In most strains of *Saccharomyces cerevisiae* the mitochondrial gene *COX1*, for subunit 1 of cytochrome oxidase, contains multiple exons and introns. Processing of *COX1* primary transcript requires accessory proteins factors, some of which are encoded by nuclear genes and others by reading frames residing in some of the introns of the *COX1* and *COB* genes. Here we show that the low molecular weight protein product of open reading frame YLR204W, for which we propose the name *COX24*, is also involved in processing of *COX1* RNA intermediates. The growth defect of *cox24* mutants is partially rescued in strains harboring mitochondrial DNA lacking introns. Northern blot analyses of mitochondrial transcripts indicate that *COX1* mRNA (6), but was subsequently shown to also play a role in *aI5* excision (30). *Mrs1p* is required for processing of introns *bI3* of *COB*, and *aI5* and *bI5* of *COX1* (31). At present the only proteins known to function in processing of a single intron are *Mss116p* (3) and *Cpb2p* (23), which target *aI5* and *bI5*, respectively.

In the course of analyzing the biochemical defects of the respiratory deficient *pet* mutant of *S. cerevisiae*, we identified a new gene, which when mutated causes the accumulation of *COX1* intermediates transcripts. This gene has been designated *COX24* in keeping with our previous convention for naming genes involved in expression of cytochrome oxidase. *COX24* corresponds to reading frame YLR204W and was previously named *QRIS* (32). The phenotype of *cox24* mutants and characterization of their mitochondrial RNAs lead us to propose that *COX24* plays an important role in processing of the *COX1* primary transcript, but like the other aforementioned processing factors, may have another function as well.

**MATERIALS AND METHODS**

**Strains and Media**—The strains of yeast used in this study are listed in Table 1. The respiratory deficient mutants of complementation group G82 were derived from *S. cerevisiae* D273-10B/A1 by mutagenesis with nitrosoguanidine or ethyl methanesulfonate (1). The following

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**Cytochrome c oxidase (COX)**

Biogenesis is a complex process that requires the expression and interaction of subunits encoded by mitochondrial and nuclear genes. In *Saccharomyces cerevisiae* at least 20 nuclear gene products (1, 2) have been shown to assist COX assembly. These proteins promote steps, ranging from processing of mitochondrial COX-specific RNAs (3, 4) and their translation (5, 6) to recruitment, formation, and addition of the metal and heme prosthetic groups present in the catalytic subunits of the complex (7–9).

*COX1p* (subunit 1) of COX is an important constituent containing the cytochrome *a* and *a*$_3$ centers. This subunit is encoded by the mitochondrial *COX1* gene, which is transcribed as a polycistronic precursor RNA containing *COX1*, *ATP8*, *ATP6*, and *ENS2* (10). Most commonly used laboratory strains have a *COX1* gene with variable but multiple introns (11). The *aI3*, *aI4*, *aI5*, and *bI5* introns of *COX1* are group I introns, whereas *aI1*, *aI2*, and *aI5*$_y$ belong to group II introns. Both types of introns have the ability to act as mobile elements with the difference that homing of group II introns depends on an RNA intermediate and homing of group I intron is a DNA-based process (12, 13). The mobility of group I introns is enhanced by endonucleases encoded in the introns themselves. *aI3* encodes the I-ScelII endonuclease that cleaves the junction of the two flanking exons, needed for its homing but in addition appears to exert a positive effect in removal of the intron (14, 15).

Splicing of the *COX1* primary transcript is assisted by protein factors referred to as maturases that are encoded by reading frames located within some of the introns of the *COX1* and *COB* genes (16–20). Excision of some intervening sequences also depends on nuclear genes such as *CBP2*, and *SLIV3* and *MSS116*, the latter two coding for RNA helicases (3, 21, 22). *Cbp2p* interacts with and stabilizes a splicing competent secondary structure of the cytochrome *b* pre-mRNA (23). *Suv3p* has been implicated in stabilizing the *COX1* transcript by regulating turnover of group I intronic RNAs (24, 25), whereas *Mss116p* has been shown to function in splicing of all mtDNA introns in *S. cerevisiae* and *Neurospora crassa* (26).

Some of the accessory factors appear to have several functions in mitochondrial RNA metabolism. *PET309*, first isolated as a *COX1* translation facilitator, also affects the stability of intron-containing *COX1* RNA (4) and was recently found to be associated with *CBP1*, a cytochrome *b* RNA stabilization and translation factor (27). *NAM2/MSL1*, the mitochondrial leucyl-tRNA synthetase, is required for excision of the *aI4* and *bI4* introns of *COB* (28, 29). Excision of the *aI5* intron of *COX1* depends on several nuclear gene products. *Mss116p* (3) and *Cpb2p* (23), which target *aI5* and *bI5*, respectively.

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**References**

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4. The abbreviations used are: COX, cytochrome *c* oxidase; *pet* mutant, respiratory deficient mutant of yeast with a mutation in a nuclear gene; *pet*- mutant, respiratory deficient mutant with either large deletions in or lacking mitochondrial DNA; mtDNA, mitochondrial DNA; HA, hemagglutinin.
| Strain     | Genotype                                      | Source       |
|------------|-----------------------------------------------|--------------|
| W303-1A    | MATa ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 | This study   |
| W303-1B    | MATa ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 | This study   |
| D73-108/A21| MATa ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 | This study   |
| W303-1B    | MATa ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 | Ref. 33      |
| IC145      | MATa ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 | Ref. 34      |
| W303-1A    | MATa ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 | ATCC 20575   |
| W303-1B    | MATa ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 | ATCC 20575   |

Note: The table continues with similar entries for other genotypes and sources of yeast strains, including genotypes such as COX24, COX14, and sources like JC3/A21 and JC11/W104.
media were used routinely to grow yeast: YPD (2% glucose, 1% yeast extract, 2% peptone), YPGal (2% galactose, 1% yeast extract, 2% peptone), and YE PG (2% ethanol, 3% glycerol, 1% yeast extract, 2% peptone).

**Cloning of COX24—** pG82/T1, a recombinant plasmid containing COX24, was isolated by transformation of C149/UL1 with a yeast genomic library consisting of partial Sau3A fragments of yeast nuclear DNA cloned in the yeast/Escherichia coli shuttle plasmid YEp13 (37). Approximately 5 × 10⁸ cells were transformed with 50 μg of the plasmid library by the method of Beggs (38).

**Disruption of COX24—** A BamHI-BglII fragment of pG82/T1 containing COX24 was transferred to pUC19. The resultant plasmid (pG82/ST10) was used to replace the COX24 coding sequence with the yeast HIS3 gene. Amplification of pG82/ST10 with bi-directional primers 5′-GGCGATCTGGGCTGTAAAACCTTCCAC and 5′-GGCA-GATCTTATTTATCTCGTCTGTGTC resulted in a clean deletion of COX24. The linear product containing COX24 5′- and 3′-flanking regions in pUC19 was digested with BglII and ligated to HIS3 on a 1-kb BamHI fragment. The cox24::HIS3 null allele was recovered from this plasmid as a BamHI-XbaI fragment and was substituted by homologous recombination (39) for the wild-type gene in respiratory competent strains W303-1B and W303-1A.

**Construction of a Hybrid Gene Expressing Cox24p Tagged with a Hemagglutinin Epitope at the Carboxy terminus—** pG82/T1 was used as a PCR template for amplification of the COX24-HA gene coding for Cox24p with 12 carboxy-terminal residues consisting of three glycine residues as spacers and the hemagglutinin tag (HA). The PCR primers used for amplification were 5′-GGCGGATCT-GTCCACCTTGCGAATATCTAC and 5′-GGCAAGCTTTCAGGGTGATCTGGGAGGGTTGTTCAGTTATGCGGCTCTCTGGATAGATTCTTCT. The PCR product was digested with a combination of BamHI and HindIII, and was cloned in the yeast/E. coli shuttle plasmids YEp352 and YIp352 (40) yielding pG82/ST16 and pG82/ST17, respectively. W303ΔCOX24 was transformed either with uncut pG82/ST16 or with pG82/ST17 linearized at the Ncol site of the URA3 marker for integration at the homologous site in chromosomal DNA (39).

**Miscellaneous Procedures—** Standard methods were used for plasmid manipulations (41). The COX24 gene and flanking regions were sequenced by the method of Maxam and Gilbert (42). Yeast mitochondria were prepared by the method of Faye et al. (43) except that Zymolyase 20T (ICN Laboratories, Aurora, OH) instead of glusulase was used to obtain spheroplasts. Spectral analyses of mitochondrial cytochromes were performed as described previously (44). Protein concentrations were determined by the method of Lowry et al. (45).

**RESULTS**

**Cloning of COX24—** C149 is one of three independent respiratory deficient strains assigned to complementation group G82 of a pet mutant collection (1). Transformation of the derivative strain C149/L1 with a yeast genomic library yielded a leucine-independent and respiratorily competent transformant (C149/L1/T1) that was used to isolate the recombinant plasmid pG82/T1. Complementation tests with subclones containing different regions of the nuclear DNA insert in this plasmid revealed that the gene responsible for restoring respiration in C149/L1 corresponds to reading frame YLR204W on chromosome XII (data not shown). The mutant W303ΔCOX24 harboring a null allele of the gene, henceforth referred to as COX24, was obtained by homologous recombination with a linear fragment of nuclear DNA in which the entire reading frame was replaced with HIS3. The identity of COX24 as the gene responsible for the respiratory defect of C149/L1 was confirmed by the lack of complementation of the point mutant by the null mutant W303ΔCOX24 and the sequence of the gene in C149/L1, which was found to have a single base (guanine) deletion in the codon corresponding to lysine at position 87 of the polypeptide chain. The deletion of this gene and flanking regions were integrated at the homologous site in chromosomal DNA (39).

**Cloning of COX24** — Approximately 5 × 10⁸ cells were transformed with 50 μg of the plasmid library by the method of Beggs (38).
indicated normal translation of cytochrome b (Fig. 1C). A partial deficiency of cytochrome b is a common property of COX mutants.

In contrast to cytochrome b, translation of Cox1p, Cox2p, and Cox3p, the three mitochondrially encoded subunits of cytochrome oxidase, was reduced. This was most evident for Cox1p, which was present in only trace amounts in the mutant. Subunit 6 (Atp6p) of the ATPase dase, was reduced. This was most evident for Cox1p, which was present to barely detectable levels in the mutant. A very substantial decrease was also seen in Cox3p. Both Cox4p and Cox5p were reduced but to a lesser extent (Fig. 1C), ruling out a defect in transcription or processing of the large polycistronic precursor RNA containing COX1, ATP8, and ATP6 (10).

The steady-state concentrations of COX subunits in the cox24 null mutant were examined by Western blot analysis with antibodies against Cox1p, Cox2p, and Cox3p, and the nuclear encoded subunits Cox4p and Cox5p. Of these five constituents, Cox1p and Cox2p were reduced to barely detectable levels in the mutant. A very substantial decrease was also seen in Cox3p. Both Cox4p and Cox5p were reduced but to a lesser extent (Fig. 1D). This pattern is characteristic of most mutants that fail to form functional COX, independent of whether the block occurs at a pre- or post-translation step of the assembly pathway (46).

W303ΔCOX24 gives rise to revertants with growth properties on non-fermentable substrates intermediate between wild-type and the mutant. Although the suppressor mutation(s) has been ascertained to be in mtDNA, for unknown reasons we have not been able to map the mutations by deletion analysis with cytoplasmic petites obtained from several such revertants. The partial restoration of growth of such a revertant (W303ΔCOX24/R2) correlates with an increase in the mitochondrial steady-state concentration of Cox1p (Fig. 1D).

Phenotype of a cox24 Mutant with Intronless Mitochondrial DNA—The lesion in the cox24 mutant appeared most likely to be in processing and/or translation of Cox1p. The parental W303 strains into which the cox24 null allele was introduced have mitochondrial genomes with COX1 introns aI1–aI4 and aI5 (47). To probe the possible involvement of Cox24p in intron processing, the cox24 null mutation was transferred to a strain lacking mitochondrial introns by a cross of W303ΔCOX24/pR (a cox24 null mutant lacking mitochondrial DNA) to the intronless strain W303/I0. Diploid cells issued from the cross yielded meiotic progeny with the cox24 null allele in an intronless mitochondrial background (aW303ΔCOX24/I0 and W303ΔCOX24/I0). These strains were able to grow on non-fermentable substrates (YPEG), albeit not as well as the wild-type strains W303-1A or W303-1A/I0 (Fig. 2A). Growth of W303ΔCOX24/I0 on glycerol/ethanol correlated with a partial restoration of Cox1p translation (Fig. 1C). Western analysis of mitochondria with a polyclonal antibody against Cox1p indicated a substantial increase in the steady-state concentration of this subunit in the mutant cells (Fig. 2B). Paradoxically, a Cox1p monoclonal antibody detected only trace amounts of Cox1p, even at very high loading of mitochondrial proteins (Fig. 2B). This suggests that the Cox1p produced in W303ΔCOX24/I0 may be different from the normal protein.

COX1 Transcript Processing in cox24 Mutants—The partial rescue of the cox24 null mutation in a strain with intronless mtDNA pointed to a
role of Cox24p in processing of the COX1 pre-mRNA. This is supported by the mitochondrial COX1 transcripts present in wild-type and mutant strains harboring mtDNAs with or without introns (Fig. 3A). Northern blots of total mitochondrial RNA hybridized to a probe from exon aE4 of COX1 indicated the accumulation of partially processed COX1 RNA intermediates in the cox24 null mutant with the original intron-containing mtDNA. In this background the mature COX1 mRNA was not detected. As expected, COX1 processing intermediates were absent in the wild-type or cox24 mutant with the intronless genome. In this background the only COX1 transcript detected by the probe had a size corresponding to the mature mRNA. This reinforces the conclusion that the cox24 mutation does not affect endonucleolytic cleavage of the primary polycistronic transcript. Northern blots of the same RNAs with an exon probe from the cytochrome b (COB) gene revealed the presence in the cox24 mutant of the mature size of COB mRNA. Although there was some accumulation of COB precursor transcripts in the mutant, the mRNA concentration was not appreciably less than in the wild type (Fig. 3B). The presence of the processed COB mRNA in the mutant is consistent with the in vivo labeling results showing normal translation of cytochrome b (Fig. 1C) and support a role of Cox24p confined to processing of the COX1 pre-mRNA.

The function of Cox24p in splicing of the COX1 precursor was assessed by hybridization of total mitochondrial RNAs with probes from introns aI1, aI2, aI3, aI4, and aI5γ. The results of these Northern analyses indicated that the cox24 mutant is able to splice group II introns aI1 and aI5γ. Both introns are stable and accumulate in the wild-type and mutant (Fig. 4). The concentration of aI2, the third stable group II intron of COX1, is significantly lower in the mutant and is present at nearly wild-type levels in the revertant W303ΔCOX24/R2. The results with probes against group I introns aI3 and aI4 are more difficult to interpret because the excised products are unstable and normally are degraded. The aI4 probe detected high molecular weight precursors that are much less abundant in wild type and are substantially reduced in the revertant. In the case of the aI3 probe, several of the partially processed intermediates seen in wild type are absent in the mutant but are partially restored in the revertant (Fig. 4). These results point to multiple roles of Cox24p in COX1 mRNA processing, including excision of both group I and group II introns.

Suppression of the cox24 Null Mutant by mtDNA with Different Intron Compositions—The COX1 processing defect was also tested by examining the suppressor activities of mitochondrial genomes differing in their intron compositions. These were introduced into a W303 derivative of the cox24 null mutant by cytoduction (49). As might be predicted, the absence of COB introns did not relieve the respiratory deficiency of the mutant (CK5112 in Fig. 5). This was also true of the cox24 mutant with a COX1 gene lacking the aI4 intron (WIO4). The cox24 mutant, however, was partially rescued by mtDNA lacking introns aI1, aI2, aI3, and aI5γ (GF134-6D). These results are consistent with Northern hybridizations with intron-specific probes, indicating impaired processing of aI2 and aI3 in the mutant (Fig. 4).

Cox1p Expression Is Inhibited in a cox14-cox24 Double Mutant—The incomplete suppression of the cox24 mutant by a mitochondrial genome devoid of introns suggested that in addition to transcript processing Cox24p may have another function, either in translation or
assembly of cytochrome oxidase. Mutations in genes required for maturation of the COX1 precursor transcript or translation of the mRNA display the absence of Cox1p among the normal complement of mitochondrial translation products. Translation of Cox1p is also compromised by mutations that prevent assembly of the subunits into the functional complex (36). Synthesis of Cox1p, in all such assembly defective mutants can be rescued when the original mutation is combined with a cox14 null mutation (36). This circumstance permits mutations that prevent formation or translation of the Cox1p mRNA to be discriminated from mutations affecting a post-translational assembly step. A regulatory model of Cox1p translation has been invoked to explain the epistatic effect of the cox14 mutation over other mutations affecting post-translational events in COX assembly (36).

This experimental paradigm was used to examine expression of Cox1p in a cox24-cox14 double mutant with intronless mtDNA. The results of in vivo translation assays indicated that not only did inclusion of the cox14 mutation fail to enhance Cox1p synthesis over and above that seen in the single cox24 null mutant, but instead exerted an inhibitory effect (Fig. 6). Because COX24 is adjacent to MSS51 (32), which is also required for Cox1p synthesis (46, 50, 51), the possibility existed that the cox24 null allele interferes with normal expression of Mss51p. This seems unlikely in view of a similar inhibition of Cox1p synthesis when the cox14 mutation was combined with the cox24 point mutation of C149 (not shown).

Cox24p Homologues Are Confined to Species of Saccharomyces—A search of the current data bases disclosed the presence of COX24 homologues within but not outside of the Saccharomyces genus. The alignment of Cox24p from S. cerevisiae, Saccharomyces paradoxus, Saccharomyces bayanus, and Saccharomyces mikatae is shown in Fig. 7A. The carboxy-terminal 39 residues, which include the premature termination codon of C149, comprise a very hydrophilic and basic domain that is identical in the four species.

Although the amino-terminal half is also conserved among the different species, there are fewer identities in this region. The protein has a centrally located putative hydrophobic membrane-spanning domain (Fig. 7B). This feature is consistent with the solubility properties of Cox24p (see below).

Cox24 Localization and Extraction Properties—To localize Cox24p, COX24 was modified so as to express the protein with a carboxyl-terminal hemagglutinin tag. This hybrid gene was introduced into a yeast strain (W303) which lacks the first three introns of COB (37, 38), and the carboxy-terminal 39 residues, which include the premature termination codon of C149, comprise a very hydrophilic and basic domain that is identical in the four species.

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brane protein extending into the intermembrane space (7). The above localization of Cox24p-HA was confirmed for the wild-type protein with an antibody raised against a 12-residue long peptide in the carboxy-terminal conserved domain (Fig. 8B).

**DISCUSSION**

Our studies show that Cox24p, encoded by reading frame YLR204W on chromosome XII, is a mitochondrial protein facing the matrix side of the inner membrane. Null mutations in this gene, for which we propose the name COX24, elicit a growth defect on non-fermentable carbon sources as a result of a deficiency in cytochrome oxidase. Assays of mitochondrial protein synthesis in whole cells and Western analysis of COX subunit polypeptides indicated that cox24 mutants are severely impaired in the synthesis of Cox1p, the heme-bearing subunit of this respiratory complex. Unlike most cytochrome oxidase mutants, which lack the absorbance bands of cytochromes a and a₃ at 605 nm, the cox24 mutant shows evidence of a weak absorption band with a maximum at 595 nm indicative of a non-native environment of heme A. At present we do not know if this absorption stems from heme A that may be associated with a small residual amount of the Cox1p present in the mutant or with some other mitochondrial protein.

The partial suppression of the cox24 null mutant by an intronless mitochondrial genome points to a role of Cox24p in intron processing. This is confirmed by Northern analysis of the mitochondrial COB and COX1 RNAs, both of which are transcribed from genes with multiple introns. Whereas processing of the COB mRNA was only marginally retarded in the mutant, the COX1 exon probe detected mainly large precursor transcripts with an almost complete absence of the mature COX1 mRNA. Hybridization of mitochondrial RNAs to specific COX1 intronic probes revealed that the mutant is blocked in processing of introns a12 and a13. The requirement of Cox24p for a12 and a13 splicing is also supported by the suppressor activity of mtDNA containing a COX1 gene with only the a14 intron.

The accumulation of high-molecular weight intermediates with the fourth (a14) intron indicated that the cox24 mutation affects either excision or stability of this group I intron. Splicing of a14 depends on the expression of a maturase encoded in the COB b14 intron, which also functions in excision of b14 itself (56). The presence in the mutant of a translatable COB mRNA excludes a deficiency of the b14 maturase as a plausible explanation for the accumulation of splicing intermediates with a14. The partial rescue of the mutant by a COX1 gene lacking all introns except the a14 intron suggests that a failure to process the upstream a2 or a13 introns may exert a polar effect on excision of a14.

Because the cox24 mutant is blocked in splicing of both group II (a12) and group I (a13) introns, it is unlikely that Cox24p is involved in catalyzing some specific step of the mechanisms by which these two different types of introns are spliced. A more plausible explanation is that Cox24p affects processing of the COX1 precursor in a more general way, perhaps by stabilizing a splicing competent conformation of the COX1 RNA similar to that proposed for Cbp2p in splicing of b15 (57).

Mutants with lesions in genes that code for proteins dedicated exclusively to splicing of introns acquire wild-type growth properties on respiratory substrates when their target introns are absent in mtDNA (58). The finding that suppression of the growth defect and restoration of COX1 synthesis in the cox24 mutant harboring an intronless mitochondrial genome is only partial, indicates that in addition to splicing of COX1 introns, Cox24p has yet another function. The phenotype of the cox14-cox24 double mutant with an intronless COX1 gene points to a possible function of Cox24p in translation of Cox1p. In previous studies the cox14 null allele was shown to restore Cox1p synthesis in mutants arrested at post-translational stages of COX assembly (36). This was not true of mutants with genetic lesions in genes such as MSSS1 and PET309 that are required for translation of Cox1p (36). The negative effect of the cox14 mutation on Cox1p translation in the double mutant argues against a post-translational role of Cox24p in assembly and is more consistent with a requirement of this protein for Cox1p translation.

Maturation of the COX1 transcript (16–20, 26, 28–31) and translation of the resultant mRNA (4, 36, 46, 50, 51) are complex events requiring the intervention of a large number of gene products. Some of these accessory proteins are organized in large complexes that are associated with the inner membranes facing the matrix where they influence different
aspects of COX1 and COB expression (27, 59). A dual role of Cox24p in mitochondrial RNA processing and translation would suggest that this protein may also be a constituent of such a multifunctional complex.

The Cox24 point mutant C149 is part of a collection of respiratory deficient pet strains that was previously used to identify 18 complementation groups defective in splicing of the COXI precursor (60). These mutants were found to exhibit a range of different phenotypes based on their patterns of COXI transcripts. One of the mutants (N266) reported in that study had a COXI RNA phenotype very similar to that of cod4 mutants. N266 had normal COB mRNA but was deficient in COXI mRNA. It accumulated splicing intermediates with aI4 and showed no evidence of aI2 processing. The similarity of the two mutants suggests that the defective protein in N266 may be functionally related to Cox24p. Further studies of this possibility will require identification of the gene, which until now has proven difficult to clone by complementation.

COX24 is transcribed from an open reading frame adjacent to, but in the opposite strand from, MSSI. Based on the presence of an ABF1 binding site between the two genes, it was speculated that their products may have related functions (32). Our results, particularly the implication of Cox24p in translation of Cox1p, lend further credence to this possibility.

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