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# Assembly of the Rotor Component of Yeast Mitochondrial ATP Synthase Is Enhanced When Atp9p Is Supplied by Atp9p-Cox6p Complexes*

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**Background:** The Atp9p rotor is an assembly module of mitochondrial ATP synthase.
**Results:** Newly translated Atp9p and Cox6p, a subunit of yeast cytochrome oxidase (COX), are present in large complexes.
**Conclusion:** Atp9p-Cox6p complexes serve as a source of Atp9p for rotor formation.
**Significance:** We propose that Cox6p enhances the efficiency of Atp9p ring formation and may also coordinate balanced expression of COX and ATP synthase.

**The Atp9p ring is one of several assembly modules of yeast mitochondrial ATP synthase. The ring, composed of 10 copies of Atp9p, is part of the rotor that couples proton translocation to synthesis or hydrolysis of ATP. We present evidence that before its assembly with other ATP synthase modules, most of Atp9p is present in at least three complexes with masses of 200–400 kDa that co-immunopurify with Cox6p. Pulse-labeling analysis disclosed a time-dependent reduction of radiolabeled Atp9p in the complexes and an increase of Atp9p in the ring form of wild type yeast and of *mss51*, *pet111*, and *pet494* mutants lacking Cox1p, Cox2p, and Cox3p, respectively. Ring formation was not significantly different from wild type in an *mss51* or *atp10* mutant. The *atp10* mutation blocks the interaction of the Atp9p ring with other modules of the ATP synthase. In contrast, ring formation was reduced in a *cox6* mutant, consistent with a role of Cox6p in oligomerization of Atp9p. Cox6p involvement in ATP synthase assembly is also supported by studies showing that ring formation in cells adapting from fermentative to aerobic growth was less efficient in mitochondria of the *cox6* mutant than the parental respiratory-competent strain or a *cox4* mutant. We speculate that the constitutive and Cox6p-independent rate of Atp9p oligomerization may be sufficient to produce the level of ATP synthase needed for maintaining a membrane potential but limiting for optimal oxidative phosphorylation.**

The mitochondrial ATP synthase of *Saccharomyces cerevisiae* is assembled from separate modules (1), one of which is a ring of 10 identical subunits of Atp9p (subunit c), a small hydrophobic subunit of the $F_0$ sector (2) that in yeast is encoded in mitochondrial DNA (mtDNA). Studies with isolated mitochondria have shown that newly translated Atp9p oligomerizes into the ring before interacting with the $F_1$ ATPase. The $F_1$-Atp9p ring assembles with another module consisting of Atp6p, Atp8p, and all the peripheral stalk subunits except OSCP (1). A similar mode of assembly has been proposed for the ATP synthase of *Escherichia coli* (3).

Assembly of yeast cytochrome oxidase (COX)[2] is also a modular process. At least two of the three mitochondrial gene products of COX that constitute the catalytic core of this respiratory complex bind to a specific subset of the eight imported subunits and various accessory proteins to form the modular intermediates that then interact with each other to produce the holoenzyme (4, 5).

Mitochondrial translation of Cox1p, one of the three core subunits of COX, is coupled to a downstream step involving its assembly with other modules of the enzyme (6–11). Similarly, translation of the Atp6p and Atp8p subunits of ATP synthase on mitochondrial ribosomes is activated by $F_1$ ATPase or partially assembled $F_1$ (12). Both regulatory mechanisms achieve a balanced expression of the mitochondrial gene product(s) relative to partner proteins derived from the nucleo-cytoplasmic system.

The mitochondrial respiratory and oxidative phosphorylation capacity is regulated by carbon source and oxygen availability. Biogenesis of respiratory complexes, including COX, is severely repressed in yeast fermenting glucose and is induced during transition to aerobic growth on carbon sources such as ethanol, glycerol, or lactate (13, 14). Derepression of respiration and oxidative phosphorylation in glucose grown cells is accompanied by transcriptional activation of a large number of nuclear genes that either directly or indirectly affect biogenesis of the respiratory chain and other catabolic pathways including the tricarboxylic acid cycle (15, 16). Glucose-responsive genes are transcriptionally regulated by the Snf1-Snf6 activator/repressor system (17) and the Hap3/4/5 complex (18). Depending on the gene, transcriptional activation can be as high as 50-fold when cells adapt from glucose to non-fermentative metabolism. Repression of the ATP synthase in cells grown on glucose is less severe (14). The ratio of the electron transfer complexes *vis à vis* one another and the ATP synthase are constant under

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[2] The abbreviations used are: COX, cytochrome oxidase; BN-PAGE, blue native PAGE; PC beads, protein C antibody beads.

**TABLE 1**
**Genotypes and sources of *S. cerevisiae* strains**

| Strain | Genotype | mtDNA | Source |
|---|---|---|---|
| W303-1A | MATa *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1* | $\rho^+$ | –[a] |
| W303-1B | MATα *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1* | $\rho^+$ | –[a] |
| MRSI°/COX1-HAC | MATα *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1arg8::HIS3* | Intronless, *COX1-HAC* | (4) |
| aW303ΔCOX6 | MATa *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cox6::URA3* | $\rho^+$ | (19) |
| W303ΔCOX6 | MATα *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cox6::URA3* | $\rho^+$ | (19) |
| W303ΔCOX4 | MATα *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cox4::URA3* | $\rho^+$ | (19) |
| aW303/COX6-HAC | MATa *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cox6::URA3 trp1::pG71/ST9* | $\rho^+$ | (4) |
| aW303ΔMSS51 | MATa *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 mss51::HIS3* | $\rho^+$ | (20) |
| aW303ΔMSS51/COX6-HAC | MATa *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cox6::URA3 mss51::HIS3 trp1::pG71/ST9* | $\rho^+$ | This study |
| aW303ΔPET111 | MATa *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 pet111::HIS3* | $\rho^+$ | (21) |
| aW303ΔPET494 | MATa *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 pet494::HIS3* | $\rho^+$ | (7) |
| aW303ΔPET494/COX6-HAC | MATa *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 pet494::HIS3 trp1::pG66/ST19* | $\rho^+$ | This study |
| aW303ΔPET111/COX6-HAC | MATa *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cox6::URA3 pet494::HIS3 trp1::pG71/ST9* | $\rho^+$ | This study |
| aW303ΔATP10 | MATa *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 atp10::LEU2* | $\rho^+$ | (22) |
| aW303ΔATP10/COX6-HAC | MATa *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cox6::URA3 apt10::LEU2 trp1::pG71/ST9* | $\rho^+$ | This study |

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given physiological conditions. This implies the existence of a mechanism(s) for adjusting the proper stoichiometry of the respiratory and ATP synthase complexes in response to the metabolic needs of the cell.

We previously reported that Atp9p translated in isolated mitochondria is pulled down with a tagged version of Cox6p, a subunit of COX (4). The presence of unassembled Cox6p and Atp9p in a common complex suggested that the two proteins might play a role in biogenesis of ATP synthase and COX, respectively. The present study was undertaken to further characterize the Atp9p-Cox6p complex and to ascertain if it has a function in oligomerization of Atp9p and in coordinating assembly of COX and ATP synthase.

We present evidence that before its incorporation into the ATP synthase, most of the newly translated Atp9p in mitochondria is present in at least three large complexes that cofractionate with Cox6p. Our evidence indicates that oligomerization of Atp9p into the ring is less efficient in mitochondria of a *cox6* mutant grown either under glucose-repressed conditions or during adaptation to respiratory growth when there is a rapid induction of respiratory and ATP synthase complexes. The relevance of these findings to ATP synthase and COX assembly is discussed.

## MATERIALS AND METHODS

*Strains and Growth Media*—The strains of *S. cerevisiae* used in this study are listed in Table 1. The compositions of solid and liquid media have been described (23). The liquid medium for glucose-repressed growth contained 0.7% yeast nitrogen base without amino acids, 0.5% yeast extract, auxotrophic requirements, and 6% glucose. The same medium was used for derepression except that the glucose concentration was reduced to 0.8%.

*Construction of Pet Mutants Expressing Tagged Cox6p*—*COX6* was PCR-amplified from W303-1A nuclear DNA with primers cox6–1 (5′-ggcgagctccatacgagccaatcag) and cox6–2 (5′-ggcggtaccggttcaagcgtagtctgggacgtcgtatgggtaagaagagcttggaaatagctc). The PCR product was digested with a combination of SacI and KpnI and ligated to the multiple cloning site of YIp349, an integrative *E. coli*/yeast vector identical to YIp351 (24) except the selectable marker is *TRP1* instead of *LEU2*. The resultant plasmid pG71/ST7 containing ~500 bp of the *COX6* 5′-UTR plus coding sequence fused in-frame at the 3′ end of the gene to the sequence coding for the HA tag. This plasmid was used as the template with primers cox6–1 and cox6-protC 5′-ggcggtacctcatttaccatcgattaatcttggatctacttgatcttctcctccagcgtagtctgggacgtcgtatgg to add the sequence coding for the protein C epitope (25) to the HA-tagged COX6. The product of the amplification was digested with a combination of SacI and KpnI and ligated to YIp349 to obtain pG77/ST9. The latter plasmid was linearized at the unique BstX site of the *TRP1* gene in the YIp349 vector and was integrated at the *trp1* locus of aW303ΔCOX6 by site-directed integration (26). aW303/COX6-HAC harboring the integrated fusion gene was verified to grow as well as wild type on respiratory substrates and to express Cox6p with a C-terminal tandem HA and protein C tags separated by two glycines (4). Linear fragments of DNA containing null alleles of *mss51*, *pet111*, *pet494*, and *atp10* were introduced into aW303/COX6 by homologous recombination at the *his3* or *leu2* loci in chromosomal DNA (26).

*Preparation of Mitochondria and Labeling of Mitochondrial Gene Products*—Mitochondria were prepared by the method of Herrmann *et al.* (27) from yeast that had been grown in rich galactose medium (YPGal) to early stationary phase and allowed to grow for an additional 2 h in fresh medium containing 2 mg/ml chloramphenicol. The chloramphenicol treatment was included to increase the pools of nuclear gene products of ATP synthase (28). Mitochondria were labeled with a mixture of [$^{35}$S]methionine and [$^{35}$S]cysteine (3000 Ci/mmol, MP Biochemicals) as described previously (4). In experiments with strains expressing Cox6p-HAC, mitochondria were extracted with digitonin and purified on protein C antibody beads (Roche Applied Sciences) as described previously (4).

*Construction of trpE-Cox6 Fusion Gene*—The sequence coding for Cox6p starting with residue 21 was amplified from purified nuclear DNA of *S. cerevisiae* W303-1A with primers cox6-10 (5′-ggcgagctcctggtgcttatcatgcaact) and cox6–11 (5′-ggcctgcagaggacaacaaattacagacgt). The product was digested with a combination of SacI and PstI and cloned into pATH20 (29) resulting in an in-frame fusion to the *trpE* gene coding for the N-terminal half of anthranylate synthase (29). The protein encoded by the hybrid *trpE-COX6* gene was expressed in *E. coli* and purified on a sizing column as described previously (29).

The purified protein was used to raise a rabbit polyclonal antibody.

*Miscellaneous Procedures*—Ligation of DNA fragments, PCR amplification of DNA, and transformation of *E. coli* were done by standard methods (30). The lithium acetate method of Schiestl and Gietz (31) was used to transform yeast. Mitochondrial proteins were separated under non-denaturing conditions by BN-PAGE on 4–13% polyacrylamide gels (32) and by SDS-PAGE (33). The second dimension in SDS-PAGE was run on 12% polyacrylamide gels. Western blots were treated with antibodies against the appropriate proteins followed by a second reaction with anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase (Sigma). Proteins were detected with SuperSignal chemiluminescent substrate kit (Pierce). Protein concentrations were estimated by the method of Lowry *et al.* (34).

## RESULTS

*Properties of the Atp9p-Cox6p Complex*—The presence of Atp9p in a complex with Cox6p was previously inferred from co-immunofractionation of newly translated but unassembled Atp9p with Cox6p C-terminally tagged with either polyhistidine or the protein C epitope (4). To exclude the possibility that the complex was formed as a consequence of the tag, native Cox6p was co-immunoprecipitated with a Cox6p-specific antibody. The results of the pulldown confirmed the presence of Atp9p in the immunoprecipitate (Fig. 1*A*).

The fraction of newly translated Atp9p in a complex with Cox6p was assessed by comparing the radioactivity in Atp9p before and after adsorption of digitonin solubilized Cox6p-HAC on protein C antibody beads (PC beads). Atp9p was labeled with [$^{35}$S]methionine/cysteine in isolated mitochondria before extraction with digitonin. The poor resolution and retardation of Atp8p and Atp9p on the SDS-PAGE gel is caused by the high concentrations of digitonin in the mitochondrial extracts (Fig. 1*B*). Nonetheless, a comparison of the radiolabel proteins in the extract and in the fraction that was not adsorbed to the PC beads suggests that a large proportion, if not all of newly translated Atp9p, is associated with Cox6p (Fig. 1*B*). This experiment also shows that the beads contain more radiolabeled Cox1p, Cox2p, Cox3p, and cytochrome *b* (Fig. 1*B*, *last lane of left panel*), consistent with their assembly into the COX and bc1 complexes, both of which are present preponderantly in the supercomplexes (35–37).

All the Atp9p recovered from the beads migrated as a monomer even though the SDS-PAGE conditions do not dissociate the Atp9p ring. The failure of SDS to dissociate the Atp9p ring is evident from the equal intensity of the ring band in the digitonin extract before and after adsorption to the protein C antibody beads (Fig. 1*B*). Cox6p-HAC in the extract was recovered quantitatively in the eluate from the beads (Fig. 1*B*, *right panel*). These results indicate that Cox6p does not interact with the Atp9p ring.

The Atp9p-Cox6p complex is stable over time in digitonin but not in 1% dodecyl maltoside.[3] To better characterize the Atp9p-Cox6p complex, mitochondria from a respiratory-competent strain and an *mss51* null mutant, both expressing Cox6p with the HAC tag, were labeled and extracted with digitonin. As a control, mitochondria of a respiratory-competent strain lacking the tagged Cox6p was similarly labeled and extracted with a digitonin. Extracts were purified on PC beads and separated by BN-PAGE. Immunoblots of the purified fractions revealed the presence of Atp9p in complexes with masses ranging from 200 to 400 kDa in both the respiratory-competent and the *mss51* mutant lacking COX (Fig. 1*C*, *right panel*). Although the protein C antibody also detected Cox6p-HAC in this region, most of the protein migrated as the monomer (Fig. 1*C*). This probably corresponds to the pool of free Cox6p-HAC, although we cannot exclude that some may have dissociated from the complex during electrophoresis on the native gel.

As expected, a large fraction of Cox6p-HAC in the respiratory-competent but not *mss51* mutant mitochondria was associated with the supercomplexes and with a band that migrated like the D4 intermediate of Cox1p, previously shown to contain Cox6p, (4). Some COX was also detected in the respiratory-competent strain with longer exposure time of the blot (Fig. 1*C*, *right panel*). Longer exposures also revealed the presence in the *mss51* mutant of Cox6p-HAC in the 200–400-kDa region. Because of the abundant D4 intermediate it was not possible to determine how much of the signal in this region of the respiratory-competent mitochondria it was contributed by the Atp9p-Cox6p complexes.

Western analysis with an antibody against the $\beta$-subunit of $F_1$ failed to detect the 200–400 kDa bands, indicating that they did not contain the $F_1$-Atp9p ring assembly intermediate of the ATP synthase (1, 38). Cox1p intermediates, some of which migrate in the 200–400-kDa region of the blue native gel, are absent in *mss51* mutants (5). The presence in the *mss51* mutant of radiolabeled Atp9p in this region, therefore, excludes it from being associated with any of the Cox1p intermediates. The absence of Cox1p as well as Cox3p intermediates is confirmed by earlier studies in which pulldown assays with different a subunit of COX failed to co-precipitate newly translated Atp9p (4, 39).

*Time-dependent Decrease of Atp9p-Cox6p Complexes*—Pulse-chase analysis of newly translated mitochondrial gene products has been useful in identifying and characterizing intermediates of the ATP synthase (1) and COX (4, 39). This approach was used to ascertain if the Atp9p-Cox6p complexes decrease during the chase period. It was not possible to study oligomerization of newly translated Atp9p in the same experiment because the ring form of this $F_0$ component is not physically associated with Cox6p and, therefore, is absent in the fraction purified on the protein C antibody beads (Fig. 1*B*). Mitochondria from the respiratory-competent strain expressing Cox6p-HAC were pulsed for 10 min and chased for 30 and 60 min after the addition of puromycin and excess unlabeled methionine and cysteine. The protein fractions eluted from PC beads were separated by BN-PAGE in the first dimension and by SDS-PAGE in the second dimension. Most of the newly translated radiolabeled Atp9p migrated as 200–400-kDa complexes in the 1st dimension (Fig. 2*A*) and as the Atp9p monomer in the second dimension (Fig. 2*B*). Moreover, the monomeric Atp9p separated into three partially overlapping bands each of which decreased progressively during the chase (Fig. 2,

[3] C.-H Su, G. P. McStay, and A. Tzagoloff, unpublished observation.

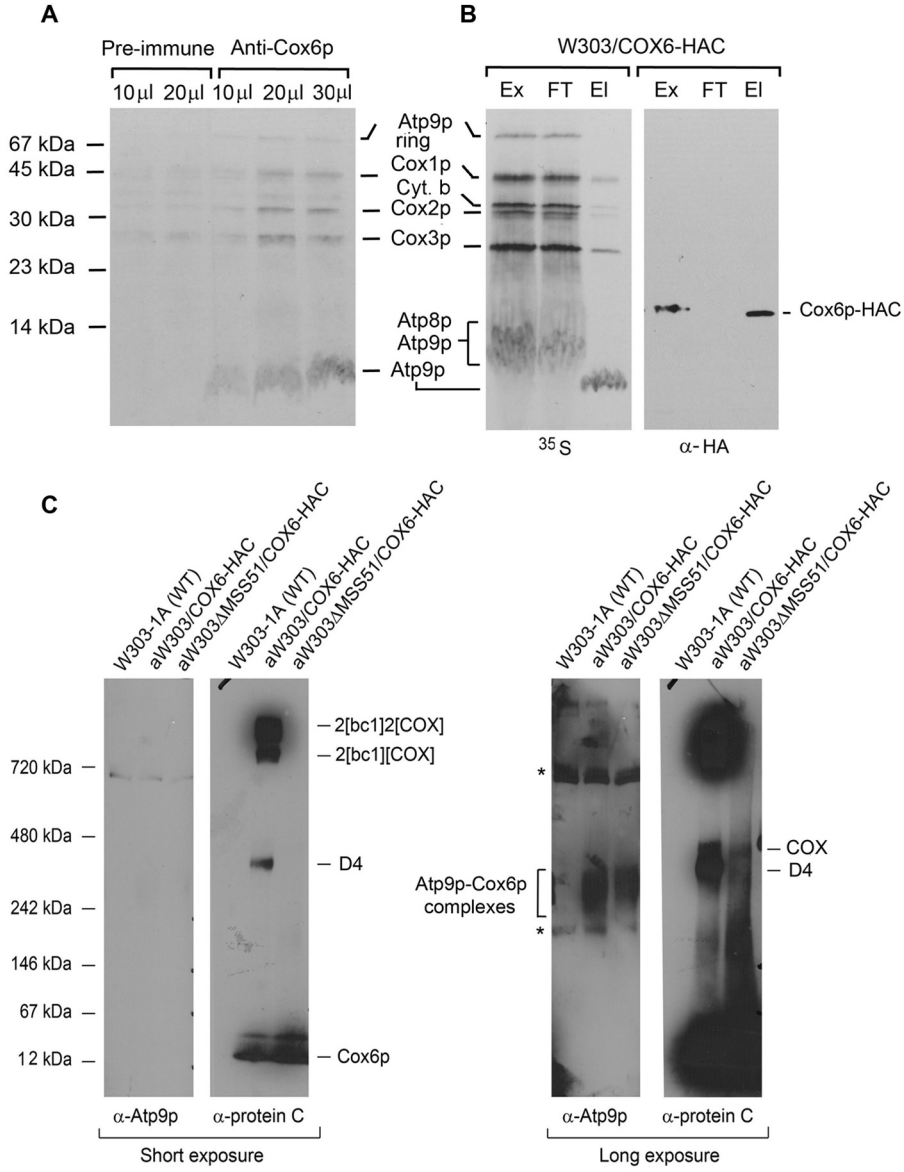


FIGURE 1. **Atp9p-Cox6 complex of yeast mitochondria.** *A*, immunoprecipitation of radiolabeled Atp9p with an antibody against Cox6p. Mitochondria (1.25 mg of protein) of the respiratory-competent strain W303-1A were labeled with [$^{35}$S]methionine/[$^{35}$S]cysteine for 20 min at 24 °C. Translation was terminated by the addition of puromycin and excess cold methionine plus cysteine. Proteins were extracted by the addition of 4% digitonin to a final concentration of 1.8%. The extract obtained after centrifugation at 100,000 × $g_{av}$ was divided into 5 equal parts and incubated for 60 min at 4 °C with the indicated volumes of pre-immune and Cox6p-specific antiserum and 40 μl of packed protein G beads. The beads were washed 3 times with buffer containing 0.5% digitonin, 10 mM Tris-Cl, pH 7, 5, and 100 mM NaCl and then eluted with 50 μl of Laemmli sample buffer (33) and separated by SDS-PAGE on a 17% polyacrylamide gel. The radiolabeled mitochondrial gene products and complexes are identified in the margins. The high concentration of digitonin in the extract and non-adsorbed fraction causes Atp8p and Atp9p bands to be smeary and to be retarded in their migration. *B*, adsorption of newly translated Atp9p and Cox6p-HAC on PC beads. Mitochondria (250 μg of protein) from the respiratory-competent strain W303/COX6-HAC were labeled and extracted with digitonin as in *A*. The extract was mixed with 30 μl of packed PC beads and rotated for 90 min at 4 °C. The beads were washed and eluted as described previously (4). Samples of the digitonin extract (*Ex*), the fraction not adsorbed to the PC beads (*FT*), and the eluate from the beads (*El*), each adjusted to the volume of the starting mitochondria, were separated by SDS-PAGE on a 17% polyacrylamide gel, transferred to nitrocellulose, and exposed to x-ray film (*left panel*). In the *right-hand* panel, the same blot was probed with a rabbit antibody against the protein C epitope (Genscript). *C*, Mitochondria (500 μg of protein) of W303-1A, aW303/COX6-HAC, and aW303ΔMSS51/COX6-HAC (a respiratory deficient *mss51* mutant expressing Cox6p-HAC) were extracted with digitonin, and Cox6p-HAC in the extract was enriched on PC beads as in *B*. The eluates from the PC beads were separated by BN-PAGE on a 4–13% polyacrylamide gel, transferred to a PVDF membrane, and reacted with antibody against Atp9p and the protein C epitope. Two different times of exposure are shown. The non-specifically adsorbed bands detected by the Atp9p antibody in the W303-1A control are marked with an *asterisk*.

*B* and *C*). Some radioactivity was associated with bands that migrated as COX and the D5 and D4 intermediates of Cox1p (4, 5, 39). In agreement with earlier studies, the two-dimensional gels showed that the chase resulted in an increase of radiolabeled products that migrated as Cox2p/cytochrome *b* and Cox3p in the regions corresponding to the two supercomplexes (4, 39). The apparent substoichiometric incorporation of label into Cox1p relative to Cox3p is explained by the dilution of radiolabel associated with newly translated Cox1p by the large steady-state pools of Cox1p intermediate complexes in mitochondria before labeling (4, 5).

The time-dependent decrease of the Atp9p-Cox6p complexes was also observed in mitochondria of a Cox1p translation-defective *mss51* mutant (Fig. 3, *B–D*) and an *atp10* mutant

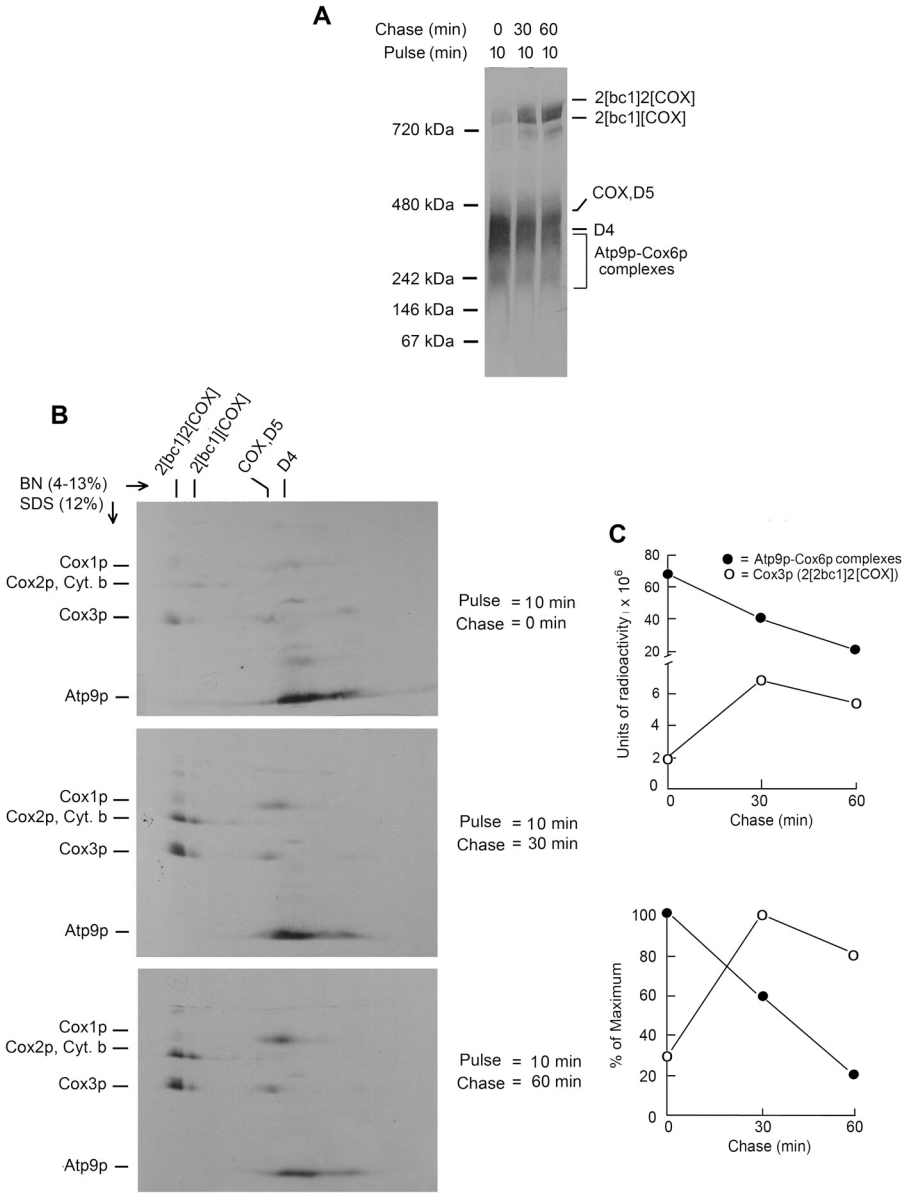


FIGURE 2. **Time-dependent loss of newly translated Atp9p from the Atp9p-Cox6p complex.** *A*, mitochondria (1.5 mg of protein) of W303/COX6-HAC were labeled for 10 min with [$^{35}$S]methionine/[$^{35}$S]cysteine as in Fig. 1*A*. Translation was terminated by the addition of puromycin and cold methionine plus cysteine. Equal samples of the translation mixture were removed immediately after the addition of puromycin and incubation for an additional 30 and 60 min at 24 °C. The samples were extracted with digitonin and purified on 60 $\mu$l of packed PC beads. One-third of the eluate was separated BN-PAGE on a 4–13% polyacrylamide gel, transferred to a PVDF membrane, and exposed x-ray film. *B*, the remaining $^2/_3$ of the eluates were separated by BN-PAGE in the first dimension followed by SDS-PAGE on 12% polyacrylamide gels in the second dimension. Proteins were transferred to nitrocellulose and exposed to x-ray film. The identities of the radiolabeled products visualized on the two-dimensional gel are indicated in the *left-hand margin* and of the supercomplexes and assembly intermediates at the top of the *0 time gel*. *Cyt. b*, cytochrome *b*. *C*, the radiolabeled spots identified as the Atp9p monomer and oligomer in the two-dimensional gel were quantified by phosphorimaging and are reported as total radioactivity measured (*top panel*) and percentage of the maximum signal (*lower panel*).

impaired in assembly of the ATP synthase but not the Atp9p ring (1) (Fig. 3, *E* and *F*). Similar experiments with mitochondria of a *pet111* and *pet494* mutant deficient in translation of Cox2p and Cox3p, respectively (40, 41), confirmed that neither mutation interfered with formation of the Atp9p-Cox6p complex. Like the *atp10* and *mss51* mutants, both the *pet111* and *pet494* mutants showed a time-dependent decrease during the chase of radiolabel in the high molecular weight complexes seen on blue native gels (not shown).

*Kinetics of Atp9p Ring Formation in Different Mutants*—Mitochondria were also pulse-chase-labeled without resorting to affinity purification of the Atp9p-Cox6p complexes. This has the advantage of allowing the decrease of radiolabeled Atp9p during the chase to be correlated with the appearance of the Atp9p ring. Mitochondria from the parental respiratory-competent haploid strain and from *mss51*, *atp10*, *cox4*, and *cox6* mutants were labeled for 3 min and chased up to an hour after the addition of excess cold methionine/cysteine. The short pulse time prevented completion of translation of the mitochondrial gene products resulting in the appearance of a smear of radioactivity in the 0 time (no chase) lane (Fig. 4*A*). This was especially true of the larger proteins, some of which could not even be distinguished as discrete bands. Translation of the partial products was completed during the first 15-min chase. The

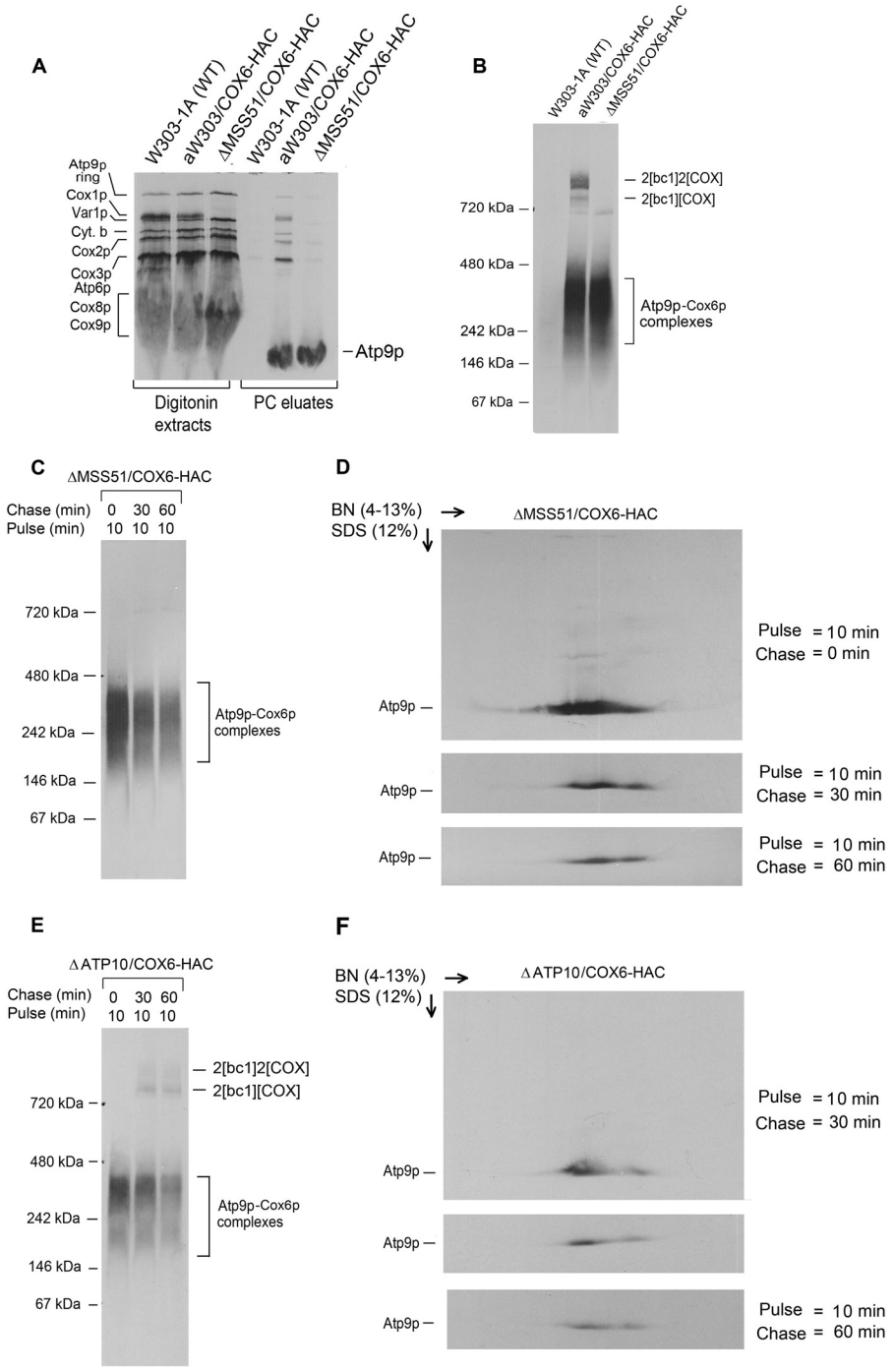


FIGURE 3. **Time-dependent loss of Atp9p from Atp9p-Cox6p complexes in *mss51* and *atp10* mutants.** *A*, W303-1A, aW303/COX6-HAC, and W303ΔMSS51/COX6-HAC mitochondria (250 $\mu$g) were labeled for 20 min with [$^{35}$S]methionine/[$^{35}$S]cysteine, extracted with digitonin, and purified on PC beads as in Fig. 2*A*. The digitonin extracts and eluates from the PC beads were separated on a 17% polyacrylamide gel by SDS-PAGE. *Cyt. b*, cytochrome *b*. *B*, the eluates from *A* were separated on a 4–13% polyacrylamide gel by BN-PAGE. *C* and *D*, pulse-chase analysis of Atp9p complexes in aW303ΔMSS51/COX6-HAC (Δ*MSS51/COX6-HAC*), a strain lacking COX and Cox1p intermediates. Mitochondria were pulsed for 10 min and chased for the indicated times as in Fig. 2*A*. The Atp9p-Cox6p complexes in the digitonin extracts were enriched on PC beads and separated in one dimension by BN-PAGE on a 4–13% polyacrylamide gel and by BN-PAGE followed by SDS-PAGE on a 12% polyacrylamide gel. Proteins were transferred to either a PVDF membrane (one dimension gel) or nitrocellulose (two dimensions) and exposed to x-ray film. *E* and *F*, mitochondria of the *atp10* mutant W303ΔATP10/COX6-HAC (Δ*ATP10/COX6-HAC*) expressing Cox6p-HAC were analyzed as in *C* and *D*.

increase of radiolabeled Atp9p in the four mutants after 15 min of chase suggests that translation of a substantial fraction, even of this small protein, had not been completed during the 3-min pulse. This was not true of the wild type strain, which perhaps because of its ability to more efficiently convert Atp9p to the ring, showed a continuous decrease of the newly translated monomer.

The wild type and *mss51* and *cox4* mutants continued to accumulate a new Atp9p ring up to 30 min of chase. The decrease of the ring in these strains seen after 60 min of chase may be due to turnover of Atp9p ring that has not been incorporated into the holoenzyme. Ring formation in both the *atp10* and *cox6* mutants continued to increase during the entire

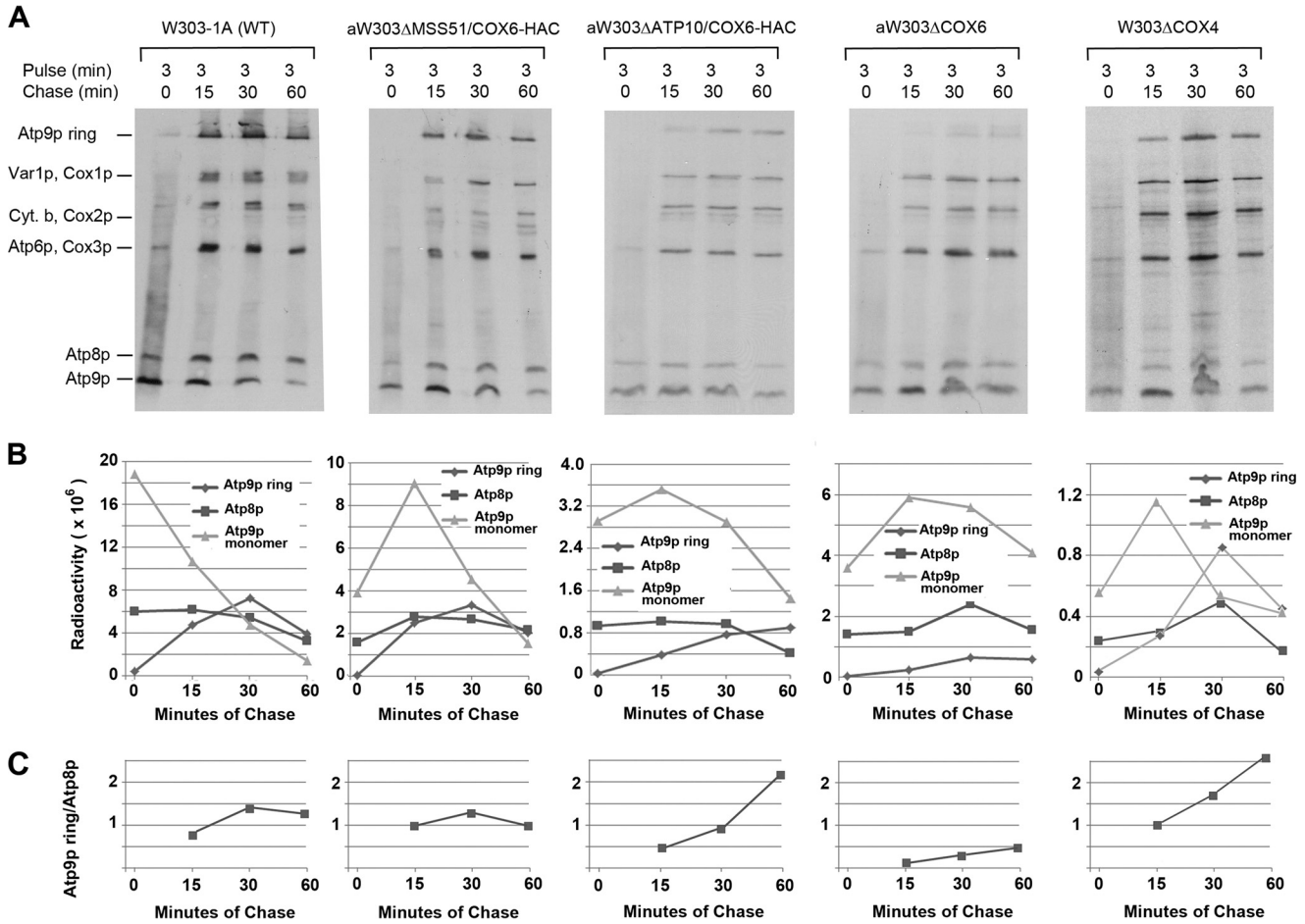


FIGURE 4. **Pulse-chase analysis of Atp9p oligomerization in a respiratory-competent and in respiratory-deficient mutants.** *A*, W303-1A, aW303ΔMSS51/COX6-HAC, W303ΔCOX4, aW303ΔCOX6, and aW303ΔATP10/COX6-HAC mitochondria (250 $\mu$g) were labeled for 3 min as in Fig. 1*A*. Excess unlabeled methionine plus cysteine was added to the translation mix, and equivalent size samples were removed immediately (0 time) and after 15, 30, and 60 min of incubation. The mitochondria were diluted into 1 ml of cold 0.6 M sorbitol and centrifuged at18,000 $\times$ $g_{av}$ for 10 min, and 10 $\mu$g of total mitochondrial proteins were separated SDS-PAGE on a 17.5% polyacrylamide gel with the running buffer adjusted to pH 8.3. Proteins were transferred to nitrocellulose and exposed to x-ray film. *Cyt. b*, cytochrome *b*. *B*, the radioactivity in the Atp9p monomer and oligomer bands and in Atp8p was quantified with phosphorimaging. For the quantifications, blots were exposed to the phosphor plate for the same time except the blot of the *cox4* mutant, which was exposed for ~5 times less time. *C*, ratios of the radioactivity in the Atp9p ring to Atp8p. The ratios are not shown for the zero time because of the high background.

period of the chase. Compared with wild type, the amount of the Atp9p ring at 30 min was estimated to be approximately 47, 20, and 8% in the *mss51*, *atp10*, and *cox6* mutant, respectively (Fig. 4*B*). The low values of the *atp10* mutant have been corrected for the 50% mitochondrial translation deficient ($\rho^{-/0}$) cells in the culture used to prepare mitochondria. Like other ATP synthase mutants this strain has lower rates of mitochondrial translation.

The ratio of radiolabel in the Atp9p ring to Atp8p, which normalizes the amount of new ring formed to the translational efficiency of the mitochondria, provides a better indication of the efficiency of this process in the different mutant backgrounds. The ratio increased to about 1–1.5 after 30 min of chase in all the strains except the *cox6* mutant in which the ratio was 0.25. Upon longer incubation the ratio in the *cox6* mutant increased to 0.5 or half that of the wild type. These results indicate that ring formation is significantly less efficient in the *cox6* mutant compared with the ATP synthase and the COX-deficient *mss51* and *cox4* mutants. This suggests that the lower rate of ring formation in the *cox6* mutant is not a general effect consequent to a deficiency of COX.

*Steady-state Concentrations of COX and ATP Synthase Constituents in Mitochondria of Glucose-repressed and Derepressed cox6 Mutant*—The efficiency of Atp9p oligomerization was also measured in mitochondria of the respiratory-competent parental strain and of the *cox6* mutant grown in 6% glucose (repressed) and derepressed after transfer at a high density (equal to the density of a stationary phase culture) to fresh medium containing 0.8% glucose in the presence or absence of chloramphenicol and grown for an additional 3.5 h. Under these conditions the glucose is exhausted in the first 20 min after which the cells begin to adapt to aerobic metabolism with only a 20–30% increase in cell mass.

The effect of the three growth conditions on expression of COX and ATP synthase was investigated by Western analysis of different subunits of each complex and by in-gel enzymatic assays. Derepression of wild type and the *cox6* and *cox4* mutants in the presence or absence of chloramphenicol caused 2–4-fold increases in the steady-state concentrations of the $\beta$ subunit of $F_1$ ATPase, Atp9p ring, and Cox6p (Fig. 5*A*). The increase of $\alpha$-ketoglutarate dehydrogenase, however, one of the most glucose-responsive mitochondrial proteins (42), was sig-

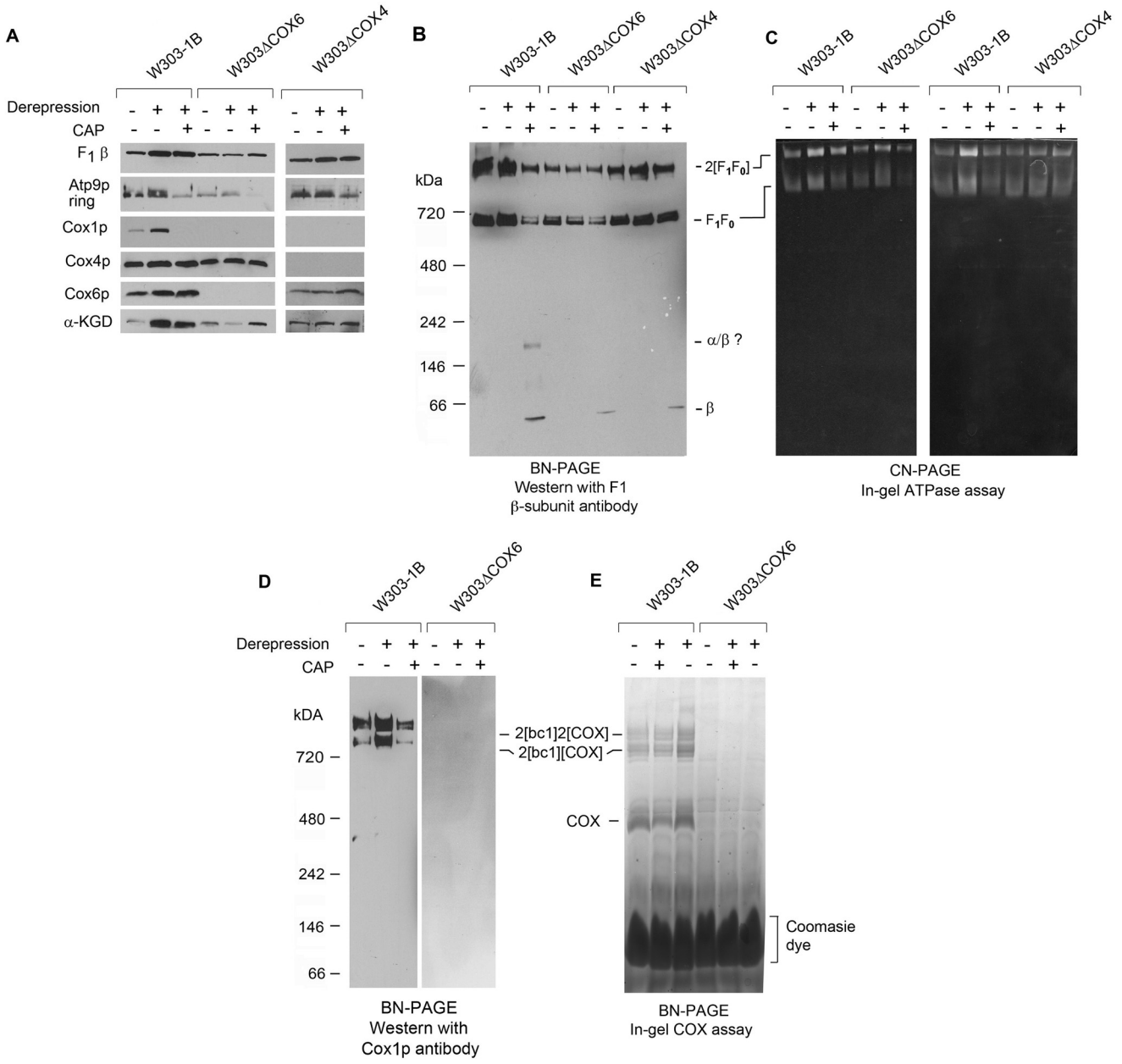


FIGURE 5. **Western analysis and in-gel activity assays of ATP synthase and COX.** *A*, the respiratory-competent strain W303-1B and the *cox6* and *cox4* null mutants W303ΔCOX6 and W303ΔCOX4, respectively, were grown to log phase in 6% glucose medium containing 6 g/liter yeast nitrogen base w/o amino acids (Difco), 0.5% yeast extract, and auxotrophic requirements. Cells were harvested, and $^1/_3$ was used to prepare mitochondria without further incubation (0 h derepression). Equal parts of the remaining $^2/_3$ of the cells were transferred to each of two flasks containing fresh medium with 0.8% glucose without and with 2 mg/ml chloramphenicol (+*CAP*). The cells were grown for an additional 3.5 h, and mitochondria (25 $\mu$g protein) from cells grown under each condition were separated by SDS-PAGE either on 12% or 15% polyacrylamide gels. Proteins were transferred to nitrocellulose and probed with antibodies against the indicated proteins. All the proteins were tested with rabbit polyclonal antibodies except Cox1p, which was reacted with a mouse monoclonal (Abcam, Cambridge, MA). Atp9p was present only as the ring in all the strains. The immunoblots of mitochondria from the *cox4* mutant were done in a separate experiment but with similar times of exposure to x-ray film. *B*, the mitochondria (250 $\mu$g of protein) were extracted with 2% final concentration of digitonin. Extracts equivalent to 31 $\mu$g of starting protein were separated BN-PAGE on 4–13% polyacrylamide gel, transferred to a PVDF membrane, and probed with an antibody against the $\beta$ subunit of $F_1$. *C*, each lane was loaded with digitonin extract equivalent of 62 $\mu$g of mitochondrial protein. Proteins were separated on a clear native gel (*CN-PAGE*). After electrophoresis of 16 h at a constant voltage of 125 V, the gel was treated for ATPase activity (44). *D*, same as *B* except that the antibody was against Cox1p. *E*, same as *C* except that in-gel cytochrome oxidase activity was assayed by staining the gel for several hours in a solution containing 0.5 mg/ml diaminobenzidine and 1 mg/ml horse heart cytochrome *c*.

nificantly greater. In contrast, inclusion of chloramphenicol in the adaptation medium resulted in a large decrease of Cox1p and Ap9p. As chloramphenicol inhibits mitochondrial translation, the decrease in Cox1p and Atp9p is likely to be a consequence of turnover of these hydrophobic proteins during the 3.5-h growth. In contrast to the wild type, the increase in the ATP synthase and COX subunits was much less in both the *cox6* and *cox4* mutants. This was also true of $\alpha$-ketoglutarate dehydrogenase (Fig. 5*A*). This suggests that the ability of yeast to adapt to oxidative phosphorylation under glucose-deprived conditions is co-regulated with the respiratory capacity of their mitochondria. The absence of Cox1p in the *cox6* and *cox4*

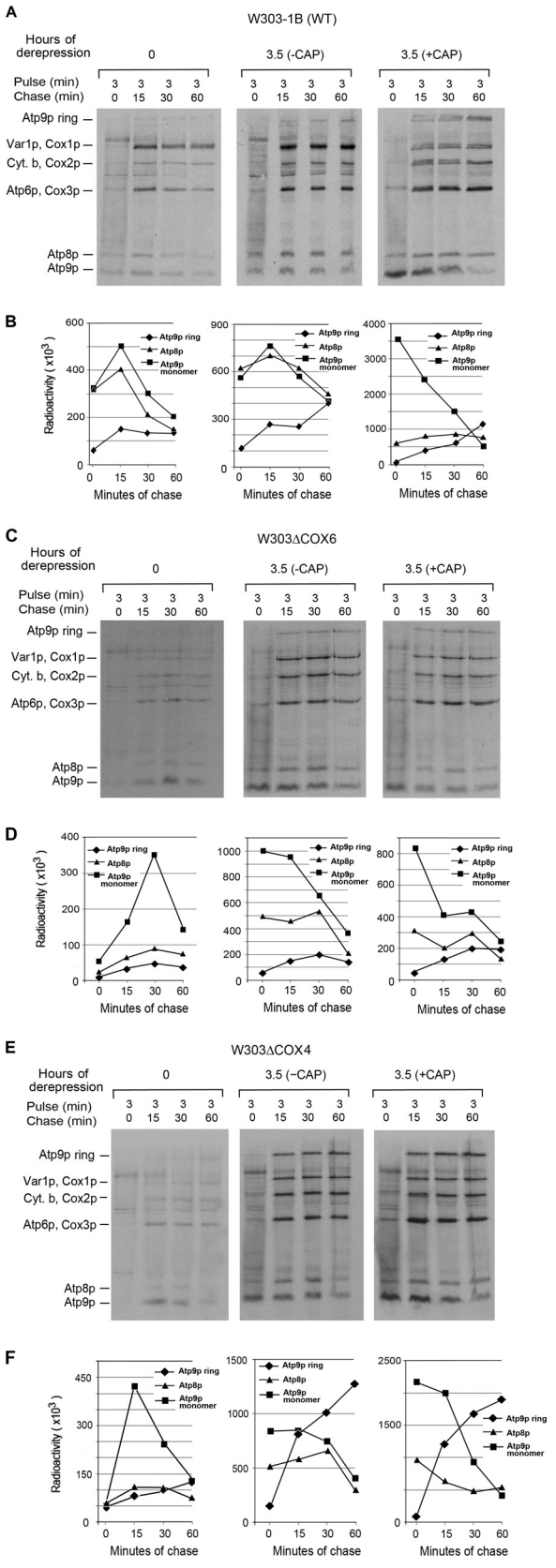


FIGURE 6. **Oligomerization of Atp9p in glucose-repressed and derepressed yeast.** *A*, the respiratory-competent strain W303-1B and the *cox6* and *cox4* null mutants W303ΔCOX6 and W303ΔCOX4 were grown as in Fig. 5*A*. Mitochondria from each culture were labeled and separated by SDS-PAGE as in Fig. 4. *Cyt. b*, cytochrome b; *CAP*, chloramphenicol. *B*, the radiolabeled bands corresponding to Atp8p, the Atp9p monomer, and Atp9 ring in *A*, *C*, and *E* were quantified with phosphorimaging and are plotted in *B*, *D*, and *F*.

mutants (Fig. 5*A*) is consistent with the previously reported strong inhibition of translation of this mitochondrial gene product in strains that are arrested in assembly of the holoenzyme (7). There was substantially less Atp9p ring in mitochondria of the glucose-repressed *cox6* mutant than in the respiratory-competent strain. Furthermore, the steady-state level of the ring was not increased during the incubation of the mutant in the low glucose medium lacking chloramphenicol (Fig. 5*A*). The even greater reduction of the Atp9p ring when chloramphenicol was included in the medium probably reflects turnover of the protein (Fig. 5*A*).

Western analysis of mitochondria from the respiratory-competent strain confirmed a 2–3-fold increase of ATP synthase after 3.5 h of derepression (Fig. 5*B*). This increase in monomeric and dimeric ATP synthase was inhibited by chloramphenicol. The native gel of mitochondria from the chloramphenicol-grown cells also revealed the presence of bands corresponding to partially and fully depolymerized $F_1$ ATPase. The cold-induced disassembly of $F_1$ (43) probably occurred during the manipulations at 4 °C of the mitochondria and digitonin extract before electrophoresis on the native gel.

Western analysis of ATP synthase separated by BN-PAGE (Fig. 5*B*) and in-gel assays of ATPase in clear native PAGE (Fig. 5*C*) confirmed the increase of ATP synthase complex in wild type and to a much lesser extent in the mutants (Fig. 5*C*). Although both *cox* mutants had less ATP synthase than the wild type, the reduction was significantly greater in the *cox6* compared with the *cox4* mutant. Consistent with the results of the Westerns of the proteins separated by SDS-PAGE, derepression of the *cox6* and *cox4* mutants induced only a marginal increase in expression of ATP synthase in either the presence or absence of chloramphenicol. The increase in Cox1 subunits in the wild type strain (Fig. 5*A*) was paralleled by an increase in COX, most of which was associated with the supercomplexes (Fig. 5, *D* and *E*).

*Pulse-chase Labeling of Mitochondria before and after Adaptation to Aerobic Metabolism*—The effect of the *cox6* mutation on ring formation during derepression was studied by pulse-chase labeling of mitochondria isolated from glucose-repressed cells before and after transfer to the derepression medium for 3.5 h. Mitochondria from the respiratory-competent glucose-repressed cells were considerably less active in translating the endogenous gene products than those obtained from the derepressed cells (Fig. 6). Mitochondrial translation was enhanced by a factor of 2–3 after derepression. As reported previously, incubation of cells in chloramphenicol is especially effective in stimulating translation of Atp9p (28). This was also true of cells derepressed in medium containing chloramphenicol (Fig. 6, *A* and *B*). The enhanced translation of Atp9p was paralleled by an increase in ring formation. After a chase up to 60 min, the largest decrease of monomeric Atp9p and increase of Atp9p ring occurred in mitochondria that had been treated with chloramphenicol during derepression (Fig. 6, *A* and *B*).

Mitochondria from glucose-repressed and derepressed *cox6* mutant cells were generally less active in translation than those of the respiratory-competent strain (Fig. 6, *C* and *D*). Although derepression led to increased ring formation, this activity was also lower than in the respiratory-competent control cells. Moreover, derepression of the *cox6* mutant in the presence of chloramphenicol did not increase either translation or oligomerization of Atp9p (Fig. 6, *C* and *D*). Even though *cox4* like *cox6* mutants are defective in COX assembly (45), the *cox4* null strain used in this study was able to efficiently convert the Atp9p monomer to the ring (Fig. 6, *E* and *F*), confirming that the compromised assembly of the ring in the *cox6* mutant is unlikely to be solely a consequence of defective respiration.

## DISCUSSION

The 10-membered Atp9p ring of *S. cerevisiae* (2) is an important assembly module of the ATP synthase. At present the mechanism by which the ring structure is formed and how this process is regulated are not well understood. Purified subunit c (Atp9p) of *E. coli* (46) and thermophilic *Bacillus* PS3 (47) have been reported to spontaneously oligomerize into rings from detergent solutions. Other studies, however, indicate that conversion of bacterial subunit c to the ring is mediated by the *uncI* gene product of *E. coli* (48, 49). Ring formation in *S. cerevisiae* has also been shown to require a protein encoded by the N-terminal half of the yeast nuclear *ATP25* gene (50). The studies reported here indicate that ring formation in yeast may be coupled to COX biogenesis through the peripheral Cox6p subunit of this respiratory complex.

We previously reported that newly translated Atp9p co-purifies with tagged Cox6p when the latter is affinity adsorbed on protein C antibody beads (4). The presence of unassembled Atp9p and Cox6 in a common complex has been confirmed in the present study with an antibody against native Cox6p, thereby excluding the possibility that the co-immunopurification of these proteins reported previously is a tag-related artifact. Two-dimensional gel electrophoresis has revealed that newly translated Atp9p is associated with at least three complexes of masses ranging between 200 and 400 kDa. Our evidence also suggests that most of the Atp9p that has not been incorporated into the ATP synthase is present in these complexes.

Atp9p associated with the high molecular weight complexes migrates as a monomer when the first dimension of the BN-PAGE gel is subjected to electrophoresis in the presence of SDS in the second dimension. Although this strongly argues against the presence of the ring form of Atp9p in the complexes, it does not exclude the possibility that they may contain partially oligomerized Atp9p. It is conceivable that unlike the ring, which is stable in SDS, linear Atp9p oligomers may be SDS-labile. This raises the possibility that the complexes may be physical platforms for Atp9p ring formation.

The function of the Atp9p-Cox6p complexes in ATP synthase assembly was examined by following the kinetics of Atp9p ring formation in mitochondria from different mutants. In pulse-chase experiments mitochondria of a respiratory-competent strain of yeast showed a progressive decrease of monomeric Atp9p and appearance of the ring form during the chase period. Most of the ring appeared in the first 30 min of chase. This was also true of an *mss51* mutant blocked in translation of Cox1p, a *cox4* mutant blocked in COX assembly, and an *atp10* mutant arrested at a late step of ATP synthase biogenesis when the $F_1$-Atp9p ring intermediate interacts with another assembly module containing Atp6p, Atp8p, and most of the peripheral stalk subunits (1). The *cox6* mutant differed from these strains in two respects. First, there was substantially less newly translated Atp9p associated with the ring in the *cox6* than in other cytochrome oxidase-deficient mutants. This was evident from the total radioactive Atp9p in the ring. Second, pulse-chase labeling of mitochondria indicated that the *cox6* mutant was less efficient in ring assembly than the parental wild type strain or in the *mss51* and *cox4* null mutant, two other COX-deficient strains. These findings indicate that Cox6p either directly or indirectly plays a role in Atp9p ring assembly.

A role of Cox6p in oligomerization of Atp9p is also supported by the results of pulse-chase labeling of mitochondria from cells transitioning from fermentative to aerobic growth. Mitochondria of glucose-repressed respiratory-competent or deficient strains were severely deficient in translation. This activity was largely restored after 3.5 h of derepression in media with or without added chloramphenicol. In agreement with previous studies, the chloramphenicol treatment was more effective in enhancing translation of Atp9p than the other mitochondria gene products (1, 28). Ring assembly was also more efficient in mitochondria of wild type than the *cox6* mutant after derepression. The inclusion of chloramphenicol in the derepression medium further increased ring formation in wild type and the *cox4* but not the *cox6* mutant. This could be the result of the 2-fold increase of Cox6p in mitochondria of the chloramphenicol-grown wild type cells and the somewhat smaller increase in the *cox4* mutant.

The increase of ATP synthase in mitochondria of derepressed cells, estimated by Western analysis of mitochondrial extracts separated by BN-PAGE, was accompanied by similar increases of ATPase activity measured by an in-gel assay. This was true of the wild type and to a lesser degree of the three COX mutants. It is noteworthy that in the absence of Cox6p there is a reduction but not absence of ATP synthase. A somewhat smaller reduction of ATP synthase was also observed in the *cox4* mutant. This suggests that Cox6p in some way helps to improve assembly of ATP synthase, most likely by enhancing the efficiency of ring formation. This does not exclude that other factors related to the respiratory activity of the cell may also influence expression of the ATP synthase.

Our evidence indicates that Cox6p and Atp9p are part of the same complexes but not necessarily that they interact with each other directly. Nor is there evidence that the association of newly translated Atp9p with these complexes depends on Cox6p. It, therefore, remains in the realm of possibility that the complexes provide a scaffold for ring formation even in the absence of Cox6p, albeit the process is less efficient.

Although our evidence supports a role of Cox6p in expression of ATP synthase, the reverse may also be true. The high instability of mtDNA in ATP synthase mutants presents an experimental obstacle in assessing COX biogenesis in *atp9* mutants. Last, we wish to stress the obvious possibility that the

enhancement of Atp9p ring formation by Cox6p could be important in modulating assembly of the ATP synthase in a manner so as to match the production of COX under different physiological conditions.

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