNA) ([*rho^0*] strains) were isolated following ethid-

* Corresponding author.

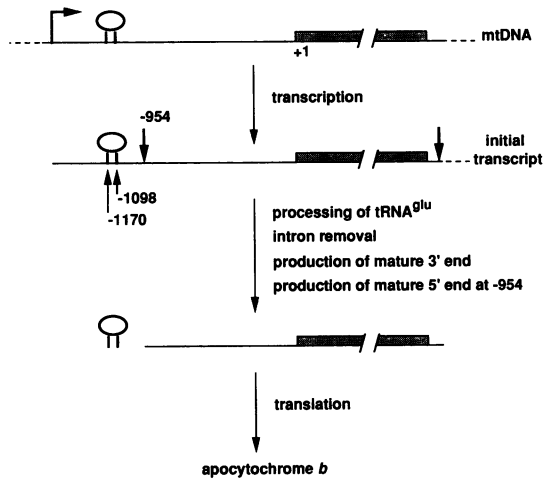

FIG. 1. Expression of the mitochondrial gene *cob*. The top line depicts a portion of the mitochondrial genome including the *cob* transcription unit. Transcription begins at the *cob* promoter (rightward arrow) and proceeds through $tRNA^{Glu}$ (open circle) and *cob* coding sequences (stippled bar). The first base pair of the *cob* coding sequence is numbered +1. Cleavage of precursor transcripts at the positions indicated by vertical arrows produces $tRNA^{Glu}$, the 3′ end of the mature *cob* mRNA, and the 5′ end of mature *cob* mRNA at nt −954. Translation of *cob* mRNA results in production of apocytochrome *b*.

ium bromide mutagenesis of the original [rho^+] strain. Strain A21 was constructed by transferring mtDNA from strain D273-10B/A21 to strain LL20/rho^0 via cytoduction (27). Strain JC3/M9410 was constructed by transferring mtDNA from strain M9410 to strain JC3/rho^0 via cytoduction. Strain aE655/Int was constructed by transferring mtDNA from strain E655/Int to strain aE655/rho^0 via cytoduction. Strain a4-35 was a spore from a cross of strain aE655/Int to strain KL14-4B/rho^0. The construction of strains carrying mitochondrial genomes with deletions in *cob* is described below. Media were YPD (1% yeast extract, 2% peptone, 2% glucose), YPG (1% yeast extract, 2% peptone, 3% glycerol), and WO (0.67% yeast nitrogen base without amino acids, 2% glucose). Solid media contained 2% agar.

Construction of plasmids and *cob* deletions. Plasmids Mb247/6-74 and Mb247/5-57 contain *cob* sequence from −1350 to +1716, a partial *Mbo*I fragment of DS400/A12 mtDNA, ligated into the *Bam*HI site of the *Escherichia coli* plasmid pBluescript (Stratagene, La Jolla, Calif.) in the 5′-to-3′ and 3′-to-5′ orientations, respectively. Deletions were created in Mb247/6-74 by digestion with *Pst*I and *Bst*EII, treatment with exonuclease III (ExoIII; Ambion, Austin, Tex.) and mung bean nuclease (Promega, Madison, Wis.), ligation, and transformation of *E. coli* XL1-blue (6). In a similar manner, deletions were created in Mb247/5-57, starting with digestion by *Pst*I and *Bam*HI. The series B deletion plasmids (p772B, p898B, p938B, and p997B) were constructed by ligating the −707 to +654 *Eco*RI fragment from an Mb247/5-57 plasmid, in which the ExoIII deletion

TABLE 1. Names and genotypes of yeast strains

Strain	Genotype or description	Reference
LL20	α [rho^+] *leu2-3 leu2-112 his3-11 his3-15* 2μm$^+$	36
LL20/rho^0	α [rho^0] *leu2-3 leu2-112 his3-11 his3-15* 2μm$^+$	29
D273-10B/A21	α [rho^{+A21}] *met6*	43
A21	[rho^{+A21}] in LL20	This study
CP1L/rho^0	*LEU2* insertion at the *Pst*I site of *CBP1* in strain LL20	29
B13L/rho^0	*LEU2* replacement of *Bam*HI fragments of *CBP1* in strain LL20	28
aM17-162-4A	**a** [rho^+ mit^-] *ade1*	45
M9410	α [rho^{+M9410} mit^-] *ade1 op1*	16
JC3/rho^0	**a** [rho^0] *kar1-1 ade2 lys2*	1
JC3/M9410	[rho^{+M9410} mit^-] in JC3	This study
DS400/A12	α [rho^-] *met6*	34
E655/Int	α [$rho^{+int4-35}$] *met6 cbp1-20*	13
aE655	**a** [rho^+] *ade1 cbp1-20*	30
aE655/Int	[$rho^{+int4-35}$] in aE655	This study
KL14-4B/rho^0	α [rho^0] *his*	48
a4-35	**a** [rho^-] *his*	This study
961A	[rho^{+961A}] in LL20	This study
948A	[rho^{+948A}] in LL20	This study
944-22	[$rho^{+944-22}$] in LL20	This study
944A	[rho^{+944A}] in LL20	This study
923A	[rho^{+923A} mit^-] in LL20	This study
707A	[rho^{+707A} mit^-] in LL20	This study
997B	[rho^{+997B} mit^-] in LL20	This study
938B	[rho^{+938B} mit^-] in LL20	This study
938B-S0	[$rho^{+938B-S0}$] revertant of 938B	This study
938B-S1	[$rho^{+938B-S0}$] in LL20	This study
938B-S2	[$rho^{+938B-S2}$] revertant of 938B	This study
938B-S3	[$rho^{+938B-S3}$] revertant of 938B	This study
898B	[rho^{+898B}] in LL20	This study
772B	[rho^{+772B}] in LL20	This study
160	[rho^{+160}] in LL20	This study
SUF63-F	[$rho^{+SUF63-F}$] in LL20	This study
SUF63-R	[$rho^{+SUF63-R}$ mit^-] in LL20	This study

TABLE 2. Oligonucleotide primers

Primer	Sequence[a]	*cob* sequence
cob1714	AGGAGTGATGGATCCCTTTGG	−1360 to −1341
cob/glu	CGGTTCGATTCCGATTAAGG	−1121 to −1102
cob1713	CGGAATTCGAATAACCTTAATCGGAAT	−1096 to −1114
cob1717	CCGAATTCATAATAATAAATACC	−961 to −943
cob1718	CCGAATTCTAATGAAAAATATATTATATA	−898 to −918
cob5B+3	CAATTATTATTATTATTATACATAAA	−826 to −854
cob2B	TCCTCGAGCAATAATTTATTGTTAATATG	−648 to −668
cob6B	AATTTTTATATTATTTATTAATATTGTT	−601 to −628
cob654A	GAATGCATTGGAATTCTATC	+668 to +649

[a] The primer sequence is in the 5'-to-3' orientation. Restriction endonuclease recognition sequences are underlined; sequence that is not identical to that of *cob* is in boldface type.

extended to −707, into the *Eco*RI sites of Mb247/6-74 plasmids with deletion endpoints at −772, −898, −938, or −997 of *cob*. Plasmid p160 was obtained by ligating the −778 to +654 *Eco*RI fragment from an Mb247/5-57 plasmid, in which the ExoIII deletion extended to −778, into the *Eco*RI site of the Mb247/6-74 plasmid with the deletion endpoint at −938.

pKS/tRNA-254 was created by polymerase chain reaction (PCR) amplification of *cob* sequence from −1350 to −1096, using primers cob1714 and cob1713 (Table 2), and ligation into *Bam*HI and *Eco*RI-digested pBluescript-KS. The series A deletions in plasmids p948A, p944A, p923A, and p707A were constructed by ligating *Eco*RI fragments from Mb247/5-57 deletion plasmids, in which the deletions extended to −948, −944, −923, or −707, into the *Eco*RI site of pKS/tRNA-254. Plasmid p961A was constructed by ligating PCR-amplified *cob* sequence from −961 to +654 (primers cob1717 and cob654A; Table 2) into the *Eco*RI site of pKS/tRNA-254. Plasmid 944-22 was created by ligating the −944 to +1716 *Hin*dIII-*Cla*I fragment from an Mb247/5-57 plasmid, in which the ExoIII deletion extended to −944, into a *Hin*dIII-plus-*Cla*I-digested Mb247/6-74 plasmid in which the ExoIII deletion extended to −1096. Plasmids pSUF63-F and pSUF63-R were constructed by ligating PCR-amplified *cob* sequence from −961 to −898 (primers cob1717 and cob1718; Table 2), into the *Eco*RI site of p707A.

Transformation of mitochondria by microprojectile bombardment. Yeast strain LL20/*rho*^{0} (α [*rho*^{0}] *leu2-3 leu2-112 his3-11 his3-15* 2μm$^+$) was cotransformed with YEp351 (a multicopy plasmid carrying the *LEU2* gene [23]) and each of the *cob* deletion plasmids by high-velocity microprojectile bombardment (25, 38). Cotransformation was performed as described previously (2, 20), with modifications. One hundred milliliters of liquid YPD medium was inoculated with 1 ml of an overnight culture of LL20/*rho*^{0}, the culture was grown at 30°C to a density of 2 × 10^8 cells per ml, and the cells were pelleted, washed once with 1.2 M sorbitol, and resuspended in 1.2 M sorbitol at a density of approximately 5 × 10^9 cells per ml. Regeneration plates (WO plus 0.75 M sorbitol, 0.75 M mannitol, 5% glucose, 60 μg of histidine per ml, and 2% agar) were spread with 0.1 ml of resuspended cells and allowed to dry at room temperature. Tungsten microprojectiles (0.75 μm; DuPont, Wilmington, Del.) were washed once with ethanol and once with water and resuspended in water at a concentration of 0.5 mg/μl. Approximately 4 μg of YEp351 plus 4 to 12 μg of the *cob* deletion plasmid (in 50 μl) were precipitated onto 50 μl of pellets by the addition of 100 μl of 2.5 M $CaCl_2$ followed by 25 μl of 1 M spermidine (free base, tissue culture grade; Sigma, St. Louis, Mo.) and incubation at room temperature for 20 min. The DNA-coated microprojectiles were pelleted, resuspended in 160 μl of ethanol, and used to bombard eight plates with the PDS-1000/He and 1300 Psi rupture disks (DuPont).

Leu$^+$ nuclear transformants appeared after 3 to 4 days of incubation at 30°C and were obtained at frequencies of one transformant per 10^5 to 10^8 cells. The nuclear transformants were grown for 10 days at 30°C and replicated on a lawn of aM17-162-4A on WO; after 2 days, the resulting diploids were replicated to YPG. Strain aM17-162-4A carries a *mit*$^-$ mutation between +74 and +504 of *cob* (35). Therefore, only those nuclear transformants which carried mitochondrial *cob* sequence yielded respiratory-competent diploids following mating to aM17-162-4A. Mitochondrial transformants (synthetic [*rho*$^-$] strains [20]) were obtained at a frequency of approximately 1 per 1,000 nuclear transformants.

Strain construction. Strains carrying recombinant mitochondrial genomes, in which a *cob* deletion present in a synthetic [*rho*$^-$] strain had been recombined into the grande mitochondrial genome, were isolated as follows. Overnight YPD cultures of the synthetic [*rho*$^-$] strain and the karyogamy-deficient strain JC3/M9410 (0.5 ml of each) were mixed and mated at 30°C without shaking for 3 h. The culture was then diluted with 2 ml of fresh YPD, grown with shaking at 30°C for 3 h, and plated for single colonies on WO plus 20 μg of histidine per ml and 20 μg of leucine per ml to select for the LL20 nucleus. Cytoductants from this cross carried one of three mitochondrial genomes: (i) the M9410 genome, which contains a deletion of *cob* sequence between −975 and −64 (cells carrying the M9410 genome lack mature *cob* mRNA and are therefore respiratory incompetent [16]), (ii) the synthetic *rho*$^-$ genome, or (iii) a recombinant genome, in which the *cob* deletion present in the synthetic *rho*$^-$ had replaced the deletion in M9410. Cytoductants which carried recombinant genomes were identified by their ability to form respiratory-competent diploids when mated to a synthetic [*rho*$^-$] tester strain, which carried a deletion of *cob* sequence from approximately −200 to +53. Recombination between the tester and the recombinant mitochondrial genomes, but not between the tester and M9410 mitochondrial genomes, restored wild-type *cob* sequence and produced respiratory-competent diploids. Yeast strains which carried recombinant mitochondrial genomes were named according to the *cob* deletion present on the mtDNA. The recombinant *cob* mitochondrial genomes were transferred to the otherwise isogenic *cbp1* strain, CP1L/*rho*^{0}, via cytoduction using the *kar1* strain JC3/*rho*^{0}.

Isolation of revertants of strain 938B. Respiratory-competent revertants of strain 938B were isolated by inoculating three separate YPD cultures with an individual colony of strain 938B, growing the cultures 1 day at 30°C, and plating

approximately 3×10^7 cells from each culture on a YPG plate. After 1 week at 30°C, several size classes of respiratory-competent colonies were evident on the YPG plates. One colony of the largest size class was chosen from each of the original cultures, yielding strains 938B-S0, 938B-S2, and 938B-S3. Strains 938B-S1 and B13L/938B-S1 were obtained by transferring the mtDNA from strain 938B-S0 to strains LL20/*rho*^{0} and B13L/*rho*^{0} via cytoduction using the *kar1* strain JC3/*rho*^{0}.

Southern and Northern blot analyses. Isolation of mtDNA and mitochondrial RNA, Southern analyses, and Northern analyses were performed as described previously (30). The *cob*-specific ^{32}P-labeled probes were obtained by random priming of *cob* sequence extending from either −1350 to +319 (for Southern analyses) or from −1350 to +654 (for Northern analyses), as recommended by the supplier of the labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) except that [α-^{32}P]dATP was substituted for [α-^{32}P]dCTP. The *oli1*-specific ^{32}P-labeled probe was obtained by random priming of *oli1* sequence extending from +179 to +797 relative to the *oli1* AUG at +1. The signals obtained from *cob* transcripts or from tRNA^{Glu} were quantitated with a Betascope (Betagen, Waltham, Mass.) and normalized to the signal obtained from *oli1* transcripts in the same strain.

PCR amplifications and sequencing of the PCR-amplified deletion junctions in strains 938B-S1, -S2, and -S3. Reaction conditions for all of the PCR amplifications described in this study were as recommended by the supplier of *Taq*I polymerase (Promega or Perkin-Elmer Cetus, Norwalk, Conn.). The primers used are listed in Table 2. The mutations present in strains 938B-S1, -S2, and -S3 were identified by PCR amplification of the region surrounding the deletion junction in each strain, using primers cob/glu and cob2B, followed by ligation of the *Taq*I-digested PCR products into the *Cla*I site of pBluescript. The inserts present in plasmid DNA isolated from four individual clones from each revertant were sequenced by using Sequenase version 2.0 and the universal T3 or T7 primers (Stratagene) as recommended by the supplier of the enzyme (United States Biochemical Corp., Cleveland, Ohio).

Primer extension analysis of mRNA 5′ ends. Primers cob5B+3 (strains A21, 961A, 944-22, 948A, and 944A; Table 2) and cob6B (strains SUF63-F, SUF63-R, 898B, 938B, 938B-S1, and 997B; Table 2) were ^{32}P labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP as recommended by the supplier of the enzyme (Promega). Five to 10 μg of total cellular RNA (isolated according to protocol in reference 7) and 5 pmol of primer were mixed and brought to a final volume of 10 μl in annealing buffer (200 mM KCl, 10 mM Tris-Cl [pH 8.3] at 42°C). The mixture was heated at 85°C for 5 min and then annealed at 58°C (cob5B+3) or 54°C (cob6B) for 90 min. The tubes were placed at 42°C, 10 μl of reaction mix (1.0 mM dGTP, 1.0 mM dCTP, 3.0 mM dATP, 3.0 mM dTTP, 2× reaction buffer, 7.0 U of avian myeloblastosis virus reverse transcriptase; the reaction buffer and enzyme were supplied by Boehringer Mannheim) was added to the annealed primer-RNA mixture, the reaction mixture was incubated at 42°C for 45 min, and the reaction was stopped by the addition of 12 μl of stop mix (95% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol). Nine microliters of each reaction mixture was loaded on a 6% polyacrylamide–7 M urea sequencing gel. Sequencing reactions served as size markers. To compare *cob* transcript levels in *cbp1* strains with those in *CBP1* wild-type strains, the signals obtained from the individual primer extension products were quantitated with a Betascope and normalized to the grams of total cellular RNA used in that primer extension reaction, as determined by A_{260}.

RESULTS

Construction of deletions in the mitochondrial *cob* gene. A previous analysis of transcripts derived from a hybrid *cob-oli1* gene led to the conclusion that *cob* sequence between the 3′ end of the tRNA^{Glu} gene (nt −1099) and nt −696 is sufficient for CBP1 function (30). To further define the sequence elements important for CBP1 function in vivo, we took advantage of the microprojectile bombardment method of mitochondrial transformation (25, 38) to create deletions within this region of *cob*.

Two series of deletions (Fig. 2A) were constructed in vitro within *cob* sequence ligated into the *E. coli* plasmid pBlueScript. The series A deletions have a common 5′ endpoint at −1096 and variable 3′ endpoints, while the series B deletions have a common 3′ endpoint at −707 and variable 5′ endpoints. With the exception of deletion 944-22, in which the deleted sequence was replaced with an extended linker sequence, deleted sequence was replaced with an *Eco*RI site.

To assess the effect of the deletions on *cob* expression in vivo, the deletions were introduced into the grande (*rho*$^+$) mitochondrial genome in two steps (described in Materials and Methods). To verify the presence of the *cob* deletions in the grande genome, mtDNA isolated from the deletion strains was restricted with *Mbo*I and *Eco*RI and analyzed by Southern blotting with a probe that extended from −1350 to +319 of *cob* (Fig. 2B). Two *Mbo*I-*Eco*RI fragments of the appropriate sizes were detected in mtDNA isolated from the mutant strains, consistent with the presence of the expected *cob* deletions (Fig. 2A). Furthermore, the deletion junctions present in several of the mitochondrial deletion genomes were identical to those in the original plasmid constructs, as determined by sequence analysis of PCR products (data not shown).

Sequence between −961 and −898 is important for *cob* transcript accumulation. As CBP1 is required for respiratory growth, the deletion of sequence necessary for CBP1 function should lead to a respiratory-deficient phenotype. Therefore, the *cob* deletion strains were tested for the ability to grow on glycerol, a nonfermentable carbon source (Fig. 3A). Strain 948A, in which *cob* sequence between −1096 and −948 had been deleted, grew on glycerol at a rate similar to that of strain A21, which has a wild-type *cob* gene. When the deletion was extended to −944 (strain 944A), the cells retained the ability to grow on glycerol but formed single colonies that were smaller than those of wild type. However, when the deletion was extended to −923 (strain 923A), the cells lost the ability to respire. The deletion of sequence between nt −707 and −898 (strain 898B) had no effect on respiratory growth, but when the deletion was extended to −938, respiratory growth was greatly inhibited, as strain 938B grew extremely slowly on glycerol. In all cases, growth on glycerol remained dependent on CBP1 (Fig. 3A). These results suggest that *cob* sequence between −948 and −898 is important for respiratory growth.

Deletions in the 5′ untranslated region of *cob* could lead to a respiratory-negative phenotype by affecting either the production of functional tRNA^{Glu} or the expression of cytochrome *b*. The presence of functional tRNA^{Glu} in the deletion strains was verified genetically by mating to strain a4-35 and testing the resulting diploids for respiratory

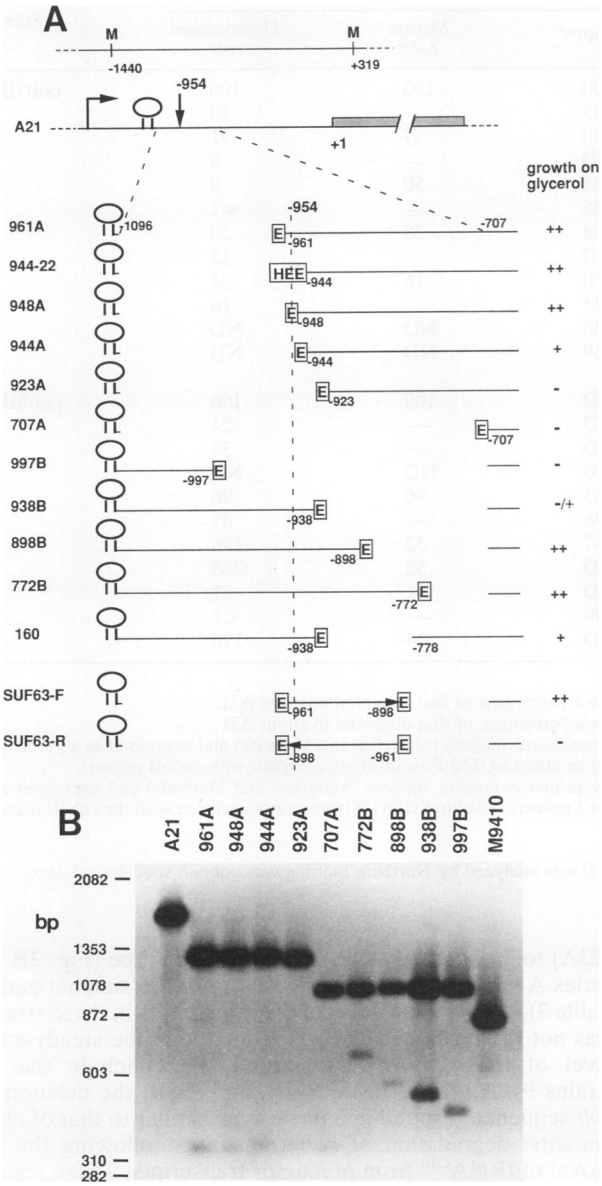

FIG. 2. Southern blot analysis of mtDNA isolated from *cob* deletion strains. (A) The *cob* region of the wild-type mitochondrial genome (A21) and the deletions constructed within the targeted region of *cob* are diagrammed. The positions of relevant *Mbo*I restriction sites (M), *Eco*RI linker sequences (boxed E), and the *Hin*dIII-*Eco*RV-*Eco*RI linker present in deletion 944-22 (boxed HEE) are indicated. The numbers at the deletion endpoints refer to the position of the first wild-type base pair at that deletion endpoint. The vertical dashed line relates the position of the cleavage at −954 to the deletion endpoints. Other symbols are as described in the legend to Fig. 1. To the right of each diagrammed deletion, the relative growth on glycerol of the *CBP1* strain carrying that *cob* deletion is given. (B) Mitochondrial DNA was isolated from *cob* deletion strains, restricted with *Eco*RI and *Mbo*I, and analyzed by Southern blotting with a probe derived from −1350 to +319 of *cob* (Materials and Methods). The positions and sizes of molecular weight markers are indicated on the left.

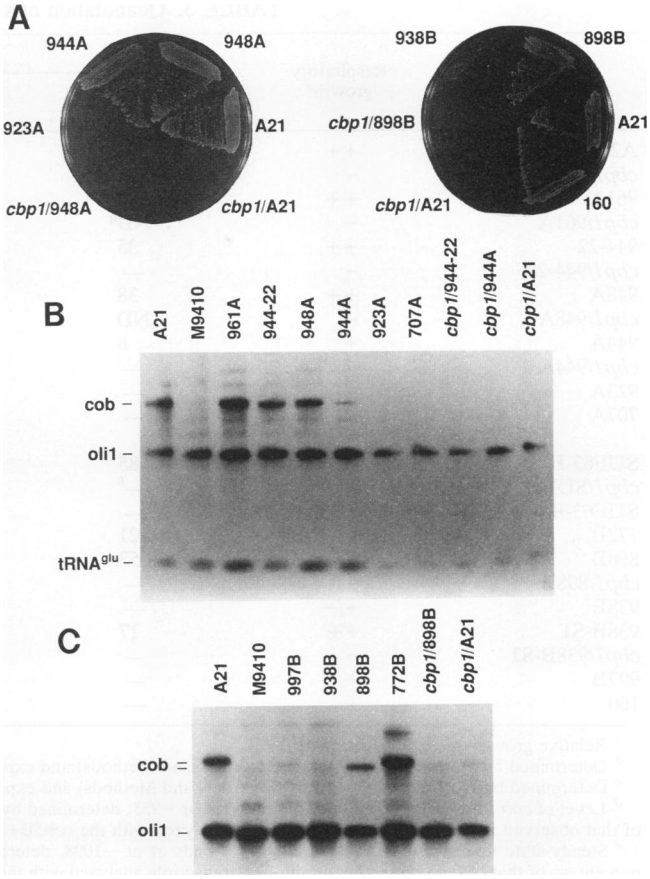

FIG. 3. Respiratory growth and *cob* transcript levels in *cob* deletion strains. (A) Series A (left) and series B (right) deletion strains were streaked on a nonfermentable carbon source (YPG) and grown at 30°C for 4 days. (B and C) Mitochondrial RNA was isolated from *cob* deletion strains and analyzed by Northern blotting (Materials and Methods) with probes derived from *cob* (−1350 to +654) and *oli1* (+179 to +797) sequences. The positions of *cob* and *oli1* transcripts and of $tRNA^{Glu}$ are indicated. The *cbp1* strain used in these experiments was CP1L (Table 1). Faint hybridization to the mitochondrial 15S rRNA, which contains sequence complementary to the *cob* probe, is observed in lanes 997B, *cbp1*/898B, and *cbp1*/A21 at a position just below that of wild-type *cob* mRNA. Data obtained by quantitation of the Northern blot signals are summarized in Table 3.

growth. Strain a4-35 carries a petite mitochondrial genome which expresses cytochrome *b* independently of *CBP1* but does not encode $tRNA^{Glu}$ (15). All of the *cob* deletion strains yielded respiratory competent diploids following mating to strain a4-35, indicating that the level of functional $tRNA^{Glu}$ in these strains is sufficient for respiratory growth (data not shown). Therefore, the mutant phenotype of deletion strains 944A, 923A, 707A, 997B, and 938B is probably due to a loss of cytochrome *b* expression.

The *cob* deletions could lead to a decrease in cytochrome *b* expression by affecting (i) steady-state levels of precursor transcripts containing both $tRNA^{Glu}$ and *cob*, (ii) steady-state levels of *cob* transcripts from which $tRNA^{Glu}$ has been removed, or (iii) translation of *cob* mRNA. To distinguish among these possibilities, the steady-state levels of $tRNA^{Glu}$ and of *cob* transcripts in the deletion strains were determined by Northern blot analysis of mitochondrial RNA (Fig.

TABLE 3. Quantitation of steady-state levels of *cob* transcripts

Strain	Respiratory growth[a]	Steady-state level				Primer
		cob transcripts[b]	$tRNA^{Glu}$[c]	Mature *cob*[d]	Unprocessed *cob*[e]	
A21	++	100	100	100	100	cob5B+3
cbp1/A21	−	—[f]	53	—	10	
961A	++	73	70	97	71	
cbp1/961A	−	ND[g]	ND	—	8	
944-22	++	35	41	50	8	
cbp1/944-22	−	—	65	—	<1	
948A	++	38	68	28	20	
cbp1/948A	−	ND	ND	—	12	
944A	+	6	50	16	24	
cbp1/944A	−	—	33	—	16	
923A	−	—	38	ND	ND	
707A	−	—	39	ND	ND	
SUF63-F	++	85	ND	100	100	cob6B
cbp1/SUF63-F	−	—[h]	ND	—	24	
SUF63-R	−	—	ND	—	35	
772B	++	121	100	ND	ND	
898B	++	53	63	96	96	
cbp1/898B	−	—	96	—	31	
938B	+/−	—	57	32	196	
938B-S1	++	17	ND	52	265	
cbp1/938B-S1	−	—	ND	—	21	
997B	−	—	100	—	<1	
160	+	—	ND	14	176	

[a] Relative growth of single colonies on YPG.
[b] Determined by Northern blot analysis (Materials and Methods) and expressed as a percentage of that observed in strain A21.
[c] Determined by Northern blot analysis (Materials and Methods) and expressed as a percentage of that observed in strain A21.
[d] Level of *cob* transcripts with 5′ ends at nt −954 or −955, determined by primer extension analysis (Materials and Methods) and expressed as a percentage of that observed in either strain A21 (transcripts analyzed with the cob5B+3 primer) or strain SUF63-F (transcripts analyzed with cob6B primer).
[e] Steady-state levels of *cob* transcripts with 5′ ends at nt −1098, determined by primer extension analysis (Materials and Methods) and expressed as a percentage of that observed in either strain A21 (transcripts analyzed with the cob5B+3 primer) or strain SUF63-F (transcripts analyzed with the cob6B primer).
[f] —, transcripts were not detected.
[g] ND, not determined.
[h] The signal observed when mitochondrial RNA isolated from strain *cbp1*/SUF63-F was analyzed by Northern blotting was not *cob* specific (see text).

3B; Table 3). The signals obtained from $tRNA^{Glu}$ and *cob* transcripts in each sample were quantitated and normalized to the signal obtained from *oli1* transcripts in the same sample (the results of quantitation are summarized in Table 3).

The steady-state level of *cob* transcripts decreased relative to that of wild type when the deletion extended from −1096 to a position downstream of nt −961 (Fig. 3B; Table 3). The level of *cob* transcripts in strain 961A was 73% of that of wild type, while in strains 944-22 and 948A, *cob* transcript levels dropped to approximately 35% of the wild-type level. In strain 944A, the steady-state level of *cob* transcripts was only 6% of that of wild type, and in strains 923A and 707A, *cob* transcripts were undetectable. In series B deletion strains (Figure 3C; Table 3), a precipitous drop in the steady-state level of *cob* transcripts was observed when the deletion extended from −707 to a position 5′ of nt −898. The steady-state level of *cob* transcripts in strain 772B was slightly greater than that of the wild type, and in strain 898B, the level of *cob* transcripts was 53% of that of the wild type. However, *cob* transcripts were undetectable in strains 938B and 997B.

The steady-state level of *cob* transcripts reflects a balance between synthesis and degradation. The production of *cob* transcripts is, in turn, dependent on the steady-state level of precursor transcripts containing both $tRNA^{Glu}$ and *cob*. As Northern blot analysis showed that $tRNA^{Glu}$ levels in strains 944A, 923A, 707A, 997B, and 938B ranged from 38% (strain 923A) to 70% (strain 961A) of that of wild type (Fig. 3B for series A strains; data for series B strains are summarized in Table 3), the very low level of *cob* transcripts in these strains was not the result of a general reduction in the steady-state level of $tRNA^{Glu}$-*cob* transcripts. We conclude that in strains 944A, 923A, 707A, 997B, and 938B, the deletion of *cob* sequence resulted in a phenotype similar to that of *cbp1* mutants: degradation of *cob* transcripts following the removal of $tRNA^{Glu}$ from precursor transcripts. These results suggest that sequence between nt −961 and −898 is important for the accumulation of *cob* mRNA.

Sequence between −961 and −898 is sufficient for CBP1 function. In the analysis of the A and B series deletion strains, all of the respiratory-competent deletion strains remained dependent on CBP1 for the accumulation of *cob* mRNA, suggesting that the region of *cob* transcripts between nt −961 and −898 is sufficient for CBP1 function. To verify that this 63 nt of the RNA is sufficient for CBP1 function, strains SUF63-F and SUF63-R, in which *cob* sequence from −961 to −898 replaced the large deletion in strain 707A (from −1096 to −707A; Fig. 2A and Materials and Methods), were analyzed for the ability to grow on glycerol (Fig. 4A) and for the accumulation of *cob* transcripts (Fig. 4B; Table 3).

Strain SUF63-F was respiratory competent and accumulated *cob* transcripts to nearly wild-type levels. Although the *cbp1* mutant strain carrying the SUF63-F mitochondrial genome was unable to respire, an RNA which migrated at

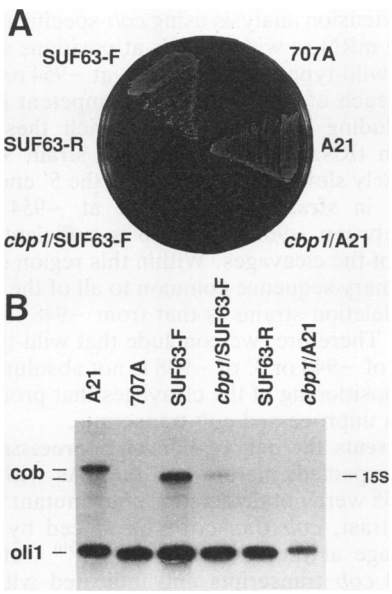

FIG. 4. Evidence that *cob* sequence from −961 to −898 is sufficient for CBP1 function. (A) SUF63-F and SUF63-R deletion strains (see Fig. 2A) were streaked on a nonfermentable carbon source (YPG) and grown at 30°C for 4 days. (B) Mitochondrial RNA isolated from strains SUF63-F and SUF63-R was analyzed by Northern blotting (Materials and Methods) with probes derived from *cob* (−1350 to +654) and *oli1* (+179 to +797) sequences. The positions of *cob* and *oli1* transcripts are indicated. That the faint band in the *cbp1*/SUF63-F lane was due to cross-hybridization of the *cob* probe to the 15S mitochondrial rRNA, which contains sequence complementary to the *cob* probe, was verified by primer extension analysis of *cbp1*/SUF63-F mitochondrial RNA (Fig. 7B). The *cbp1* strain used in these experiments was CP1L (Table 1). Data obtained by quantitation of the Northern blot signals are summarized in Table 3.

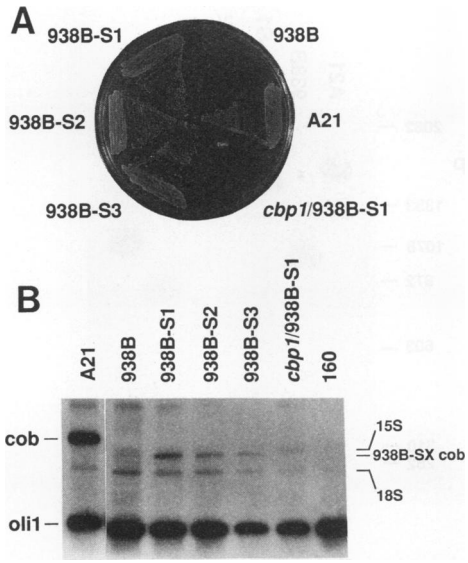

FIG. 5. Analysis of suppressors of the *cob* deletion in strain 938B. (A) Strains 938B-S1, -S2 and -S3, independently derived revertants of *cob* deletion strain 938B, were isolated as described in Materials and Methods. The revertants were streaked on a nonfermentable carbon source (YPG) and grown at 30°C for 4 days. (B) Mitochondrial RNA was isolated from the three respiratory-competent revertants of strain 938B and from strain 160 (diagrammed in Fig. 2A). The mitochondrial RNA was analyzed by Northern blotting (Materials and Methods) with probes derived from *cob* (−1350 to +654) and *oli1* (+179 to +797) sequences. The positions of wild-type *cob* transcripts and *oli1* transcripts are indicated on the left. The positions of *cob* transcripts in deletion strains 938B-S1, -S2, and -S3 and of the cross-hybridizing 15S and 18S rRNAs are indicated on the right. In this nondenaturing gel system, the cytoplasmic 18S rRNA migrates at a faster rate than does the mitochondrial 15S rRNA. The *cbp1* allele present in *cbp1*/938B-S1 is B13L-1 (Table 1). Data obtained by quantitation of the Northern blot signals are summarized in Table 3.

the same rate as the mature SUF63-F mRNA was detected in this strain. Primer extension analysis confirmed that this RNA was not *cob* specific (see Fig. 7B) and was most likely the mitochondrial 15S rRNA, which migrated at the same rate as the SUF63-F *cob* mRNA and contains stretches of sequence complementary to the *cob* probe. *cob* mRNA was undetectable in strain SUF63-R, and the strain was unable to respire, eliminating the possibility that the accumulation of *cob* transcripts in strain SUF63-F was due to the *Eco*RI linkers flanking the defined 63 nt (Fig. 2A). These results lead to the conclusion that sequence between −961 and −898 is sufficient for CBP1 function and therefore for any interaction that occurs between CBP1 and *cob* transcripts.

Suppression of the mutant phenotype of strain 938B by a mitochondrial point mutation. In previous studies, analyses of mitochondrial mutations that suppress *pet* mutations have provided information concerning the functions of the wild-type *PET* gene products (15, 22, 37). We reasoned that the nature of mitochondrial mutations that restore growth on glycerol to respiratory-incompetent *cob* deletion strains might provide information about the sequence requirements for the accumulation of *cob* transcripts. Therefore, respiratory-incompetent *cob* deletion strains 997B, 923A, SUF63-R, and 707A, and strain 938B, which grew extremely slowly on glycerol, were plated on glycerol to select for revertants. Only strain 938B yielded faster-growing colonies on glycerol. Three independently derived revertants (938B-S0, -S2 and -S3; see Materials and Methods) showed growth on glycerol similar to that of wild type (Fig. 5A). When mtDNA from strain 938B-S0 was transferred via cytoduction to a strain without mtDNA, the resulting strain (938B-S1) was respiratory competent (Fig. 5A), indicating that the reversion was due to a mitochondrial mutation. Northern blot analysis showed that the steady-state level of *cob* transcripts in the revertants was 5 to 17% of that of wild type, an increase over the level of *cob* transcripts in the original strain, 938B (Fig. 5B; Table 3). The accumulation of *cob* transcripts and the ability of the revertants to grow on glycerol remained *CBP1* dependent (Fig. 5).

Southern analysis (Fig. 6B) revealed that the *Eco*RI linker site (−937 to −931) was mutated in all three revertants, and sequence analysis (Materials and Methods; data not shown) verified that the only sequence change was at position −935, which changed the wild-type A to T (Fig. 6B). As sequence 3′ of −938 in the revertants differs from that present in wild type, we hypothesized that wild-type primary sequence 3′ of −938 is not absolutely required for the accumulation of *cob* transcripts.

To further examine the effect of changing the sequence 3′ of −938 on the accumulation of *cob* transcripts, strain 160 was constructed by deleting sequence between −938 and −778. In strain 160, sequence 3′ of −938 differed from that in either wild type or strain 938B (Fig. 2A and 6B). Strain 160 was respiratory competent but formed single colonies on

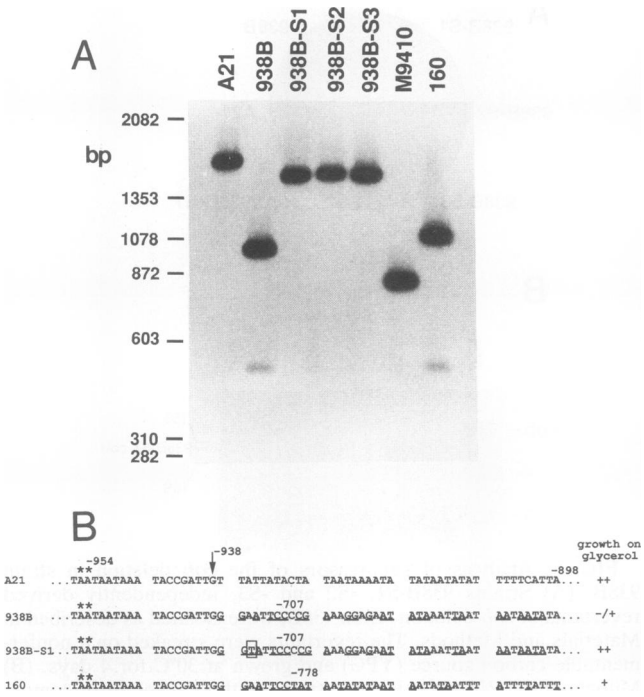

FIG. 6. Evidence that 938B suppressors are mitochondrial mutations. (A) mtDNA isolated from the three revertants of strain 938B was restricted with *Eco*RI and *Mbo*I and analyzed by Southern blotting with a probe derived from −1350 to +319 of *cob* (Materials and Methods). The positions and sizes of molecular weight markers are indicated on the left. (B) Sequences between −956 and −898 in *cob* deletion strains 938B, 938B-S1, and 160 are compared with the analogous wild-type sequence (A21). The arrow is above position −938, the 5′ deletion endpoint in strains 938B, 938B-S1, and 160. The numbers refer to the first wild-type base pair at each deletion endpoint (the minus signs are directly above the deletion endpoints). Underlined sequence differs from the analogous wild-type sequence. The boxed T at position −935 of the 938B-S1 sequence indicates the point mutation identified in all three respiratory-competent revertants of strain 938B. The relative growth of single colonies of each strain on the nonfermentable carbon source, glycerol, is indicated on the right. Asterisks denote the positions of the 5′ ends of mature *cob* mRNAs in that strain as determined by primer extension analysis (Fig. 7B and text).

glycerol that were smaller than those of wild type (Fig. 3A). The steady-state level of *cob* transcripts in strain 160 could not be determined by Northern blot analysis (Fig. 5B), but primer extension analysis indicated that mature *cob* mRNA was present at a steady-state level sevenfold lower than that of wild type (Fig. 7B; Table 3). Together, the results obtained from strains 938B, 938B-S1, and 160 lead to the conclusion that while changes in primary sequence 3′ of −938 do affect the steady-state level of *cob* transcripts, wild-type primary sequence 3′ of −938 is not absolutely required for *cob* transcript accumulation and therefore for CBP1 function.

Mutant precursor transcripts are cleaved at the same positions as in wild type to produce mature mRNA. As the sequence surrounding the cleavage site at −954 was changed in several of the respiratory-competent deletion mutants (Fig. 7A), it was possible that the position of the cleavage producing mature *cob* mRNA had been altered from that in wild type. Therefore, the positions of the 5′ ends of *cob* transcripts present in the deletion strains were determined by primer extension analysis using *cob*-specific primers (Fig. 7B). Mature mRNAs with 5′ ends at positions analogous to those of the wild-type mature mRNA, at −954 or −955, were detected in each of the respiratory-competent *cob* deletion strains, including strain 944A, in which these cleavages occur within $tRNA^{Glu}$ (Fig. 7A), and strain 938B, which grew extremely slowly on glycerol. As the 5′ ends of mature *cob* mRNA in strain SUF63-F are at −954 and −955, sequence between −961 and −898 is sufficient for correct positioning of the cleavages. Within this region of the RNA, the only primary sequence common to all of the respiratory-competent deletion strains is that from −948 to −938 (Fig. 6B and 7A). Therefore, we conclude that wild-type primary sequence 5′ of −948 or 3′ of −938 is not absolutely required for correct positioning of the cleavages that produce mature mRNA from unprocessed *cob* transcripts.

CBP1 prevents the degradation of unprocessed *cob* transcripts. As expected, mature *cob* mRNAs with 5′ ends at −954 or −955 were not detected in *cbp1* mutant strains (Fig. 7B). In contrast, *cob* transcripts produced by endonucleolytic cleavage at the 3′ end of $tRNA^{Glu}$ (referred to as unprocessed *cob* transcripts and indicated with the letter "e" in Fig. 7B) were detectable in both the *CBP1* and *cbp1* strains (Fig. 7B). However, quantitation of the primer extension products indicated that regardless of the mitochondrial genome, the steady-state levels of the unprocessed transcripts were lower in the *cbp1* strains than in the corresponding *CBP1* strains (Table 3). For example, the steady-state levels of the unprocessed *cob* transcripts in *cbp1* strains carrying the wild-type, 961A, or 944A mitochondrial genome were 10, 11, and 66%, respectively, of that in the *CBP1* strain carrying the same mitochondrial genome. These data indicate that CBP1 prevents the degradation of unprocessed *cob* transcripts.

DISCUSSION

The product of the nuclear *CBP1* gene is required post-transcriptionally for the accumulation of mitochondrial *cob* mRNA, which encodes cytochrome *b* (15). In *cbp1* mutant strains the $tRNA^{Glu}$-*cob* unit is transcribed, but *cob* mRNA does not accumulate and the cells are respiratory deficient. To elucidate the sequence requirements for CBP1 function, we used the technique of microprojectile bombardment for transformation of yeast mitochondria (20, 25) to introduce deletions within the −1098 to −696 interval of *cob* previously defined as sufficient for CBP1 function (30). Using this approach, we determined that a 63-nt region of *cob* transcripts, from −961 to −898, is sufficient both for CBP1 function and for correct positioning of the cleavages at nt −954 and −955 that produce the mature 5′ ends of *cob* mRNAs.

Two observations argue that CBP1 does not require *cob* sequence downstream of −700. First, a deletion of *cob* sequence between −682 and −4 had no effect on the accumulation of *cob* transcripts (30a). Second, a fusion of *cob* sequence 5′ of −696 to a heterologous mitochondrial gene resulted in the production of a hybrid transcript that accumulated in a CBP1-dependent manner (30).

CBP1 could act indirectly to allow the accumulation of *cob* mRNA by regulating the activity of a second, as yet unidentified protein involved in *cob* mRNA turnover. However, as previous studies have demonstrated that CBP1 is imported into mitochondria (47), we favor a simpler model in which CBP1 interacts directly with *cob* transcripts. CBP1 could act directly to allow the accumulation of *cob* mRNA by any one

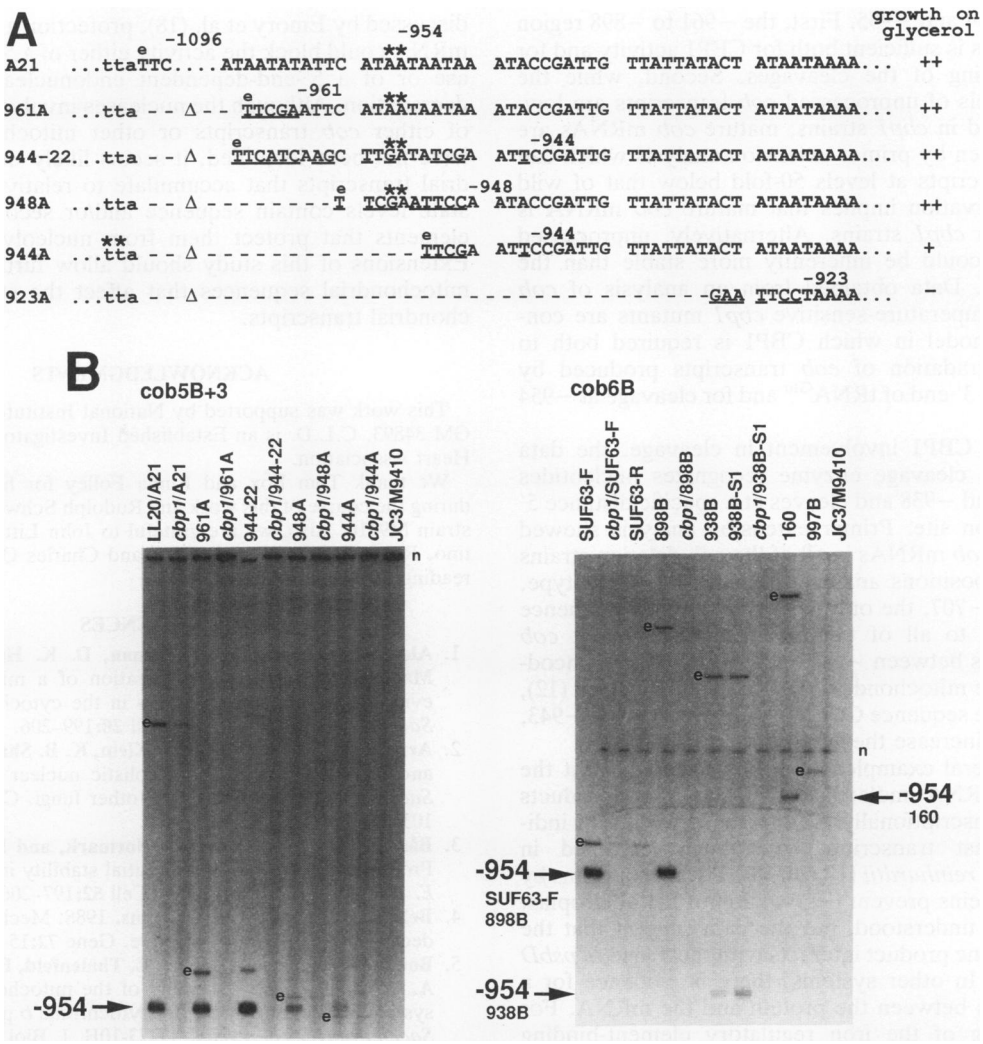

FIG. 7. 5′ ends of *cob* transcripts in *cob* deletion strains. (A) Sequences surrounding the cleavage site at −954 in series A *cob* deletion strains are compared with the analogous wild-type sequence (A21). The lowercase letters denote the last three nucleotides of the $tRNA^{Glu}$ gene. The 5′ ends (determined by primer extension analysis) of mature *cob* mRNAs (asterisks) and of *cob* transcripts produced by endonucleolytic cleavage at the 3′ end of $tRNA^{Glu}$ (e) are indicated. Deleted sequences are replaced with dashed lines to emphasize the fact that following cleavage at the 3′ end of $tRNA^{Glu}$, unprocessed *cob* transcripts in the series A deletion strains differ at their 5′ ends. All other symbols are as described for Fig. 6B. (B) RNA isolated from strains carrying either wild-type (A21) or *cob* deletion mitochondrial genomes was analyzed by primer extension with the ^{32}P-labeled cob5B+3 or cob 6B primer (Materials and Methods). The primer extension products were separated on a 6% polyacrylamide–7 M urea sequencing gel. Primer extension products corresponding to transcripts with 5′ ends at positions analogous to nt −954 are indicated with arrows. Primer extension products corresponding to unprocessed *cob* transcripts resulting from endonucleolytic cleavage at the 3′ end of $tRNA^{Glu}$ are indicated with the letter "e". The *cbp1* strains used in these experiments were the same as those used for Northern blot analyses (Fig. 3 to 5). As the *cob*-specific binding sites for the primers have been deleted in strain JC3/M9410, mitochondrial RNA isolated from this strain served as a negative control for the primer extension reactions. The data obtained by quantitation of the primer extension products are summarized in Table 3.

or some combination of the following mechanisms: (i) preventing degradation of unprocessed *cob* transcripts by binding and/or modification, (ii) preventing degradation of mature *cob* mRNA by binding and/or modification, or (iii) cleaving precursor transcripts at −954 or −955 to produce mature *cob* mRNA that is resistant to degradation.

The analysis of *cob* transcripts in both wild-type and *cob* deletion strains indicated that CBP1 is required for the wild-type accumulation of unprocessed *cob* transcripts produced by endonucleolytic cleavage at the 3′ end of $tRNA^{Glu}$. Primer extension analysis showed that the steady-state levels of either wild-type or mutant unprocessed *cob* transcripts were lower in *cbp1* strains than in *CBP1* strains (Table 3). The production of these transcripts is dependent on the steady-state level of the initial $tRNA^{Glu}$-*cob* transcript and on the rate of endonucleolytic cleavage at the 3′ end of $tRNA^{Glu}$. As $tRNA^{Glu}$ levels do not appear to be dependent on CBP1, we conclude that the low steady-state levels of unprocessed *cob* transcripts in *cbp1* strains are not the result of lower rates of production of these RNA molecules. Therefore, consistent with model i, CBP1 prevents the degradation of the unprocessed *cob* transcripts. These data do not rule out the possibility that CBP1 also prevents the degradation of mature *cob* mRNA and/or enhances the cleavage of precursor transcripts to produce mature mRNA.

Two observations are suggestive of a role for CBP1 in the

cleavages at −954 and −955. First, the −961 to −898 region of *cob* transcripts is sufficient both for CBP1 activity and for correct positioning of the cleavages. Second, while the steady-state levels of unprocessed *cob* transcripts are lowered 2- to 13-fold in *cbp1* strains, mature *cob* mRNAs are undetectable, even by primer extension analysis which can detect *cob* transcripts at levels 50-fold below that of wild type. This observation implies that mature *cob* mRNA is never formed in *cbp1* strains. Alternatively, unprocessed *cob* transcripts could be inherently more stable than the mature mRNAs. Data obtained from an analysis of *cob* transcripts in temperature-sensitive *cbp1* mutants are consistent with a model in which CBP1 is required both to prevent the degradation of *cob* transcripts produced by processing at the 3′ end of $tRNA^{Glu}$ and for cleavage at −954 or −955 (40).

Regardless of CBP1 involvement in cleavage, the data suggest that the cleavage enzyme recognizes nucleotides between −948 and −938 and cleaves at a specific distance 5′ of the recognition site. Primer extension analysis showed that the mature *cob* mRNAs in all of the *cob* deletion strains had 5′ ends at positions analogous to those of wild type. However, 5′ of −707, the only wild-type primary sequence that is common to all of the respiratory-competent *cob* deletion strains is between −948 and −938. As the noncoding regions of the mitochondrial genome are 95% A+T (12), recognition of the sequence CCG at nucleotides −944, −943, and −942 could increase the specificity of cleavage.

There are several examples of proteins that prevent the degradation of RNA molecules. Nuclear gene products required posttranscriptionally for the accumulation of individual chloroplast transcripts have been identified in *Chlamydomonas reinhardtii* (17, 26, 39). The mechanisms by which these proteins prevent degradation of the chloroplast mRNAs are not understood, but the data suggest that the nuclear *NAC2* gene product interacts with the 5′ end of *psbD* transcripts (26). In other systems, there is evidence for a direct interaction between the protein and the mRNA. For example, binding of the iron regulatory element-binding protein to sequences in the 3′ untranslated region of human transferrin receptor mRNA controls the rate of degradation of the mRNA (33). In *E. coli*, specific endoribonucleases cleave the unstable polycistronic *papBA* and bacteriophage T4 transcripts to produce monocistronic mRNAs that are resistant to degradation (3, 31). CBP1 may protect unprocessed *cob* transcripts by binding in a way similar to that of the iron regulatory element-binding protein and may cleave precursor *cob* transcripts to produce a stable mature mRNA, as does the endoribonuclease that cleaves *papBA* transcripts.

Another factor affecting the rate at which an RNA molecule is degraded is secondary structure within the RNA. In *E. coli* (for review, see reference 4) and in *C. reinhardtii* (42) or higher plant chloroplasts (41), stem-loop structures at the 3′ end of mRNAs protect against 3′-to-5′ exonucleolytic degradation. Also, a stem-loop structure at the 5′ end of the *ompA* mRNA in *E. coli* appears to prevent cleavage by a 5′ end-dependent endonuclease that initiates degradation of the message (9, 18). Changes in the primary sequence of *cob* RNA 5′ of −948 or 3′ of −938 lowered, but did not abolish, accumulation of the transcripts. One explanation for this observation is that involvement of these sequences in secondary structure is important for the stability of *cob* transcripts.

How might protein binding to or secondary structure within an mRNA protect the molecule from degradation? As discussed by Emory et al. (18), protection at the 5′ end of an mRNA could block the activity either of a 5′-to-3′ exonuclease or of a 5′-end-dependent endonuclease that initiates degradation. Although the nucleases involved in degradation of either *cob* transcripts or other mitochondrial mRNAs have not been identified, it seems likely that all mitochondrial transcripts that accumulate to relatively high steady-state levels contain sequence and/or secondary structural elements that protect them from nucleolytic degradation. Extensions of this study should allow further definition of mitochondrial sequences that affect the stability of mitochondrial transcripts.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grant GM 34893. C.L.D. is an Established Investigator of the American Heart Association.

We thank Tom Fox and Linda Folley for helpful discussions during the course of this work and Rudolph Schweyen for providing strain M9410. Also, we are grateful to John Little, Donald Copertino, Robin Staples, Roy Parker, and Charles Quinton for critical reading of the manuscript.

REFERENCES

1. **Alexander, N. J., P. S. Perlman, D. K. Hanson, and H. R. Mahler.** 1980. Mosaic organization of a mitochondrial gene: evidence from double mutants in the cytochrome *b* region of *Saccharomyces cerevisiae*. Cell **20**:199–206.
2. **Armaleo, D., G.-N. Ye, T. M. Klein, K. B. Shark, J. C. Sanford, and S. A. Johnston.** 1990. Biolistic nuclear transformation of *Saccharomyces cerevisiae* and other fungi. Curr. Genet. **17**:97–103.
3. **Båga, M., M. Göransson, S. Normark, and B. E. Uhlin.** 1988. Processed mRNA with differential stability in the regulation of *E. coli* pilin gene expression. Cell **52**:197–206.
4. **Belasco, J. G., and C. F. Higgins.** 1988. Mechanisms of mRNA decay in bacteria: a perspective. Gene **72**:15–23.
5. **Bonitz, S. G., G. Homison, B. E. Thalenfeld, F. G. Nobrega, and A. Tzagoloff.** 1982. Assembly of the mitochondrial membrane system: processing of the apocytochrome *b* precursor RNAs in *Saccharomyces cerevisiae* D273-10B. J. Biol. Chem. **257**:6268–6274.
6. **Bullock, W. O., J. M. Fernandez, and J. M. Short.** 1987. XL1-Blue: a high efficiency plasmid transforming recA *Escherichia coli* strain with beta-galactosidase selection. BioTechniques **5**:376–378.
7. **Caponigro, G., D. Muhlrad, and R. Parker.** A small segment of the *MATα1* transcript promotes mRNA decay in yeast: a stimulatory role for rare codons. Submitted for publication.
8. **Chen, J.-Y., and N. C. Martin.** 1988. Biosynthesis of tRNA in yeast mitochondria: an endonuclease is responsible for the 3′-processing of tRNA precursors. J. Biol. Chem. **263**:13677–13682.
9. **Chen, L.-H., S. A. Emory, A. L. Bricker, P. Bouvet, and J. G. Belasco.** 1991. Structure and function of a bacterial mRNA stabilizer: analysis of the 5′ untranslated region of *ompA* mRNA. J. Bacteriol. **173**:4578–4586.
10. **Christianson, T., J. C. Edwards, D. M. Mueller, and M. Rabinowitz.** 1983. Identification of a single transcriptional initiation site for the glutamic acid tRNA and *cob* genes in yeast mitochondria. Proc. Natl. Acad. Sci. USA **80**:5564–5568.
11. **Christianson, T., and M. Rabinowitz.** 1983. Identification of multiple transcriptional initiation sites on the yeast mitochondrial genome by *in vitro* capping with guanylytransferase. J. Biol. Chem. **258**:14025–14033.
12. **de Zamaroczy, M., and G. Bernardi.** 1987. The AT spacers and the *var1* genes from the mitochondrial genomes of *Saccharomyces cerevisiae* and *Torulopsis glabrata*: evolutionary origin and mechanism of formation. Gene **54**:1–22.
13. **Dieckmann, C. L., and B. Gandy.** 1987. Preferential recombination between GC clusters in yeast mitochondrial DNA. EMBO J. **6**:4197–4203.

14. **Dieckmann, C. L., G. Homison, and A. Tzagoloff.** 1984. Assembly of the mitochondrial membrane system: nucleotide sequence of a yeast nuclear gene (*CBP1*) involved in 5′ end processing of cytochrome *b* pre-mRNA. J. Biol. Chem. **259:** 4732–4738.
15. **Dieckmann, C. L., T. J. Koerner, and A. Tzagoloff.** 1984. Assembly of the mitochondrial membrane system: *CBP1*, a yeast nuclear gene involved in 5′ end processing of cytochrome *b* pre-mRNA. J. Biol. Chem. **259:**4722–4731.
16. **Dobres, M., S. Gerbl-Rieger, C. Schmelzer, M. W. Mueller, and R. J. Schweyen.** 1985. Deletions in the *cob* gene of yeast mtDNA and their phenotypic effect. Curr. Genet. **10:**283–290.
17. **Drapier, D., J. Girard-Bascou, and F.-A. Wollman.** 1992. Evidence for nuclear control of the expression of the *atpA* and *atpB* chloroplast genes in *Chlamydomonas*. Plant Cell **4:**283–295.
18. **Emory, S. A., P. Bouvet, and J. G. Belasco.** 1992. A 5′-terminal stem-loop structure can stabilize mRNA in *Escherichia coli*. Genes Dev. **6:**135–148.
19. **Folley, L. S., and T. D. Fox.** 1991. Site-directed mutagenesis of a *Saccharomyces cerevisiae* mitochondrial translation initiation codon. Genetics **129:**659–668.
20. **Fox, T. D., J. C. Sanford, and T. W. McMullin.** 1988. Plasmids can stably transform yeast mitochondria lacking endogenous mtDNA. Proc. Natl. Acad. Sci. USA **85:**7288–7292.
21. **Grivell, L. A.** 1989. Nucleo-mitochondrial interactions in yeast mitochondrial biogenesis. Eur. J. Biochem. **182:**477–493.
22. **Hill, J., P. McGraw, and A. Tzagoloff.** 1985. A mutation in yeast mitochondrial DNA results in a precise excision of the terminal intron of the cytochrome *b* gene. J. Biol. Chem. **260:**3235–3238.
23. **Hill, J. E., A. M. Myers, T. J. Koerner, and A. Tzagoloff.** 1986. Yeast/*E. coli* shuttle vectors with multiple unique restriction sites. Yeast **2:**163–167.
24. **Hollingsworth, M. J., and N. C. Martin.** 1986. RNase P activity in the mitochondria of *Saccharomyces cerevisiae* depends on both mitochondrion and nucleus-encoded components. Mol. Cell. Biol. **6:**1058–1064.
25. **Johnston, S. A., P. Q. Anziano, K. Shark, J. C. Sanford, and R. A. Butow.** 1988. Mitochondrial transformation in yeast by bombardment with microprojectiles. Science **240:**1538–1541.
26. **Kuchka, M. R., M. Goldschmidt-Clermont, J. van Dillewijn, and J.-D. Rochaix.** 1989. Mutation at the *Chlamydomonas* nuclear *NAC2* locus specifically affects stability of the chloroplast *psbD* transcript encoding polypeptide D2 of PS II. Cell **58:**869–876.
27. **Lancashire, W. E., and J. R. Mattoon.** 1979. Cytoduction: a tool for mitochondrial genetic studies in yeast. Mol. Gen. Genet. **170:**333–344.
28. **Liu, Y., and C. L. Dieckmann.** 1989. Overproduction of yeast viruslike particles by strains deficient in a mitochondrial nuclease. Mol. Cell. Biol. **9:**3323–3331.
29. **Mayer, S. A., and C. L. Dieckmann.** 1989. The yeast *CBP1* gene produces two differentially regulated transcripts by alternative 3′-end formation. Mol. Cell. Biol. **9:**4161–4169.
30. **Mittelmeier, T. M., and C. L. Dieckmann.** 1990. *CBP1* function is required for stability of a hybrid *cob-oli1* transcript in yeast mitochondria. Curr. Genet. **18:**421–428.
30a. **Mittelmeier, T. M., and C. L. Dieckmann.** Unpublished data.
31. **Mudd, E. A., P. Prentki, D. Belin, and H. M. Krisch.** 1988. Processing of unstable bacteriophage T4 gene 32 mRNAs into a stable species requires *Escherichia coli* ribonuclease E. EMBO J. **11:**3601–3607.
32. **Mueller, D. M., and G. S. Getz.** 1986. Steady state analysis of mitochondrial RNA after growth of yeast *Saccharomyces cerevisiae* under catabolite repression and derepression. J. Biol. Chem. **261:**11816–11822.
33. **Müllner, E. W., B. Neupert, and L. C. Kühn.** 1989. A specific mRNA binding factor regulates the iron-dependent stability of cytoplasmic transferrin receptor mRNA. Cell **58:**373–382.
34. **Nobrega, F. G., and A. Tzagoloff.** 1980. Assembly of the mitochondrial membrane system: complete restriction map of the cytochrome *b* region of mitochondrial DNA in *Saccharomyces cerevisiae* D273-10B. J. Biol. Chem. **255:**9821–9827.
35. **Nobrega, F. G., and A. Tzagoloff.** 1980. Assembly of the mitochondrial membrane system: DNA sequence and organization of the cytochrome *b* gene in *Saccharomyces cerevisiae* D273-10B. J. Biol. Chem. **255:**9828–9837.
36. **Orr-Weaver, T. L., J. W. Szostak, and R. J. Rothstein.** 1981. Yeast transformation: a model system for the study of recombination. Proc. Natl. Acad. Sci. USA **78:**6354–6358.
37. **Rödel, G., A. Körte, and F. Kaudewitz.** 1985. Mitochondrial suppression of a yeast nuclear mutation which affects the translation of the mitochondrial apocytochrome *b* transcript. Curr. Genet. **9:**641–648.
38. **Sanford, J. C., T. M. Klein, E. D. Wolf, and N. Allen.** 1987. Delivery of substances into cells and tissues using a particle bombardment process. Particulate Sci. Technol. **5:**27–37.
39. **Sieburth, L. E., S. Berry-Lowe, and G. W. Schmidt.** 1991. Chloroplast RNA stability in *Chlamydomonas*: rapid degradation of *psbB* and *psbC* transcripts in two nuclear mutants. Plant Cell **3:**175–189.
40. **Staples, R. R., and C. L. Dieckmann.** The characteristics of new temperature-sensitive *cbp1* strains of *S. cerevisiae* imply that CBP1 is involved in processing of cytochrome *b* mRNA. Submitted for publication.
41. **Stern, D. B., H. Jones, and W. Gruissem.** 1989. Function of plastid mRNA 3′ inverted repeats: RNA stabilization and gene-specific protein binding. J. Biol. Chem. **264:**18742–18750.
42. **Stern, D. B., E. R. Radwanski, and K. L. Kindle.** 1991. A 3′ stem/loop structure of the *Chlamydomonas* chloroplast *atpB* gene regulates mRNA accumulation *in vivo*. Plant Cell **3:**285–297.
43. **Tzagoloff, A., A. Akai, and F. Foury.** 1976. Assembly of the mitochondrial membrane system: modified form of the ATPase proteolipid in oligomycin-resistant mutants of *Saccharomyces cerevisiae*. FEBS Lett. **65:**391–396.
44. **Tzagoloff, A., and C. L. Dieckmann.** 1990. *PET* genes of *Saccharomyces cerevisiae*. Microbiol. Rev. **54:**211–225.
45. **Tzagoloff, A., F. Foury, and A. Akai.** 1976. Assembly of the mitochondrial membrane system: cytoplasmic mutants of *Saccharomyces cerevisiae* with lesions in enzymes of the respiratory chain and in the mitochondrial ATPase. Mol. Gen. Genet. **149:**33–42.
46. **Tzagoloff, A., and A. M. Myers.** 1986. Genetics of mitochondrial biogenesis. Annu. Rev. Biochem. **55:**249–285.
47. **Weber, E. R., and C. L. Dieckmann.** 1990. Identification of the *CBP1* polypeptide in mitochondrial extracts from *Saccharomyces cerevisiae*. J. Biol. Chem. **265:**1594–1600.
48. **Wolf, K., B. Dujon, and P. P. Slonimski.** 1973. Mitochondrial genetics: multifactorial mitochondrial crosses involving a mutation conferring paromomycin-resistance in *Saccharomyces cerevisiae*. Mol. Gen. Genet. **125:**53–90.