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Suppressor Analysis of Mutations in the 5′-Untranslated Region of *COB* mRNA Identifies Components of General Pathways for Mitochondrial mRNA Processing and Decay in *Saccharomyces cerevisiae*

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ABSTRACT

The cytochrome *b* gene in *Saccharomyces cerevisiae*, *COB*, is encoded by the mitochondrial genome. Nuclear-encoded Cbp1 protein is required specifically for *COB* mRNA stabilization. Cbp1 interacts with a CCG element in a 64-nucleotide sequence in the 5′-untranslated region of *COB* mRNA. Mutation of any nucleotide in the CCG causes the same phenotype as *cbp1* mutations, *i.e.*, destabilization of both *COB* precursor and mature message. In this study, eleven nuclear suppressors of single-nucleotide mutations in CCG were isolated and characterized. One dominant suppressor is in *CBP1*, while the other 10 semidominant suppressors define five distinct linkage groups. One group of four mutations is in *PET127*, which is required for 5′ end processing of several mitochondrial mRNAs. Another mutation is linked to *DSS1*, which is a subunit of mitochondrial 3′ → 5′ exoribonuclease. A mutation linked to the *SOC1* gene, previously defined by recessive mutations that suppress *cbp1 ts* alleles and stabilize many mitochondrial mRNAs, was also isolated. We hypothesize that the products of the two uncharacterized genes also affect mitochondrial RNA turnover.

GENE expression in yeast mitochondria is a coordinated process that requires the functions of both nuclearly and mitochondrially encoded proteins. Mitochondrial *COB* mRNA, which encodes cytochrome *b*, is a good model with which to study this type of fine regulation in *Saccharomyces cerevisiae*. Nuclear-encoded protein factors specific to the *COB* transcript have been shown to be required for processing of introns, production of the 5′ end of the mRNA, mRNA stability, and mRNA translation. In this study, we have used genetic suppressor analysis to uncover factors involved in general pathways of mRNA decay in yeast mitochondria.

The nuclear-encoded protein Cbp1 stabilizes *COB* mRNA but none of the other six mitochondrial mRNAs (Dieckmann *et al.* 1984). *COB* mRNA is undetectable in *cbp1* mutants and thus the mutants are respiratory-deficient; they can grow on a fermentable carbon source, such as glucose, but not on a nonfermentable carbon source, such as glycerol. Our recent study supports the idea that Cbp1 protein physically interacts with the RNA to protect it from nucleolytic degradation (Chen and Dieckmann 1997). We showed that a CCG element at positions −944 to −942 of the *COB* 5′-untranslated region (5′-UTR; with start codon AUG of *COB* defined as +1) plays a critical role in *COB* mRNA stabilization. Mutation of any single nucleotide of this CCG eliminates *COB* mRNA accumulation and reduces the level of pre-*COB* RNA fivefold, a phenotype equivalent to that of *cbp1* mutants. Because the single-nucleotide mutations affect *COB* mRNA levels drastically, the respiratory growth of the mutants on glycerol was affected. Mutant CAG (C<u>C</u>G → C<u>A</u>G) is like mutant AAU (<u>CCG</u> → <u>AAU</u>), and does not grow at all on glycerol medium at any temperature. However, mutants ACG (<u>C</u>CG → <u>A</u>CG) and CCU (CC<u>G</u> → CC<u>U</u>) grow slowly at 25 and 30°, and faster-growing pseudorevertants arise spontaneously (Chen and Dieckmann 1997).

In this article, we have characterized 11 of these pseudorevertants. The suppressor mutations are nuclearly encoded. One is a dominant, single nucleotide mutation in *CBP1* (Chen and Dieckmann 1997). The other 10 suppressors are semidominant and fall into five different linkage groups. One of the linkage groups is defined by *pet127* mutations. *PET127* functions in 5′ processing and turnover of mitochondrial RNAs (Wiesenberger and Fox 1997). Another suppressor is linked to *DSS1*. *DSS1* encodes one of the three protein components comprising the 3′ → 5′ exoribonuclease activity in yeast mitochondria (referred to as mtEXO; Dmochowska *et al.* 1995). A mutation linked to the *SOC1* locus was also recovered. *SOC1* was originally defined by recessive mutations that suppress *cbp1 ts* alleles (Staples and Dieckmann 1994). *soc1* mutations increase the stability of many of the mitochondrial mRNAs. The other two linkage groups define loci of unknown function.

MATERIALS AND METHODS

Strains and media: The *S. cerevisiae* strains used in this study are listed in Table 1. The media in which yeast or *Escherichia*

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TABLE 1

Names and genotypes of yeast strains

Strain	Genotype or description	Reference
LL20/*rho*^{0}	α [*rho*^{0}] *leu2-3 leu2-112 his3-11 his3-15* 2μm$^+$	Mayer and Dieckmann (1989)
aLL20/*rho*^{0}	LL20/*rho*^{0} switched to *MAT***a** by *HO*	This study[a]
αu20/*rho*^{0}	α [*rho*^{0}] *ura3::LEU2 his3-11 his3-15* 2μm$^+$	This study
au20/*rho*^{0}	**a** [*rho*^{0}] *ura3::LEU2 his3-11 his3-15* 2μm$^+$	This study
JC3/*rho*^{0}	**a** [*rho*^{0}] *kar1-1 ade2 lys2*	Alexander *et al.* (1980)[b]
JC7/*rho*^{0}	α [*rho*^{0}] *kar1-1 leu2*	Alexander *et al.* (1980)
SUF63-F	α [*rho*$^{+SUF63\text{-}F}$] in LL20	Mittelmeier and Dieckmann (1993)
ACG	α [*rho*$^{+ACG}$] in LL20	Chen and Dieckmann (1997)
sup-a1	α [*rho*$^{+ACG}$] with *pet127*-S310 mutation	This study
sup-a2	α [*rho*$^{+ACG}$] with D533Y in *CBP1*	This study
sup-a3	α [*rho*$^{+ACG}$] with supa-3 mutation in LL20	This study
sup-a4	α [*rho*$^{+ACG}$] with supa-4 mutation in LL20	This study
sup-a5	α [*rho*$^{+ACG}$] with supa-5 mutation linked to *SOC1*	This study
CCU	α [*rho*$^{+CUU}$] in LL20	Chen and Dieckmann (1997)
sup-u6	α [*rho*$^{+CCU}$] with *pet127*-S533 mutation	This study
sup-u7	α [*rho*$^{+CCU}$] with *pet127*-S345 mutation	This study
sup-u8	α [*rho*$^{+CCU}$] with *pet127*-S131 mutation	This study
sup-u9	α [*rho*$^{+CCU}$] with sup-u9 mutation in LL20	This study
sup-u10	α [*rho*$^{+CCU}$] with sup-u10 mutation in LL20	This study
sup-u11	α [*rho*$^{+CCU}$] with sup-u11 mutation linked to *DSS1*	This study
D273-10B/A21	α [*rho*$^{+A21}$] *met6 E*^{R} *O*^{R} *P*^{R}	Tzagoloff *et al.* (1976)
CB11	**a** [*rho*$^{+A21}$] *ade1*	ten Berge *et al.* (1974)
M17-162	α [*rho*$^+$ *mit*$^-$] *met6*	Tzagoloff *et al.* (1976)
aM17-162-4A	**a** [*rho*$^+$ *mit*$^-$] *ade1*	Tzagoloff *et al.* (1976)
aRSY29/*rho*^{0}	**a** [*rho*^{0}] *ade1 leu2-3 leu2-112 ura3-52 soc1-1*	Staples and Dieckmann (1994)
pet127/rho^0	**a** [*rho*^{0}] *pet127::URA3* in **a**u20/*rho*^{0}	This study
suv3/rho^0	**a** [*rho*^{0}] *suv3::URA3* in **a**u20//*rho*^{0}	This study
dss1/rho^0	**a** [*rho*^{0}] *dss1::URA3* in **a**u20/*rho*^{0}	This study

[a] American Type Culture Collection (ATCC) reference code 201578.
[b] ATCC reference code 201577.

coli strains were grown were as described previously (Chen and Dieckmann 1997).

Isolation of spontaneous pseudorevertants: ACG and CCU mutant strains were patched on YEPD plates and incubated at 30° overnight. The patches were replicated onto YEPG plates and incubated at 30° for 6–10 days until faster-growing colonies arose in the patches. Five independent colonies were recovered from each of the ACG and CCU strains and purified. The pseudorevertants isolated from ACG were named "sup-a" followed by numbers 1 to 5; those from CCU were named "sup-u" followed by numbers 6 to 10. The pseudorevertants were grown in liquid YEPD medium overnight, diluted, and plated for single colonies on YEPD plates, which after 2 days incubation at 30° were replica-plated to YEPG medium. Over 95% of the colonies were able to grow on glycerol. This test guarantees that suppression is not due to heteroplasmic elements with mitochondrial DNA rearrangements (Dieckmann *et al.* 1984; Müller *et al.* 1984). Suppression is otherwise not maintained on nonselectable media because of segregation of the suppressing, rearranged molecule from the *grande* mitochondrial DNA.

Yeast mating type switches and changes of genetic markers: A switch of mating type from *MAT*α to *MAT***a** was implemented by transformation with the *HO* gene. Plasmid pRS413/*HO* was constructed, with the sequence of *HO* from −1360 to +2510 (Russell *et al.* 1986) ligated into pRS413 (Sikorski and Hieter 1989). Transformants were selected on histidine dropout plates. The mating types were tested by mating with testers D237-10B/A21 (*MAT*α) and CB11 (*MAT***a**). Only those that could mate with D237-10B/A21, but not with CB11, were *MAT***a.** To get rid of the *HO* plasmid after the mating type was switched, the strains were plated for single colonies on YEPD after overnight growth in liquid YEPD. His$^-$ colonies were identified by replica-plating on histidine dropout plates. The new strains were named "**a**" followed by their original names.

To enable the selection of diploids when the isogenic pseudorevertants were crossed to each other, the auxotrophies of the *MAT*α *leu2 his3* strains were changed to *MAT*α *ura3 his3* by the following method. Plasmid U::L-F was constructed with *LEU2* inserted into the *Sca*I site of *URA3*, which is carried in the pUC18 (Norrander *et al.* 1983) backbone. "F" refers to *LEU2* in the same transcriptional orientation as *URA3*. A total of 1 μg of U::L-F was digested with *Hin*dIII and the digestion mixture was directly transformed into the pseudorevertants. Transformants were selected on leucine dropout plates. To confirm the disruption of the *URA3* locus, chromosomal DNAs were prepared as described previously (Elion and Warner 1986) except that the zymolyase buffer contained 1.2 m sorbitol, 75 mm KPO_4 (pH 7.5), 2.5% β-mercaptoethanol, 0.2 mm EDTA (pH 7.5), and 1 mg of zymolyase (Seikagaku Corp., Ijamsville, MD). A total of 2 μg of each sample was digested with *Hin*dIII and separated on a 1% agarose gel. The Nytran membrane, onto which the DNA was transferred, was probed

with a random-priming-labeled *URA3-Hin*dIII fragment. All strains showed a shifted *URA3::LEU2* fragment (3.3 kb) compared with the wild-type *URA3-Hin*dIII fragment (1.2 kb). Pseudorevertants with changes of the auxotrophies have "αu" added to their original names, respectively. **a**LL20/*rho*^{0} (*MAT***a** *leu2 his3*), αu20/*rho*^{0} (*MAT*α *ura3 his3*), or **a**u20/*rho*^{0} (*MAT***a** *ura3 his3*) are derivatives of LL20/*rho*^{0} (*MAT*α *leu2 his3*).

Backcrosses and linkage analysis: The αu pseudorevertant strains were crossed with **a**LL20/*rho*^{0}. The diploids were selected on glucose medium without any amino acid (WO) + histidine medium and then grown on YEPG plates to test the dominance of the suppressor mutations by examining the respiratory capability of the *sup*/+ diploids at 30 and 25°. To examine whether each pseudorevertant strain contains a single nuclear mutation, the resulting diploids were sporulated in 1% potassium acetate solution at room temperature. After zymolyase digestion, the tetrads were dissected by micromanipulation. After 2 days, spores were tested for auxotrophies and respiration, at 30° for [*rho*$^{+ACG}$] and 33° for [*rho*$^{+CCU}$].

To cross the 10 pseudorevertants with each other, the *MAT***a** *leu2 his3* strains were mated with the *MAT*α *ura3 his3* strains. Diploids were selected on WO + histidine medium and then sporulated. From 20 to 40 tetrads were examined for each of the crosses. To determine the linkage between "sup-a" suppressors and "sup-u" suppressors, αu sup-u strains were made [*rho*^{0}] (Fox *et al.* 1991) and then crossed with **a** sup-a strains containing [*rho*$^{+ACG}$]. The resulting diploids were sporulated as above.

Allele specificity tests of the suppressors: To examine whether the suppressors cause a respiratory-deficiency phenotype of their own, to examine whether sup-a1, -a2, -a3, -a4, and -a5 could suppress [*rho*$^{+CCU}$] or [*rho*$^{+CAG}$], and to examine whether sup-u6, -u7, -u8, -u9, and -u10 could suppress [*rho*$^{+ACG}$] or [*rho*$^{+CAG}$], all 10 pseudorevertants were made [*rho*^{0}] by ethidium bromide-mutagenesis (Fox *et al.* 1991). Lack of mitochondrial DNAs was confirmed by ultraviolet fluorescence microscopy of cells suspended in 1 μg/μl of 4′, 6′-diamidino-2-phenylindole (DAPI). Using karyogamy-deficient strains carrying the wild-type mitochondrial genome or genomes with different mutations in their *COB* genes (JC3/*rho*$^+$, JC3/*rho*$^{+ACG}$, JC3/*rho*$^{+CAG}$, and JC3/*rho*$^{+CCU}$ strains), the nuclear genomes containing the 10 different suppressors were combined with various mitochondrial genomes by cytoduction (Berlin *et al.* 1991).

Suppression of single-nucleotide *COB* mutants by overexpression of *CBP1*: Plasmid pG60/T31 (Dieckmann *et al.* 1984), which carries the entire *CBP1* gene in the yeast 2μ vector YEp13, was transformed into the ACG, CAG, and CCU mutant strains. YEp13 with no insert was transformed as a control. Transformants were selected on leucine dropout plates, replicated onto YEPG plates, and grown at 30°.

Suppression of single-nucleotide *COB* mutants by *soc1* and *pet127*: To obtain strains with the mitochondrial genomes of ACG, CAG, and CCU in the mutant *soc1* and *pet127* nuclear backgrounds, the mitochondrial genomes [*rho*$^{+ACG}$], [*rho*$^{+CAG}$], and [*rho*$^{+CCU}$] were transferred into aRSY29/*rho*^{0} (Staples and Dieckmann 1994) and *pet127*/*rho*^{0} via cytoduction using strains JC3/*rho*^{0} and JC7/*rho*^{0}.

Linkage analysis of the suppressors with *SOC1*, *PET127*, *SUV3*, and *DSS1* loci: Because *SOC1* is not yet cloned, the relationship of the suppressors to *SOC1* was carried out by examining respiration of the diploids generated by crossing the suppressors (*MAT*α *leu2 his3 sup* [*rho*^{0}]) with a *soc1*[*rho*^{0}] strain [**a**RSY29/*rho*^{0}] (Staples and Dieckmann 1994). Diploids were selected on WO + leucine medium, replicated onto YEPG plates, and grown at 30°. Diploids were sporulated and tetrads dissected.

To examine whether a wild-type copy of *PET127*, *SUV3*, or *DSS1* could complement the suppressor functions, plasmids pGW694 (Wiesenberger and Fox 1997), YEp24(T1), and pAD15 (Dmochowska *et al.* 1995) were transformed into the pseudorevertants, respectively. Transformants were selected on uracil or leucine dropout plates and then grown on glycerol plates (without uracil or leucine supplied) at 30°. To examine whether the suppressors are linked to the *PET127*, *SUV3*, or *DSS1* loci, pseudorevertants were crossed with *pet127*/*rho*^{0}, *suv3*/*rho*^{0}, and *dss1*/*rho*^{0} separately. Diploids were selected and sporulated. The isogenic *pet127*/*rho*^{0} strain was obtained by disruption of the *PET127* gene in the **a**u20/*rho*^{0} strain. Plasmid p(Δpet127) was constructed by ligating the *URA3* gene into *Sal*I- and *Xba*I-digested pGW694, which truncates *PET127* on the 5′ end. The *pet127::URA3* fragment was amplified by PCR with primers PET127-U2 (5′-cagggcacttgagagagcac-3′) and PET127-L5 (5′-aagcgaatggtgtgatgaaatc-3′) and transformed into the **a**u20/*rho*^{0} strain. Plasmid Yep351-SUV3ΔU (D. G. Roberts and C. L. Dieckmann, unpublished results) was cut with *Ban*I, and the 2.2-kb fragment containing a *suv3::URA3* disruption allele was used to transform **a**u20/*rho*^{0} to obtain the *suv3*/*rho*^{0} strain. Plasmid pKS(Δdss1), which carries a *dss1::URA3* disruption allele in the pKS vector, was linearized, and transformed into **a**u20/*rho*^{0} to make the isogenic *dss1*/*rho*^{0} strain. Ura$^+$ transformants were selected in each of the three transformations on medium lacking uracil. Disruption of *PET127*, *SUV3*, and *DSS1* was confirmed by Southern blot (data not shown).

Southern blot analysis and sequencing: Mitochondrial DNAs were prepared from the 10 pseudorevertants (Bonitz *et al.* 1980). Approximately 1 μg of each sample was digested with *Mbo*I. The fragments were separated on a 1% agarose gel and transferred to Nytran membrane, which was probed with a random-priming-labeled *COB* −1350 → +319 fragment (Chen and Dieckmann 1994). The *COB* sequence from −1360 to −648 from each of the 10 pseudorevertants was amplified by PCR and cloned into pBluescript KS (Stratagene, La Jolla, CA) and sequenced as previously described (Chen and Dieckmann 1997).

Primer extension analysis: Total cellular RNAs were isolated from SUF63-F and all of the pseudorevertants (Caponigro *et al.* 1993). Primer extension reactions and signal strength analyses were performed as described previously (Chen and Dieckmann 1997).

PCR, T/A cloning, and sequencing: To examine the *PET127* genes in the pseudorevertants bearing group II suppressors, chromosomal DNAs were prepared from these strains. The *PET127* genes were amplified by PCR using primers PET127-U2 (5′-cagggcacttgagagagcac-3′) and PET127-L2 (5′-cccaacgc tgactactgtct-3′). The PCR products were directly ligated to the pGEM-T Easy (Promega, Madison, WI) vector. The cloned *PET127* genes from the pseudorevertants were sequenced (UA facility), with primers PET127-U2, PET127-L2, PET127-U3 (5′-cgctacaaaaattgagagata-3′), and PET127-L3 (5′-cctcgtactcaggt gaca-3′).

RESULTS

Single-nucleotide mutations in *COB* mRNA allow recovery of spontaneous pseudorevertants: In a previous study of the *COB* 5′-UTR, a 64-nucleotide sequence was defined as sufficient for *COB* mRNA stabilization (Mittelmeier and Dieckmann 1993). The SUF63-F strain has only the −962 to −898 sequence of the leader retained in a deletion from −1098 to −707 and has wild-type levels of *COB* mRNA. This strain has been used as the wild type in the present study. Within the 64-

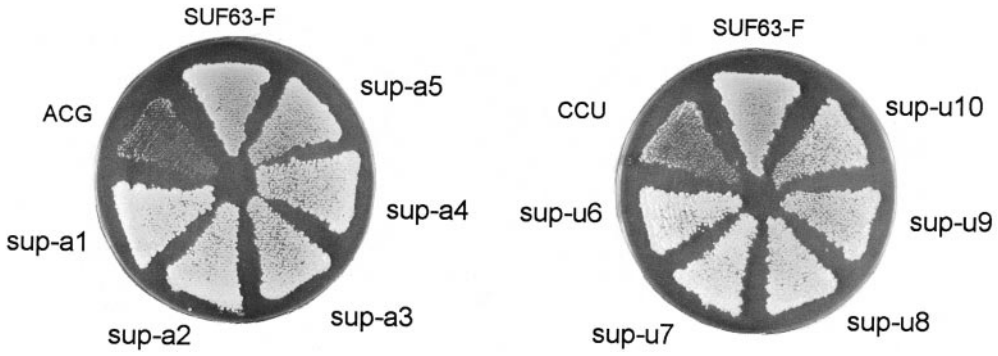

Figure 1.—Respiratory growth of the spontaneous pseudorevertants of the ACG and CCU mutant strains. All strains were streaked on YEPD plates and after overnight incubation replicated onto YEPG plates. Pictures were taken after 4 days of incubation on YEPG at 30°. SUF63-F is the wild-type control strain.

nucleotide *cis*-element, the sequence CCG located at positions −944 to −942 was shown to be particularly important, because mutation of any of the 3 nucleotides leads to degradation of *COB* messages and thus respiratory deficiency (Chen and Dieckmann 1997). The AAU mutant, in which the CCG is mutated to AAU, and the CAG mutant, with CCG mutated to CAG, disallow growth on glycerol medium at all temperatures. However, the ACG mutant, with CCG mutated to ACG, and the CCU mutant, with CCG mutated to CCU, grow slowly on medium containing a nonfermentable carbon source and give rise to faster-growing colonies (Chen and Dieckmann 1997). Ten independent strains carrying suppressors were isolated, 5 from the ACG mutant, sup-a1, sup-a2, sup-a3, sup-a4, and sup-a5, and 5 from the CCU mutant, sup-u6, sup-u7, sup-u8, sup-u9, and sup-u10 (Figure 1). All 10 suppressors were shown to be mitotically stable (see materials and methods); suppression was not lost after overnight growth in rich glucose medium, which is nonselective for respiration. This implies that the suppressors are stable and heritable genomic mutations.

To determine whether the suppressor mutations were nuclear or in the mitochondrial genome, the mitochondrial genomes of the 10 pseudorevertants were transferred into the wild-type LL20 nuclear background via cytoduction using a karyogamy-deficient strain. The resulting strains were as respiratory-deficient as the original ACG and CCU mutants (data not shown). Thus, the suppressor mutations were in the nuclear genome.

To test dominance, the pseudorevertants (*MAT*α *ura3 his3*) were outcrossed to the **a**LL20/*rho*^{0} strain and respiration of the diploids was examined. As shown in Figure 2, only the sup-a2/+[*rho*$^{+ACG}$] diploids showed respiratory competence similar to that of the haploid, which implies that sup-a2 contains a dominant suppressor. One might have expected that the +/+[*rho*$^{+ACG}$] and +/+[*rho*$^{+CCU}$] diploids would grow similarly to the respective +[*rho*$^{+ACG}$] and +[*rho*$^{+CCU}$] haploid strains. However, the diploids grew more slowly than the haploids on glycerol (*i.e.*, +/+[*rho*$^{+ACG}$] < +[*rho*$^{+ACG}$] and +/+[*rho*$^{+CCU}$] < +[*rho*$^{+CCU}$]). Upon combination of any one of the other nine suppressors with wild-type (*sup*/+), a level of growth between that of the respiratory-incompetent +/+ diploids and diploid suppressor strains *sup*/*sup* was observed. Thus, except for sup-a2, all of the other nine suppressors are semidominant (+/+ <*sup*/+ <*sup*/*sup*).

To examine whether each of the pseudorevertants contained single nuclear mutations, the diploids were sporulated and 20–40 tetrads were dissected for each diploid. The tetrads were grown on YEPD and replica-plated onto YEPG medium. Eight of the diploid strains yielded tetrads that were 2:2 for respiration, demonstrating that these eight pseudorevertants contain single nuclear mutations. The majority of the sup-u9 diploids that were dissected showed 2:2 segregation; however, one diploid yielded two unlinked suppressors. The second suppressor was isolated from a nonparental ditype (NPD) tetrad of this sup-u9 diploid, and was named sup-u11. sup-u11 showed very similar features to the other five CCU pseudorevertants, containing a single nuclear semidominant mutation (Figure 2). Because the diploids do not respire well, we believe that the second suppressor arose during the selective growth in the acetate-containing sporulation medium, which requires the ability to respire.

Eleven suppressors define six linkage groups: Because the mutations behaved in a dominant or semidominant fashion, it was not possible to use complementation analysis to group the suppressors. Thus, linkage analysis was required for determining groupings. To be able to cross the pseudorevertants to each other, the original *MAT*α strains were converted to isogenic *MAT***a** strains by mating-type switching. To facilitate the selection of diploids on minimal medium, the *URA3* locus of each of the *MAT*α strains was disrupted by insertion of *LEU2* (see materials and methods). Diploids of *MAT*α *ura3 his3 sup1* × *MAT***a** *leu2 his3 sup2* were selected on WO + histidine medium. The diploids from all crosses were respiratory competent, which is consistent with our observation that the suppressors are semidominant. The diploids were sporulated and tetrads were dissected. After scoring the tetrads for respiration from all crosses, the 11 suppressors were categorized into six linkage groups (Table 2). The dominant sup-a2 suppressor was shown to be a *cbp1* mutation and has been described in detail previously (Chen and Dieck-

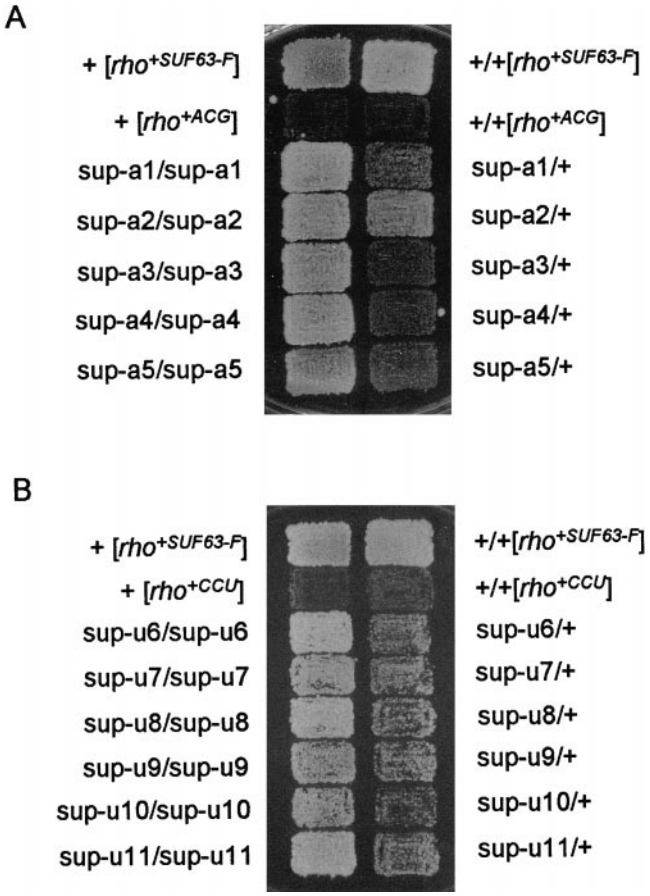

Figure 2.—Dominance test of the suppressors. The 11 suppressor strains, wild-type control strain SUF63-F, and the ACG and CCU mutant strains derived from SUF63-F were crossed to **a**LL20/*rho*^{0}. All haploid and diploid strains were streaked on YEPD plates, and then replicated onto YEPG plates after overnight incubation. Pictures were taken after 6 days of incubation at 30° on YEPG. All strains are named by nuclear genome, followed by mitochondrial genome. (A) The mitochondrial genomes are [*rho*$^{+ACG}$] if not specified. The plate was incubated at 28° instead of 30° to better show the growth difference between +/+[*rho*$^{+ACG}$] diploids and sup/+[*rho*$^{+ACG}$] diploids. (B) The mitochondrial genomes are [*rho*$^{+CCU}$] if not specified.

mann 1997). None of the other 10 suppressors was linked to *CBP1*, and they fell into five different linkage groups.

Suppressors show some allele specificity and have no phenotype of their own: One possibility for gain-of-function suppressors is that they may have a phenotype of their own when separated from the original mutation (Adams and Botstein 1989). The semidominant suppressors do not have a respiratory-deficient phenotype of their own when combined with a wild-type mitochondrial genome (Table 3). This result is consistent with the hypothesis that these suppressors are loss-of-function mutations.

Some dominant reciprocal suppressors are also allele specific. To examine whether the suppressors of [*rho*$^{+ACG}$] can suppress [*rho*$^{+CAG}$] and [*rho*$^{+CCU}$] and whether the suppressors of [*rho*$^{+CCU}$] can suppress [*rho*$^{+ACG}$] and [*rho*$^{+CAG}$], different "sup" nuclear backgrounds were combined with different mitochondrial mutant genomes by cytoduction. The respiratory growth at 30° of all new strain combinations is summarized in Table 3. All of the [*rho*$^{+ACG}$] suppressors can suppress [*rho*$^{+CCU}$]; all of the [*rho*$^{+CCU}$] suppressors can suppress [*rho*$^{+ACG}$], though most of the suppressors are unable to suppress [*rho*$^{+CAG}$]. Thus, the suppression of *COB* 5′-UTR mutations by the suppressors isolated here is partially but not completely allele-specific. This result is also consistent with these mutations being loss-of-function alleles.

Overexpression of *CBP1* suppresses the ACG and CCU mutations but not the CAG mutation: Previous genetic evidence showed that Cbp1 interacts with *COB* mRNA (Chen and Dieckmann 1997). We wondered whether the interaction with mutated *COB* mRNA could also be suppressed by increasing the level of wild-type Cbp1 protein *in vivo*. The pG60/T31 plasmid, which contains the *CBP1* gene in the 2μ YEp13 backbone, was transformed into the [*rho*$^{+ACG}$] (ACG), [*rho*$^{+CAG}$] (CAG), and [*rho*$^{+CCU}$] (CCU) strains. Transformation of YEp13 alone into these strains was used as a control. Figure 3 shows growth of the transformants on glycerol medium. Approximately 10-fold overexpression of *CBP1* suppressed the respiratory deficiency of the [*rho*$^{+ACG}$] and [*rho*$^{+CCU}$] mutants, but not the [*rho*$^{+CAG}$] mutant. Consistent with our previous findings, this is another piece of evidence that the Cbp1 protein interacts with *COB* mRNA. Presumably the binding affinity between Cbp1 and *COB* 5′-UTR is lowered because of the mutations

TABLE 2

Linkage groups of suppressors of *COB* 5′-UTR mutations

Linkage group	Suppressor strains	Locus	Dominance
I	sup-a2	*CBP1*	Dominant
II	sup-a1, sup-u6, sup-u7, and sup-u8	*PET127*	Semidominant
III	sup-a3, sup-a4, and sup-u9	?	Semidominant
IV	sup-a5	*SOC1* ?	Semidominant
V	sup-u10	?	Semidominant
VI	sup-u11	*DSS-1* ?	Semidominant

TABLE 3

Allele specificity as shown by respiratory growth of different combinations of nuclear (sup) and mitochondrial (*rho*) mutations

	Nuclear										
Mitochondrial	LL20	sup-a1	sup-a2	sup-a3	sup-a4	sup-a5	sup-u6	sup-u7	sup-u8	sup-u9	sup-u10
rho^{+}	++	++	++	++	++	++	++	++	++	+	++
rho^{+ACG}	−*	++	++	++	++	++	++	++	++	+	+
rho^{+CAG}	−	+*	−	−	−	−	−*	−*	−*	−	−*
rho^{+CCU}	+*	++	++	++	++	++	++	++	++	++	++

$^{++}$, growth observed after 2–4 days of incubation; +, growth observed after 5–8 days of incubation; −, no growth observed after 10 days of incubation; *, faster-growing colonies observed; −*, growth like "+" could be observed at 25°.

in CCG, but the affinity is strong enough that binding can be restored by increasing the level of Cbp1 severalfold in the cell.

RNA analysis reveals processing defects in some pseudorevertant strains: To begin to analyze the function of the suppressors we examined *COB* mRNA in these strains. Mitochondrial function was induced by growing the pseudorevertants in YEPG media, and total cellular RNAs were prepared. To compare *COB* RNA processing in the pseudorevertants (all lanes labeled sup) with that in the wild-type strain (lanes labeled SUF63-F), primer extension reactions were used to examine the ratio of *COB* pre-RNA to mature message (Figure 4A). Strains containing group II suppressors (sup-a1, sup-u6, sup-u7, sup-u8) showed a striking RNA phenotype; *COB* precursor accumulated to a level 4- to 5-fold higher than that in the wild-type strain. However, no mature *COB* message was detected. This implies that *COB* pre-RNA can be translated because the strains respire (discussed below). Strains with suppressors in the other linkage groups accumulated both *COB* precursor and mature message, however, at levels 2- to 5-fold lower (precursor) and 7- to 33-fold lower (mature) than those in wild type (Table 4). Figure 4B shows the primer extension assay

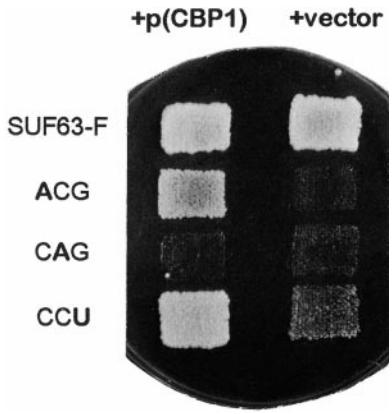

Figure 3.—Suppression of the ACG, CAG, and CCU mutations by overexpression of *CBP1*. Plasmid p60/T31, which contains *CBP1* in the 2μ vector YEp13 [lane "p(CBP1)"] and an empty YEp13 (lane "vector"), was transformed into control strain SUF63-F, and the mutants ACG, CAG, and CCU. The picture was taken after 6 days of incubation at 30° on YEPG.

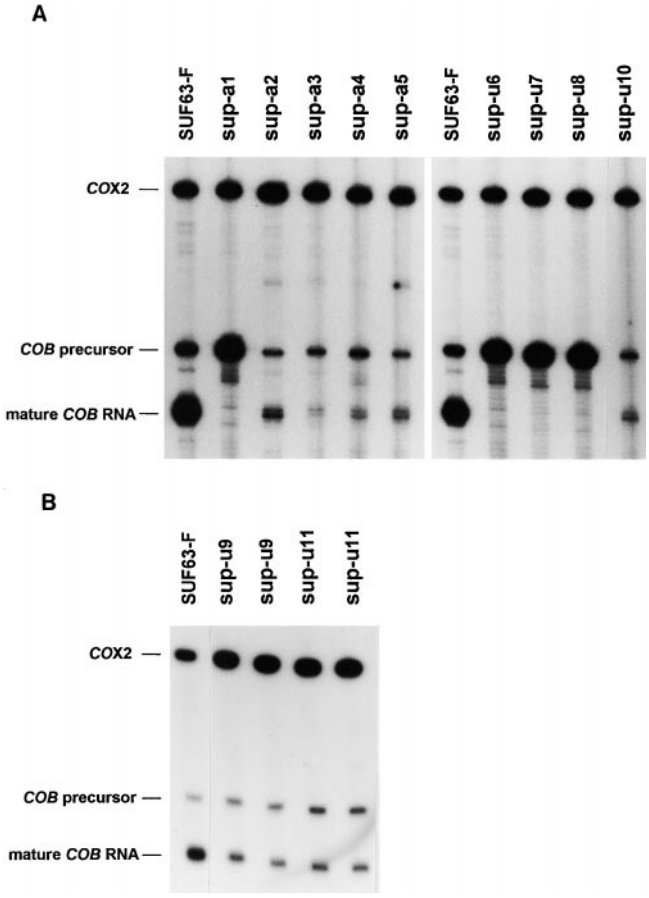

Figure 4.—Accumulation of *COB* pre-RNAs and mature messages in the suppressor strains analyzed by primer extension assay. Total cellular RNAs were prepared from each strain. A total of 10 pmol of COB6B and COX4242 primers was end-labeled and hybridized in a saturation ratio to 8 μg of RNA. cDNAs were synthesized by reverse transcriptase and separated on a sequencing gel. Signals from *COX2* mRNA were used for normalization. (A) Strains sup-a1, -a2, -a3, -a4, -a5, and sup-u6, -u7, -u8, -u10. (B) Strains sup-u9 and sup-u11 shown are segregants from the NPD tetrad.

TABLE 4

Levels of *COB* RNAs in the suppressor strains analyzed by primer extension assay

Strain	*COB* precursor	Mature *COB* RNA
SUF63-F	100[a]	100
sup-a1	405 ± 30	–[b]
su-a2	20 ± 5	8 ± 3
sup-a3	30 ± 5	4 ± 1
sup-a4	48 ± 5	7 ± 2
sup-a5	31 ± 3	8 ± 1
sup-u6	499 ± 30	–
sup-u7	444 ± 50	–
sup-u8	521 ± 70	–
sup-u9	38 ± 10	13 ± 3
sup-u10	42 ± 3	8 ± 1
sup-u11	69 ± 11	14 ± 1

Numbers are obtained from the averages and standard deviations of five gels.

[a] *COB* precursor is ~25–30% of the mature *COB* RNA in the SUF63-F strain.

[b] Values are as low as the background level, which is ~3% of the RNA level in the wild-type strain.

for the four spores from an NPD tetrad of sup-u9 (group III) and sup-u11 (group VI).

Survey of RNA turnover genes: Because the phenotype of the group II suppressor strains was indicative of a loss-of-function in 5′ trimming of *COB* pre-mRNA, we decided to survey genes encoding known components of mitochondrial mRNA processing and turnover machinery as likely candidates mutated in our pseudorevertants. *PET127* was a good candidate because it was shown to have a role in RNA 5′ trimming and turnover of a broad range of mitochondrial transcripts including *COB* (Wiesenberger and Fox 1997). *pet127* mutants accumulate *COB* pre-RNA and have no mature message, similar to the group II suppressor strains in this study. We also tested to see whether our suppressors are in *SUV3* or *DSS1*. Along with a third, 75-kD protein, Suv3 and Dss1 constitute a mitochondrial 3′ → 5′ exonuclease (mtEXO) activity (Dmochowska *et al.* 1995; Margossian *et al.* 1996). Another good candidate is encoded by the nuclear *SOC1* gene. *soc1* mutations suppress $cbp1^{ts}$ mutations and cause accumulation of precursor and mature *COB* mRNA in levels similar to those of groups III–VI suppressors. *soc1* mutations increase RNA accumulation in mitochondria generally (Staples and Dieckmann 1994). Therefore, all the candidates described above play a role in general RNA turnover pathways in yeast mitochondria.

Group II suppressors are *pet127* mutations: *PET127* was originally identified by a suppressor of a mutation in the mitochondrial translational activator, Pet122, which is required for translation initiation of *COX3* (Haffter and Fox 1992). Recently it was found that Pet127 is required for proper 5′ trimming and turnover of mitochondrial RNAs (Wiesenberger and Fox 1997). We found that our group II suppressor strains had a phenotype similar to that of the *pet127*/*cox3* double mutants in that study. To examine the possibility that group II suppressors were in the *PET127* locus, plasmid pGW694, which carries a wild-type copy of *PET127* on the Yplac33 backbone, was transformed into all 11 suppressor strains containing the *URA3::LEU2* disruption. Transformants of linkage group II strains were as respiratory-deficient as the original [rho^{+ACG}] and [rho^{+CCU}] mutants, whereas transformants of the other groups maintained respiratory competence.

To test whether a mutant *pet127* nuclear background would suppress mutations in CCG, [rho^{+ACG}], [rho^{+CAG}], and [rho^{+CCU}] mitochondrial genomes were combined with a *pet127* nuclear genome via cytoduction. Respiratory growth of recombined strains *pet127*/rho^{+ACG}, *pet127*/rho^{+CAG}, and *pet127*/rho^{+CCU} are shown in Figure 5A. The *pet127* mutation did suppress the respiratory deficiency of the ACG and CCU mutants and allowed very slow growth of the CAG mutant at 30° (observed after 7 days of incubation).

To examine whether group II suppressors are indeed linked to *PET127*, they were crossed to an isogenic *pet127*/rho^0 strain. The diploids were sporulated, and 20 tetrads were scored from each cross. All tetrads segregated $4^+:0^-$ for respiration, implying that group II suppressors are tightly linked to *PET127*.

To examine the molecular nature of the *pet127* genes in group II suppressor strains, the genes were amplified by PCR, cloned, and sequenced. Compared with the wild-type *PET127* gene in the LL20 strain, all four group II suppressor strains were found to contain mutations in *PET127*. sup-a1 contained a single nucleotide deletion at position +585 (with A of start codon AUG defined +1), which results in a premature termination at codon 310 of the wild-type protein. sup-u6 contained a single nucleotide mutation of G → T at position +1597, which changes codon 533 from Glu (GAA) to a stop codon (UAA). sup-u7 contained a single nucleotide deletion at position +997, which results in a peptide truncated at codon 345. Finally, sup-u8 contained three mutations; the most upstream one was a G → T mutation at +391, which changes codon 131 from Glu to a stop codon. These data show that all group II suppressor strains contain either nonsense mutations or frameshift mutations in *PET127*, which are likely knockouts of gene function.

The suppressors are not *suv3* mutations: *SUV3* was originally identified by a nuclear suppressor that affects a variety of post-transcriptional processes in yeast mitochondria (Zhu *et al.* 1989) and was recently identified as encoding a helicase component of the mitochondrial 3′ → 5′ exonuclease (mtEXO; Margossian *et al.* 1996). *suv3* mutations lead to pleiotropic mitochondrial RNA defects including accumulation of excised group I in-

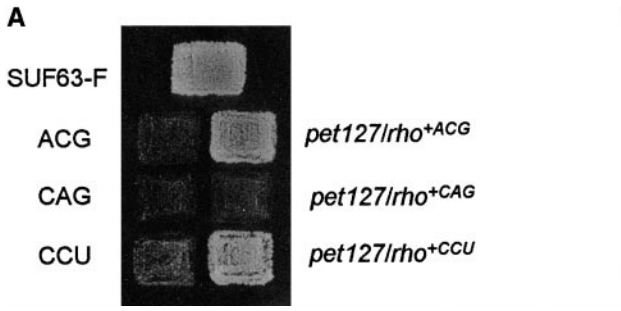

Figure 5.—Suppression of the ACG, CAG, and CCU mutations in *COB* by *pet127* (A) and *soc1* (B) nuclear mutations. Pictures were taken after 4 days of incubation at 30° on YEPG. (A) Respiratory growth of the strains *pet127*/*rho*$^{+ACG}$, *pet127*/*rho*$^{+CAG}$, and *pet127*/*rho*$^{+CCU}$. The *pet127*/*rho*$^{+CAG}$ strain started to show light growth after 7 days (data not shown). (B) Respiratory growth of the strains *soc1*/*rho*$^{+ACG}$, *soc1*/*rho*$^{+CAG}$, and *soc1*/*rho*$^{+CCU}$. Growth of *soc1*/*rho*$^{+CAG}$ could not be observed after 10 days of incubation (data not shown).

trons, unspliced aI5β- or bI3-containing precursors, and reduced *COB* and *COX1* mRNA levels (Conrad-Webb *et al.* 1990). We did observe intron bI4- and bI5-containing processing intermediates in some of the group II and group III *cob* suppressor strains (Northern blot not shown), consistent with the observations of Conrad-Webb *et al.* (1990). To study whether any of the suppressors might be in the *SUV3* locus, plasmid YEp24(T1), which contains a wild-type *SUV3* fragment in the YEp24 backbone, was transformed into all 11 pseudorevertants. Transformation did not cause any difference in respiratory growth of the strains, which implies that none of the suppressors is in the *SUV3* locus. To confirm the transformation data, a *suv3rho*^{0} strain was mated to all of the suppressor strains. The diploids respired only as well as the ACG and CCU mutant strains when mated to wild-type *rho*^{0}. Even though the diploids respired very weakly, 27–32 tetrads were dissected in crosses to representatives of groups III, IV, and V. No linkage was observed to the *SUV3* locus.

The sup-u11 suppressor is tightly linked to *DSS1*: *DSS1* was identified as a multicopy suppressor of a *suv3* disruption strain that cannot stably maintain mitochondrial genomes (Dmochowska *et al.* 1995). The Dss1 protein encodes another of the three protein components of the mtEXO complex and has sequence homology to *E. coli* 3′ → 5′ exonuclease RNaseII. Plasmid pAD15, which carries a wild-type copy of the *DSS1* gene on the YEp434 backbone, was transformed into all 11 suppressor strains. Introducing a wild-type copy of the *DSS1* gene partially affected respiratory growth of group III and group VI suppressors, but did not affect suppressors in the other groups. Crosses of group III suppressors to the isogenic *dss1*/*rho*^{0} strain did not yield respiratory-competent diploids, which implies that group III suppressors are not *dss1* mutations. However, the cross of sup-u11 to *dss1*/*rho*^{0} yielded respiratory-competent diploids. All 40 tetrads from this cross showed 2:2 segregation for respiration. Because knockout mutations in *DSS1* lead to loss of mitochondrial DNA (Dmochowska *et al.* 1995), the results of the tetrad analysis indicate that sup-u11 is tightly linked to *DSS1*.

Group IV suppressor is a *soc1* mutation: *soc1* mutations were identified previously as nuclear suppressors of *cbp1*^{ts} mutations (Staples and Dieckmann 1994). *soc1* behaves as a true recessive suppressor of the *cbp1*^{ts} mutations. *cbp1*^{ts} *soc1* mutants showed accumulation of both *COB* precursor and mature message, though both in reduced levels compared with those in the wild-type strain. To examine whether any of the suppressors in the present study were *soc1*, the 10 semidominant suppressor strains were crossed with **a**RSY29/*rho*^{0}, which is a *soc1* mutant strain containing no mitochondrial DNA. All diploids showed the codominance phenotype associated with crosses of the suppressor strains collected in this study to each other. The diploids were sporulated and tetrads dissected from representatives of groups III, IV, and V. A total of 28 tetrads segregated 4:0 respiratory-competent to respiratory-deficient progeny, indicating that sup-a5 is tightly linked to *SOC1*.

To examine whether a *soc1* allele originally isolated as a suppressor of a *ts cbp1* mutation can suppress CCG *cob* mutations, *soc1*/*rho*$^{+ACG}$, *soc1*/*rho*$^{+CAG}$, and *soc1*/*rho*$^{+CCU}$ strains were made via cytoduction. Respiratory growth of these strains is shown in Figure 5B. *soc1* can indeed suppress the ACG and CCU mutations in *COB* mRNA, but not the CAG mutation. This finding strengthens the idea that *SOC1* plays a role in mitochondrial RNA decay.

DISCUSSION

Yeast mitochondrial RNAs are different from yeast cytoplasmic RNAs. They do not have the m^{7}Gppp cap structure at the 5′ ends, and polyadenylation of 3′ ends has not been reported. They do have long AU-rich 5′ and 3′ UTRs and a common motif at the 3′ end, a AAUAA(U/C)AUUCUU dodecamer sequence (Osinga *et al.* 1984). Little is known about the turnover pathways for mitochondrial RNAs in yeast. A general 3′ → 5′ exonucleolytic decay pathway is suggested by the presence of both a degradative enzyme and a common protection mechanism against its action. Three polypeptide units comprise the 3′ → 5′ exonuclease activity, two of which are encoded by *SUV3* and *DSS1* (for a review, see Margossian and Butow 1996). A three-polypeptide

protein complex binds to the 3′ dodecamer sequence of each of the mitochondrial mRNAs and protects them from 3′ → 5′ degradation (Min and Zassenhaus 1993). In addition to the 3′ → 5′ pathway, there are emerging hints that a 5′ end-dependent pathway may also be important for mRNA maturation and decay. In a recent study by Wiesenberger and Fox (1997), and in our current study, *pet127* nulls were recovered as suppressors of both *cox3* and *cob* 5′-UTR mutations that render the individual mRNAs unstable. A careful analysis showed that a *pet127* knockout affects the stability and 5′ end processing of many mitochondrial mRNAs and 15S rRNA (Wiesenberger and Fox 1997). Whether 3′ → 5′ or 5′ end-dependent degradation is the major pathway of mitochondrial mRNA turnover has not been determined. Such an analysis will require pulse-chase experiments and analysis of decay intermediates.

COB mRNA clearly requires a specific stabilization system at the 5′ end in addition to the general 3′ end one. The nuclear-encoded protein Cbp1 is required uniquely for *COB* mRNA accumulation by protecting the 5′ end. By deletion assay and site-directed mutagenesis (Mittelmeier and Dieckmann 1993; Chen and Dieckmann 1997), we located a *cis*-element in the 5′-UTR of *COB* that is important for Cbp1-dependent mRNA accumulation. All of our data support the hypothesis that Cbp1 recognizes and interacts with a CCG-containing element. Mutation of any of these three nucleotides leads to degradation of *COB* mRNA and respiratory-deficient cell growth. Nevertheless, two different single-nucleotide mutations in CCG allow suppressors to arise spontaneously.

To identify compensatory mutations in Cbp1 or other proteins that specifically interact with mutated *COB* 5′-UTR, and also to begin to identify components of the general 5′ end-dependent degradation pathway, we used a genetic analysis of the spontaneous suppressors to identify factors that affect mRNA stability as an alternative to biochemical approaches, *e.g.*, affinity chromatography and gel retardation assays, which have been used widely and successfully to identify RNA-binding proteins and enzymes (Lee *et al.* 1983; Sun and Antony 1996). Because none of the ACG and CCU suppressors are rearrangements of mitochondrial DNA, and all are nuclear mutations, genetic analysis of these suppressors had the potential to reveal protein-RNA interactions, protein-protein interactions, and novel proteins.

As it has been difficult to obtain purified Cbp1 protein, we have not been able to show direct binding of this protein with *COB* mRNA yet. Discovery of a dominant suppressor of a *cob* mutation in *CBP1* in the present study provided the first evidence that Cbp1 interacts directly with *COB* mRNA (Chen and Dieckmann 1997). That a 5- to 10-fold overexpression of *CBP1* suppresses two of the CCG mutations strengthens the idea that Cbp1 physically interacts with *COB* mRNA. Our model is that the conditional ACG and CCU mutations cause a decrease in the binding efficiency between Cbp1 and *COB* mRNA, and that overexpression or mutation of Cbp1 overcomes the reduction in affinity. This suppressor system is similar to suppression of mutations in actin, *ACT1*, by mutations in the actin-binding protein Sac6 (Adams and Botstein 1989).

Interestingly, this suppressor analysis revealed not only an allele of *CBP1*, a specific protector of *COB* mRNA, but also loci that affect mitochondrial mRNAs in general. Suppressor sup-u11 was found to be tightly linked to *DSS1*, which encodes a subunit of the 3′ → 5′ exonuclease. Studies are being continued to identify the *dss1* mutation in the sup-u11 strain. Because knockout mutations in *DSS1* cause loss of the mitochondrial genome, the sup-u11 mutation is likely a missense mutation rather than a nonsense or frameshifting mutation. The linkage between sup-u11 and *DSS1* strongly implies that the 3′ → 5′ exonuclease is important in mitochondrial mRNA decay. It is curious that impairment of the 3′ → 5′ exonuclease is implicated in suppression of a 5′-UTR mutation. Is there direct communication between the 5′- and 3′-UTRs, or is dysfunction of any major turnover enzyme able to raise the concentration of the mutant mRNA?

It was interesting to have isolated *pet127* mutations as suppressors of *COB* mRNA mutations in this study. Only recently was it discovered that a *pet127* knockout mutation has a pleiotropic effect on the processing and turnover of several mitochondrial RNAs (Wiesenberger and Fox 1997). It was proposed that Pet127 plays a surveillance role in RNA metabolism (*i.e.*, to remove unnecessary or incorrectly processed RNAs) or acts as a general RNA degradation factor. Our findings in this study favor the model that Pet127 is involved in a general 5′ end-dependent RNA decay pathway in yeast mitochondria. The RNA phenotype of the *pet127* suppressor strains revealed another unexpected phenomenon; they contain unprocessed pre-*COB* RNA only, but they respire as well as wild type. Thus, it is likely that the *COB* precursor is being translated in these strains. Provided with *COB* pre-RNA at a level as high as that of the mature *COB* message in a wild-type strain, the group II suppressor strains generate enough cytochrome *b* protein to be able to respire. In *cbp1* mutants, which also do not contain any mature *COB* RNA, the *COB* precursor content is 20-fold lower than in the *pet127* strains and is not enough to support respiration. Such levels of mature mRNA support respiratory growth. Either the precursor is translated less efficiently than the mature message, or Cbp1 is also required for translation.

A third previously characterized component important for mitochondrial turnover, Soc1, was also identified in the current study. *soc1* mutations allow accumulation of higher than normal levels of many mitochondrial mRNAs and stabilize *COB* mRNA manyfold in a strain with a *ts cbp1* allele (Staples and Dieckmann 1994). The *COB* transcript pattern of groups I and III–VI suppressor

strains is the same as the transcript pattern for *soc1* suppression of *cbp1*^{ts} at the restrictive temperature (Staples and Dieckmann 1994). Both *COB* pre-RNA and mature message accumulate to levels lower than those in the wild-type strain. The suppressors do not greatly increase the steady-state level of *COB* precursor, but permit the return of a low level of mature *COB* mRNA. Identification of the two unknown genes defined by the groups III and V suppressors will likely reveal key components of the mitochondrial RNA turnover machinery.

An interesting point in this study is that all of the suppressors described are semidominant (Figure 2). A prominent consequence of semidominance is that the suppressor phenotypes are additive in diploids; *i.e.*, *sup1*+/+*sup2* respires better than either *sup1*/+ or +/*sup2*. We propose two models for the semidominance feature. In model A, the Sup protein has multiple functions. In a *sup* strain, one of the functions is lost, but the other is not; *e.g.*, the Sup protein encoded by *sup* in a *sup*/+ diploid has lost endo-/exonuclease activity, but it still retains mRNA-binding activity, which allows competition with or interruption of the binding and activity of wild-type Sup protein. In model B, the dosage of the Sup protein in the cell is a limiting factor. Because there is twice as much wild-type Sup protein in +/+ cells as in *sup*/+ cells, +/+ mRNAs degrade faster than *sup*/+ mRNAs, and *sup*/+ mRNAs degrade faster than *sup*/*sup* mRNAS. Model B explains why *sup*/+ diploids grow more like + haploids, rather than +/+ diploids, and explains the additive feature shown by the double suppressor mutants. Both models may be represented in the collection of the 10 semidominant suppressors. Because Δ*pet127* is a null and acts as a suppressor, and the other *pet127* suppressors are likely to be nulls, the mechanism of suppression for the group II mutations is most likely described by model B.

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