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CARACTERIZACIÓN DEL PAPEL DEL EXTREMO CARBOXILO TERMINAL DE LA PROTEÍNA Cox1 EN LA REGULACIÓN NEGATIVA DE SU SÍNTESIS

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Dedicatorias

A mis padres Celia y Filemón que han sido mi ejemplo a seguir y a quienes les debo todo lo que soy.

A mis hermanos Carmen, Juan y Cecilia y a mi sobrina Zara, sin ustedes mi vida estaría vacía.

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CONTENIDO

Abreviaturas

APS	Sigla en inglés de "Amonium Persulfate"
BN-PAGE	Gel de acrilamida-bisacrilamida no desnaturalizante.
CcO	Citocromo *c* oxidasa.
Cox1	Subunidad 1 de citocromo *c* oxidasa.
Cox1ΔC15	Subunidad 1 de citocromo *c* oxidasa que carece de los últimos 15 aminoácidos del extremo carboxilo terminal (SPPAVHSFNTPAVQS).
Cox1ΔC11	Subunidad 1 de citocromo *c* oxidasa que carece de los últimos 11 aminoácidos del extremo carboxilo terminal (VHSFNTPAVQS).
Cox1ΔC5	Subunidad 1 de citocromo *c* oxidasa que carece de los últimos 5 aminoácidos del extremo carboxilo terminal (PAVQS).
Cox2	Subunidad 2 de citocromo *c* oxidasa.
EDTA	Siglas en inglés de "Ethylenediaminetetraacetic acid disodium".
HEPES	Siglas en inglés de "4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid".
ioTL	Traducción *in organello* (en mitocondrias aisladas).
ivTL	Traducción *in vivo* (en células completas).
mtDNA	DNA mitocondrial.
mRNA de *COX1*	RNA mensajero de *COX1.*
PMSF	Siglas en inglés de "Phenylmethanesulfonyl fluoride"
PVDF	Siglas en inglés de "Polyvinylidene fluoride".
SDS	Siglas en inglés de "Sodium dodecyl sulfate".
SDS-PAGE	Gel de acrilamida-bisacrilamida desnaturalizante, con SDS.
TLCK	Siglas en ingles de "N_α-Tosyl-L-lysine chloromethyl ketone hydrochloride".
UTR	Región no traducible.

Nomenclatura genética (Amberg et al., 2005)

- Los alelos dominantes se escriben con las primeras tres letras del nombre del gen en mayúsculas, seguidas por un número, todo en itálicas, por ejemplo, *LEU2* o *COX1*.

- Los alelos recesivos se escriben en minúsculas e itálicas, por ejemplo, *leu2* o *cox2-62*.

- La designación fenotípica corresponde a la designación del gen seguida de un símbolo positivo o negativo, ejemplo, Leu$^+$ y Leu$^-$.

- Las proteínas se representan con la primera letra en mayúscula y el resto en minúscula, por ejemplo, Leu2.

- La inserción de un gen (*LEU2*) en el locus de otro gen (*ARG8*), sin que se interrumpa la función de ninguno de los dos genes, *ARG8::LEU2*.

- La inserción de un gen (*LEU2*) en el locus de otro gen (*ARG8*), en donde se interrumpe la función del segundo, *arg8::LEU2*

- Cepa rho+ (ρ^+)   Cepa de levadura con DNA mitocondrial completo.

- Cepa rho- (ρ^-)   Cepa de levadura con DNA mitocondrial incompleto.

- Cepa rho0 (ρ^0)   Cepa de levadura sin DNA mitocondrial.

- Cepa rho- sintética (ρ^{-s})   Cepa de levadura obtenida al bombardear a una cepa ρ^0 con un plásmido con las diferentes mutantes de genes mitocondriales.

Resumen

La citocromo *c* oxidasa (CcO) es el último aceptor de electrones de la cadena respiratoria mitocondrial. En la levadura *Saccharomyces cerevisiae* está formada por once subunidades, de las cuales Cox1, Cox2 y Cox3 son codificadas en el DNA mitocondrial y por lo tanto sintetizadas por los ribosomas mitocondriales y forman el centro catalítico de la enzima. La transcripción y traducción de las subunidades mitocondriales está acoplada a la inserción en la membrana interna mitocondrial. Cox1 es la subunidad más grande de la CcO. Ésta consiste de doce cruces transmembranales, donde se localizan los grupos hemo *a* y a_3 y el centro Cu_B, los cuales forman parte del sitio catalítico de la enzima. Cox1 tiene un papel central en la biogénesis de la CcO. Varios estudios han demostrado que el marcaje radioactivo de Cox1 disminuye drásticamente cuando el ensamblaje de la CcO se ve afectado. Hasta la fecha los mecanismos que regulan negativamente la síntesis de Cox1 no están bien entendidos.

La biogénesis de Cox1 depende principalmente de cinco proteínas: Pet309, la cual activa la traducción del mRNA de *COX1*, al actuar en su extremo 5´UTR; Mss51, tiene un papel similar a Pet309, pero también se une a Cox1 recién sintetizada; Cox14, interactúa con Mss51 y con Cox1 recién sintetizada y estabiliza el complejo Cox1-Mss51. Finalmente, Shy1 y Coa1 interaccionan con Cox1 recién sintetizada y regulan el ensamblaje postraduccional.

El objetivo de este trabajo fue entender los mecanismos que regulan negativamente la expresión de Cox1. Encontramos que la traducción del mRNA de *COX1* disminuye cuando existen mutaciones que afectan el ensamblaje de la CcO. Mediante manipulación del DNA mitocondrial demostramos que esta regulación requiere de la presencia en *cis* de las regiones UTR del mRNA de *COX1* y de la región codificante. Cox1 tiene un extremo carboxilo terminal expuesto a la matriz mitocondrial. Al eliminar los últimos 15 aminoácidos del extremo carboxilo terminal de Cox1 determinamos que esta región es importante para la regulación traduccional de la proteína. Esta región de Cox1 es necesaria para estabilizar la asociación de Mss51 y Cox14, dos factores involucrados en la traducción de Cox1. Finalmente, encontramos que Pet54 es un nuevo factor que facilita la síntesis de Cox1.

Abstract

The cytochrome *c* oxidase (CcO), the last electron acceptor of the mitochondrial respiratory chain, is formed by eleven subunits in the yeast *Saccharomyces cerevisiae*. Cox1, Cox2 and Cox3 are the subunits encoded by mitochondrial DNA and are the catalytic core of the CcO. Transcription and translation of the mitochondrial encoded subunits seems to be coupled to insertion of newly made proteins into the inner membrane. The largest subunit of CcO is Cox1, constituted by twelve transmembrane regions, where hemes a - a_3 and the site Cu_B are located and reduction of oxygen to water occurs. Cox1 has a central role in the start of the assembly of CcO. Several studies have demonstrated that mutations that affect CcO assembly significantly reduce radioactive labelling of Cox1.

To date, the mechanism controlling this down-regulation is not well understood. Cox1 synthesis and insertion is known to be regulated by at least five proteins: Pet309, which activates the translation of the *COX1* mRNA by acting on the 5´UTR; Mss51 has a similar role to Pet309 but also interacts with newly made, unassembled Cox1; Cox14 interacts with Mss51 and with newly made Cox1, sensing the assembly and stabilizing the Cox1-Mss51 complex. Shy1 and Coa1 interact with newly synthesized Cox1 and regulate the post-translational CcO assembly. The aim of this study was to understand the mechanisms that down-regulate expression of Cox1.

We found that *COX1* mRNA translation is decreased by mutations disturbing CcO assembly. By mitochondrial DNA mutagenesis we demonstrated that this regulation appears to require the presence in *cis* of the *COX1* mRNA untranslated regions and the coding sequence. In addition, Cox1 has a large carboxyl terminal region of 56 amino acids, this portion of the protein faces the mitochondrial matrix. By deletion of the last 15 amino acids of the Cox1 C-terminal region we found that regulation of Cox1 synthesis depends on this region. This region of Cox1 is necessary for stable association of Mss51 and Cox14, two factors involved in Cox1 biogenesis. Finally, we found that Pet54 is a new factor that facilitates the Cox1 synthesis.

Introducción

Saccharomyces cerevisiae como modelo de estudio

S. cerevisiae es una levadura anaeróbica facultativa. Esto significa que puede crecer en fuentes de carbono fermentables o en ausencia de oxígeno. El hecho de que la respiración no sea esencial para su viabilidad hace a este organismo un modelo ideal para estudiar los mecanismos genéticos y bioquímicos responsables del funcionamiento mitocondrial. De manera tradicional, todas aquellas mutantes de levadura que no respira, son llamadas mutantes "pet" (Tzagoloff and Dieckmann, 1990).

El DNA mitocondrial (mtDNA) de esta levadura puede ser manipulado genéticamente, lo cual ha facilitado la disección de los mecanismos de expresión del genoma mitocondrial. Dada la facilidad de manipulación bioquímica y genética y la disponibilidad de la secuencia de los genomás nuclear y mitocondrial (Foury et al., 1998), los análisis funcionales de sus genes y proteínas son relativamente fáciles (Bonnefoy and Fox, 2002).

Aunque la levadura es un organismo relativamente sencillo, muchas de las proteínas descubiertas en ésta tienen homólogos mitocondriales en humanos. Por esto, parte del trabajo en este organismo ha contribuido al entendimiento de enfermedades debidas a deficiencias en la citocromo *c* oxidasa (CcO) ocasionadas por mutaciones en genes nucleares que afectan la biogénesis mitocondrial (Foury and Kucej, 2002; Zee and Glerum, 2006).

La mitocondria y su genoma

En la mitocondria se realizan procesos como el metabolismo de aminoácidos y lípidos, la homeostasis de iones, la síntesis de grupos hemo y la apoptosis, entre muchas otras funciones. Sin embargo, a pesar de participar en una gran cantidad de procesos bioquímicos, sólo unos pocos componentes moleculares de esas vías son codificados por el mtDNA (Burger et al., 2003).

Es el organelo encargado de la mayor parte de la producción de ATP en casi todos los organismos eucariontes. Aunque se describe como un organelo de forma esférica con un tamaño de 1 a 2 μm de diámetro, es muy flexible estructuralmente, ya que se encuentra formando una red en el citoplasma que es continuamente remodelada

por mecanismos de fusión y fisión membranal (Mannella, 2008). Este organelo tradicionalmente se ha dividido en cuatro regiones: membrana externa, espacio intermembranal, membrana interna y matriz mitocondrial. La membrana externa es permeable a moléculas de 3 kDa o menores debido a la presencia de canales no selectivos formados principalmente de porina (Manon et al., 1998). La membrana interna forma pliegues hacia la matriz mitocondrial, llamados crestas mitocondriales (Scheffler, 2001); esta membrana es la barrera más selectiva del contenido mitocondrial. En la matriz mitocondrial se encuentra su propio material genético y la maquinaria necesaria para expresar éste genoma (Dujon, 1981).

El mtDNA de *S. cerevisiae* es una molécula de 75 a 80 kpb, dependiendo del contenido de intrones de cada cepa. Contiene los genes de las subunidades I, II y III de la citocromo *c* oxidasa (*COX1*, *COX2* y *COX3* respectivamente), el gen del citocromo *b* (*COB*) (Slonimski and Tzagoloff, 1976), los genes de las subunidades 6, 8 y 9 de la ATP sintasa (*ATP6*, *ATP8* y *ATP9* respectivamente), el gen de una proteína de la subunidad pequeña ribosomal (*VAR1*), los genes de los rRNAs *21S* y *15S* y de 24 tRNAs, así como un RNA (*ENS2*) que es componente de una enzima semejante a la RNasa-P (Costanzo and Fox, 1990; Foury et al., 1998).

Hasta donde se sabe, la regulación en la expresión genética mitocondrial se lleva a cabo principalmente a nivel traduccional. La traducción de los mRNA mitocondriales no depende de secuencias Shine-Dalgarno, sino que participan activadores traduccionales codificados en el núcleo e importados a la mitocondria. Estos activadores traduccionales se encuentran asociados a la membrana interna mitocondrial y actúan de manera específica sobre el extremo 5´ no traducido (5´UTR) de cada mRNA mitocondrial (Fox, 1996a; Fox, 1996b; Perez-Martinez et al., 2008), para activar la síntesis de las proteínas mitocondriales. Existen númerosos reportes que apoyan la hipótesis de que los activadores traduccionales promueven la síntesis de las proteínas mitocondriales cerca de su lugar de inserción en la membrana interna mitocondrial (Brown et al., 1994; Costanzo and Fox, 1988; Manthey and McEwen, 1995; Manthey et al., 1998; Mulero and Fox, 1993a; Mulero and Fox, 1993b; Perez-Martinez et al., 2003). También, se ha reportado que los ribosomás mitocondriales están unidos a la membrana interna del lado de la matriz. De esta manera se facilita la inserción co-traduccional de las subunidades de los complejos de la cadena respiratoria en la membrana interna mitocondrial (Szyrach et al., 2003, Jia, 2003 #95).

Citocromo *c* oxidasa (CcO) (E. C. 1.9.3.1)

La CcO es el último complejo enzimático de la cadena respiratoria. Acopla la transferencia de electrones entre el citocromo *c* y el oxígeno molecular a la translocación de protones a través de la membrana interna de la mitocondria (Hatefi, 1985). La CcO de la mayoría de procariontes consiste únicamente de tres subunidades, mientras que la CcO de eucariontes consiste de once a trece subunidades, dependiendo del organismo. (Michel et al., 1998; Ostermeier et al., 1997).

Los electrones entran a la CcO a través de un sitio Cu_A localizado en Cox2. Éstos son transferidos al centro catalítico embebido en Cox1, el cual contiene un hemo *a* y un centro hemo a_3-Cu_B. Es en este último sitio donde el oxígeno se une y se reduce a agua. Cox1 se encuentra rodeada por las subunidades nucleares, las cuales son importantes para el ensamblaje y la función de la enzima, así como para la regulación catalítica (Michel et al., 1998; Tsukihara et al., 1996) (Figura 1).

El complejo de la CcO de levadura consiste de once subunidades (Michel et al., 1998). Ocho subunidades son codificadas por genes nucleares, sintetizadas por ribosomás citoplásmicos e importadas a la mitocondria. Las otras tres subunidades Cox1, Cox2 y Cox3 son codificadas en el genoma mitocondrial y sintetizadas por los ribosomás mitocondriales (Fox, 1996b). Estas subunidades forman el núcleo catalítico de la enzima, son las más grandes y están altamente conservadas desde bacterias hasta mamíferos (Tsukihara et al., 1996) (Figura 1).
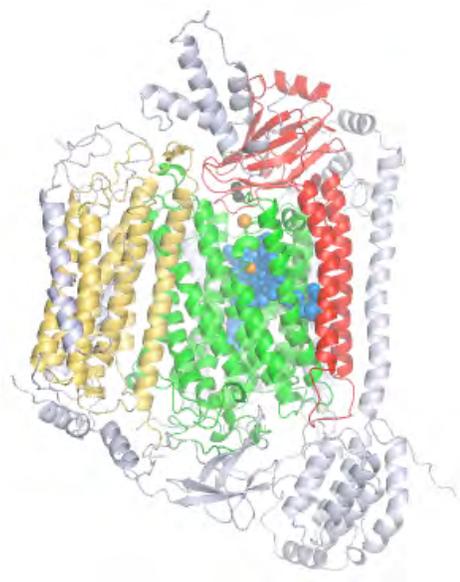
Figura 1. Estructura cristalina del monómero de la CcO de bovino. Se muestran en color los cofactores y las tres subunidades codificadas en el mtDNA y que forman el núcleo catalítico de la enzima. Cox1 en verde, Cox2 en rojo, Cox3 en amarillo, los grupos hemo en azul, los átomos de Cu_A y Cu_B en negro y anaranjado, respectivamente. Las subunidades nucleares, coloreadas en gris claro, regulan la actividad y estructura de la enzima. Modificado de (Tsukihara et al., 1996).

En los últimos años el estudio de la biogénesis de la CcO ha sido de gran interés ya que defectos en el ensamblaje de esta enzima son una de las principales causas de enfermedades mitocondriales en humanos (Fontanesi et al., 2006; Foury and Kucej, 2002).

Biogénesis de la subunidad 1 de la CcO (Cox1)

Cox1 es la subunidad más grande e hidrofóbica de la CcO. Ésta cruza doce veces la membrana interna mitocondrial. En las regiones transmembranales se encuentran embebidos dos centros redox, uno formado por un grupo hemo *a* y otro por un grupo *hemo a_3* y un átomo de Cu_B. Es la subunidad más conservada, clave en el ensamblaje de la CcO y parte fundamental del sitio catalítico de la enzima (Tsukihara et al., 1996). La Cox1 de *S. cerevisiae* presenta un extremo amino terminal de 6 aminoácidos y un extremo carboxilo terminal soluble que consta de 56 aminoácidos, ambos expuestos hacia la matriz mitocondrial (Figura 2). La biogénesis de Cox1 es el primer paso en el proceso de ensamblaje de la CcO (Horan et al., 2005; Nijtmans et al., 1998).
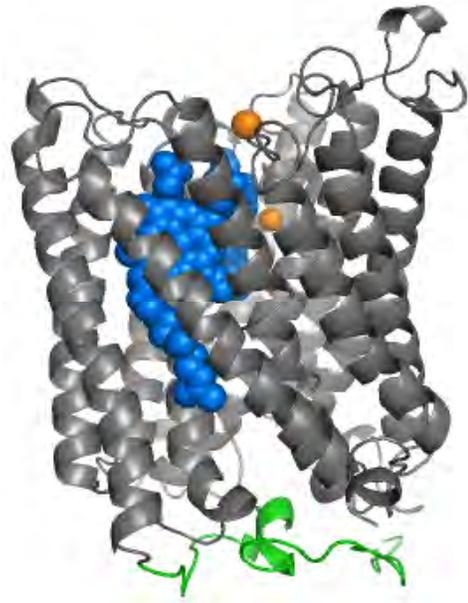
Figura 2. Modelo cristalino de Cox1 de bovino. Se muestran en gris los doce cruces transmembranales de Cox1. En verde el extremo carboxilo terminal de la proteína. En azul los grupos hemo *a* y *a_3* y en naranja los átomos de Cu_B, parte del sitio catalítico. Modificado de (Tsukihara et al., 1996).

En *S. cerevisiae* el gen *COX1* es transcrito como un RNA precursor policistrónico (Figura 3), el cual contiene los genes *COX1, ATP8, ATP6* y *ENS2* que codifican a la subunidad 1 de la CcO, las subunidades 8 y 6 de la ATPasa, y un RNA que es parte de la endonucleasa mitocondrial, respectivamente. Este transcrito policistrónico es procesado entre los cistrones *COX1* y *ATP8,* liberando así el transcrito *COX1* del policistrón *ATP*8*-ATP*6*-ENS*2. En algunas cepas de *S. cerevisiae* la región codificante de *COX1* contiene intrones, los cuales deben ser removidos para que la proteína funcional Cox1 pueda ser traducida. Por lo tanto, la expresión del gen *COX1* involucra varios pasos de procesamiento del RNA, para así traducir el mRNA de *COX1* maduro (Figura 3) (Dieckmann and Staples, 1994; Lipinski et al., 2010).
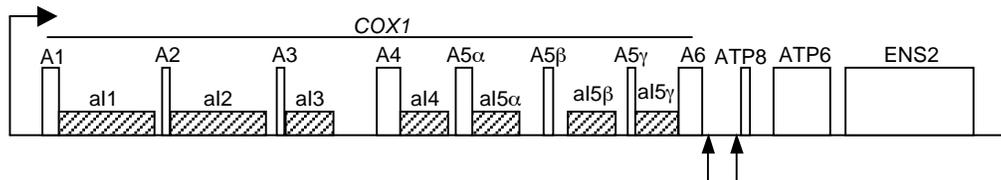
Figura 3. Unidad de transcripción de *COX1*, *ATP8*, *ATP6* y *ENS*2. Exones (□) de COX1 (numerados como A1-A6) y de *ATP8, ATP6* y *ENS2*; intrones (▨) denominados aI1-aI5γ. Las flechas indican sitios de procesamiento en el extremo 3'-UTR de *COX1*. Modificado de (Dieckmann and Staples, 1994).

Factores que participan en la síntesis o ensamblaje de Cox1

La expresión del gen *COX1* es muy compleja y poco comprendida. Se han identificado y caracterizado un gran número de proteínas que facilitan la expresión de este gen. Estas proteínas ayudan al procesamiento del mRNA de *COX1*, al ensamblaje de los grupos hemo, a la inserción de los cofactores metálicos y a la translocación e inserción a la membrana interna mitocondrial (Barros et al., 2006; Broadley et al., 2001; Costanzo and Fox, 1988; Costanzo et al., 1986; Fontanesi et al., 2006; Haffter et al., 1990; Herrmann and Funes, 2005).

Las proteínas Pet309 y Mss51 activan la traducción de Cox1 (Manthey and McEwen, 1995; Perez-Martinez et al., 2003; Siep et al., 2000) al actuar específicamente sobre el mRNA de *COX1*. Se cree que Pet309 ayuda a la identificación del lugar de inicio de la traducción por parte del ribosoma (Fox, 1996b; Tavares-Carreon et al.,

2008). Mss51 y Cox14 se unen a Cox1 recién sintetizada para facilitar y sensar la inserción y/o ensamblaje en la membrana interna (Barrientos et al., 2004; Perez-Martinez et al., 2003). Recientemente se ha propuesto que Cox24 participa en el procesamiento del transcrito de *COX1* y probablemente participa en la traducción del mismo (Barros et al., 2006). Shy1 y Coa1 participan en el ensamblaje de la CcO y se asocian a Mss51; esta asociación depende de la presencia de Cox14 (Barrientos et al., 2002; Mick et al., 2007; Pierrel et al., 2007). Hasta el momento éstas son las proteínas reportadas que participan específicamente en el proceso de traducción-inserción de Cox1 en la membrana interna (Figura 4).
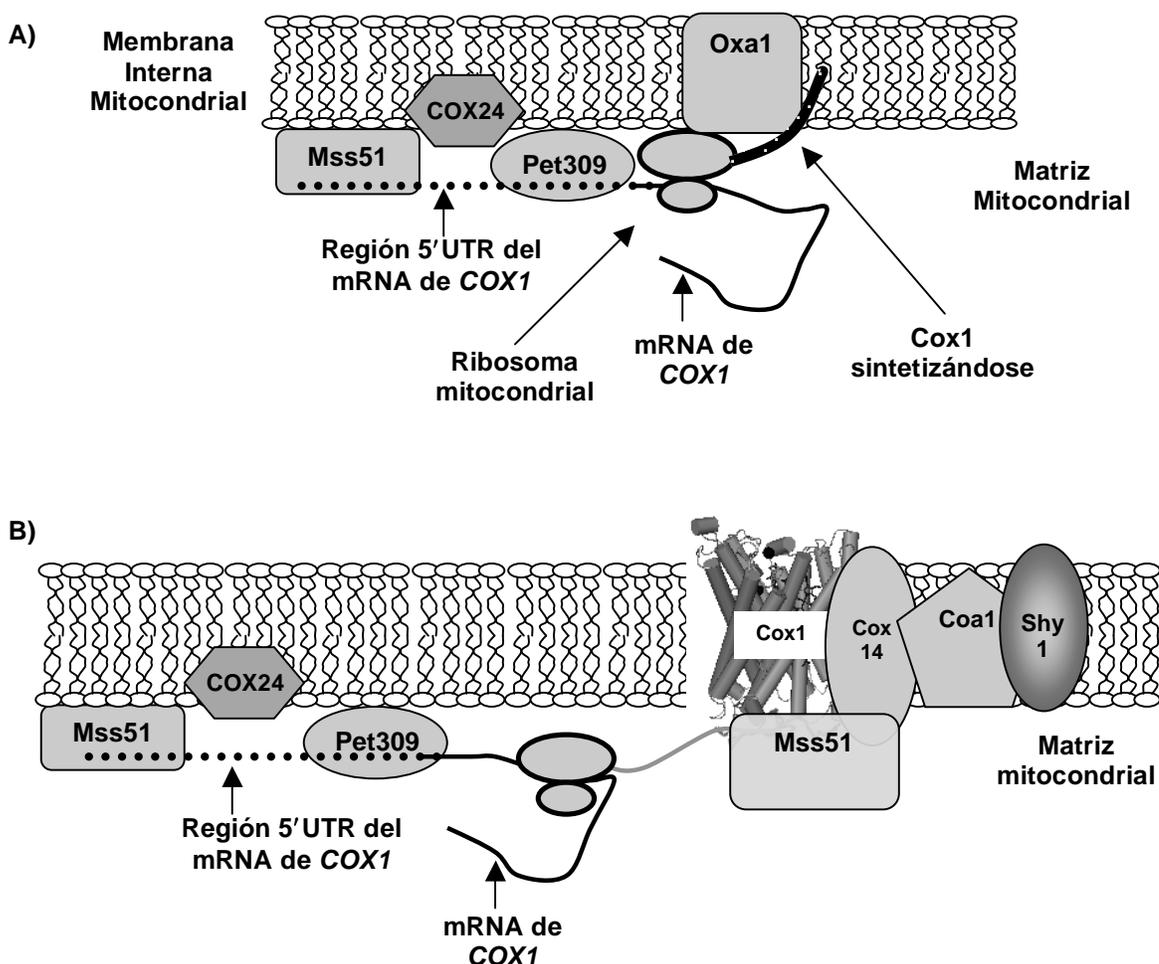
Figura 4. Modelo de acción de las proteínas involucradas en la biogénesis de Cox1. **(A)** Cox24 participa en el procesamiento del transcrito de *COX1*. Pet309 y Mss51 activan la traducción del mRNA de *COX1*. Oxa1 es una proteína de "uso general" que facilita la inserción de Cox1 a la membrana interna mitocondrial. **(B)** Mss51, Cox14, Shy1 y Coa1 se unen a Cox1 recién sintetizada para facilitar la inserción y/o ensamblaje en la membrana interna.

Pet309 es una proteína de aproximadamente 106 kDa y es el activador traduccional de *COX1*. Mutantes nulas de *S. cerevisiae* en esta proteína no pueden procesar el pre-mRNA de *COX1* y tampoco pueden traducir el RNA maduro. Pet309 es el único activador traduccional de levadura descrito que presenta dominios PPR (por las siglas en inglés de "Pentatrico Peptide Repeat"). Los dominios PPR son motivos que consisten de 35 aminoácidos que usualmente aparecen en repeticiones de 2 a 26 en serie en una proteína. Evidencia genética sugiere que los motivos PPR se unen a secuencias específicas en el RNA ayudando a la maduración y expresión del RNA en organelos (Shikanai, 2006). Datos genéticos y bioquímicos indican que Pet309 actúa específicamente con la región 5´-UTR de *COX1* para así promover la traducción de Cox1 (Manthey and McEwen, 1995); Tavares-Carreon, sin publicar). Trabajo previo de nuestro grupo mostró que Pet309 es una proteína periférica de membrana interna mitocondrial. Se ha propuesto que esta localización es importante para ayudar a que el inicio de la síntesis de Cox1 esté cerca de su sitio de inserción en la membrana interna mitocondrial (Tavares-Carreon et al., 2008).

Mss51 es una proteína periférica de membrana (Siep et al., 2000) de aproximadamente 51 kDa y se ha propuesto como otro activador traduccional de Cox1 que actúa en el extremo 5´UTR del mRNA (Perez-Martinez et al., 2009). Mutantes nulas de *S. cerevisiae* en este gen no sintetizan Cox1; sin embargo, a diferencia de lo que se observa en mutantes de Pet309, el mRNA de *COX1* es tan estable como en la cepa silvestre (Zambrano et al., 2007). Por otro lado, se ha reportado que Mss51 interactúa físicamente con Cox1 recién sintetizada y que es limitante para la traducción de *COX1* (Perez-Martinez et al., 2003; Perez-Martinez et al., 2009).

Cox14 es de aproximadamente 8 kDa. Aunque en su estructura se predice un cruce trasmembranal, no se tiene clara su asociación con la membrana interna mitocondrial (si es proteína integral o periférica de membrana) (Barrientos et al., 2004; Glerum et al., 1995). Mutantes nulas de *S. cerevisiae* en este gen sintetizan Cox1, pero carecen de CcO funcional. Esto sugiere que Cox14 participa en el ensamblaje de Cox1 y tiene un papel de regulador negativo de la síntesis de Cox1 cuando existen defectos en el ensamblaje de la CcO (Barrientos et al., 2004). También se ha reportado que Cox14 interactúa físicamente con Mss51 y con Cox1 recién sintetizada. La asociación de Cox14 con Mss51 depende de la presencia de Cox1; de igual manera la unión entre Mss51 y Cox1 depende de Cox14 (Perez-Martinez et al., 2009).

Cox24, una proteína integral de membrana, es de aproximadamente 12 kDa. Mutantes nulas de *S. cerevisiae* en este gen no sintetizan Cox1, ya que no se acumula el transcrito de *COX1* maduro. Sin embargo, se ha propuesto que tiene un papel adicional en la traducción o pasos postraduccionales (Barros et al., 2006); García-Villegas, datos sin publicar de nuestro laboratorio).

Shy1 es una proteína integral de membrana, de aproximadamente 45 kDa. En mutantes nulas de este gen el marcaje radioactivo de Cox1 disminuye. Se cree que Shy1 facilita la formación del grupo hemo a_3-Cu_B de Cox1 (Barrientos et al., 2002; Khalimonchuk et al., 2009; Mick et al., 2007).

Coa1 es una proteína de aproximadamente 22 kDa. Mutantes nulas de *S. cerevisiae* en este gen sintetizan Cox1 y presentan una actividad muy disminuida de la CcO. Se ha reportado que Coa1 se asocia a Mss51, Cox14 y Shy1 así como a Cox1 recién sintetizada. Aunque no se conoce claramente su función, se cree que Coa1 facilita la formación del complejo Cox1-Cox14-Shy1 y así facilita la inserción de los grupos hemo (Mick et al., 2007; Pierrel et al., 2007). Se piensa que esta proteína actúa después de Mss51 (Khalimonchuk et al., 2009).

Se ha propuesto que la inserción de cofactores a Cox1 se da de manera co-traduccional (Carr and Winge, 2003) y que las subunidades Cox5 y Cox6 forman un subcomplejo de ensamblaje junto con Cox1, siendo probablemente Cox6 la primera subunidad en unirse a Cox1, formando un centro de nucleación sobre el cual se ensamblan las otras subunidades (Horan et al., 2005).

Justificación

La biogénesis de Cox1 es muy compleja y poco entendida. En diversos estudios se ha observado que el marcaje radioactivo con [35S]-Met de Cox1 disminuye fuertemente en mutantes nucleares o mitocondriales que afectan el ensamblaje de la CcO, sin que estas mutaciones estén en el gen mitocondrial *COX1* (Figura 5) (Barrientos et al., 2004; Cabral and Schatz, 1978; Glerum and Tzagoloff, 1997). Incluso se observa que al afectar la formación de otros complejos, como la ATPsintasa, la síntesis de Cox1 disminuye drásticamente (Rak et al., 2007). Hasta la fecha no se ha entendido a que se debe esta disminución en el marcaje radioactivo, no se sabe si se debe a una menor traducción o a una alta degradación proteolítica.
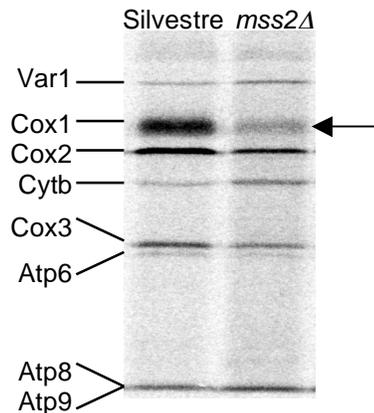
Figura 5. Traducción de proteínas mitocondriales *in vivo* en presencia de cicloheximida, un inhibidor de la síntesis de proteínas citoplásmicas. En la cepa que porta la mutación que afecta el ensamblaje de la CcO (*mss2Δ*) se observa una disminución en el marcaje radioactivo de la proteína Cox1 (señalado con la flecha). Se muestran las ocho proteínas codificadas en el genoma mitocondrial. Las proteínas se separaron en un gel de acrilamida-SDS al 16%/0.15% tipo Laemmli, el gel se secó y se reveló por autorradiografía.

Como se mencionó anteriormente, Cox1 es una proteína hidrofóbica embebida en la membrana interna mitocondrial. La región más grande de la proteína que se encuentra expuesta a la matriz mitocondrial es el extremo carboxilo terminal, el cual consiste de 56 aminoácidos. Creemos que esta región podría ser importante en la regulación de su propia síntesis, ya que podría ser el sitio de reconocimiento de proteínas periféricas como Mss51.

Hipótesis y objetivos

Hipótesis

Debido a que Cox1 es una proteína clave en el ensamblaje de la CcO cuyo marcaje disminuye cuando existen defectos en el ensamblaje de la CcO y que además contiene un extremo carboxilo terminal soluble, expuesto hacia la matriz mitocondrial, se propone que éste podría ser el sitio de interacción de factores como Mss51. Creemos que los extremos UTR del mRNA de *COX1* están participando en esta regulación negativa de la síntesis.

Objetivo general

Estudiar el mecanismo o mecanismos por los cuales la síntesis de Cox1 se regula a través de su extremo carboxilo terminal y los extremos UTR, en presencia de defectos en el ensamblaje de la CcO.

Objetivos particulares

- Determinar si la disminución en el marcaje radioactivo de Cox1 se debe a una menor traducción.

- Analizar si los extremos UTR del mRNA de *COX1* participan en la regulación negativa de su traducción.

- Generar mutaciones en el extremo carboxilo terminal de Cox1 y analizar el efecto en la regulación negativa de su síntesis.

- Generar y analizar mutantes de diferentes subunidades y chaperonas del ensamblaje de la CcO para determinar qué tan general es el efecto observado.

- Estudiar si las mutantes en el carboxilo terminal de Cox1 interactúan con Mss51 y Cox14 en cepas silvestres y en cepas con defectos en el ensamblaje de CcO.

Materiales y métodos

Ver el Apéndice 2 para la lista de cepas y el Apéndice 3 para las lista de oligonucleótidos y plásmidos utilizados en este trabajo

Medios de cultivo

YPD (1 litro)

Extracto de levadura	10 g
Peptona	20 g
Dextrosa	20 g
Adenina	0.03 g

Para medio sólido agregar 20 g de agar. Esterilizar en autoclave.

YPR (1 litro)

Extracto de levadura	10 g
Peptona	20 g
Rafinosa	20 g
Adenina	0.03 g

Para medio sólido agregar 20 g de agar. Esterilizar en autoclave.

YPGal (1 litro)

Extracto de levadura	10 g
Peptona	20 g
Galactosa	20 g
Adenina	0.03 g

Para medio sólido agregar 20 g de agar. Esterilizar en autoclave.

YPEG (1 litro)

Extracto de levadura	10 g
Peptona	20 g
Glicerol	30 ml
Etanol	30 ml (adicionar después de esterilizar y cuando el medio este a una T= 50 °C)

| Adenina | 0.03 g |
| Agar | 20 g |

Esterilizar en autoclave.

Gal/-Met (1 litro)

Base nitrogenada sin aminoácidos	6.7 g
Dextrosa	20 g
Mezcla de suplementos -Met	0.75 g

Para medio sólido agregar 20 g de agar. Esterilizar en autoclave.

-Ura/Sorbitol (1 litro)

Base nitrogenada de levadura	6.7 g
Glucosa	50 g
Sorbitol	1 M
Adenina	100 µg/ml
Mezcla de suplementos -Ura	0.79 g
Agar	30 g

Generación de mutantes nucleares (Amberg et al., 2005)

Nota: Ver la lista de oligonucleótidos en el Apéndice.

Las mutantes nucleares de eliminación de proteínas por casetes de resistencia a kanamicina (*KanMX4*) o de marcadores de auxotrofía (*LEU2* o *URA3*) se construyeron por PCR y recombinación homóloga.

El gen de interés se amplificó por PCR a partir de DNA total de levadura utilizando un juego de oligonucleótidos específicos para cada gen (Ver Apéndice). Los productos de PCR se separaron en un gel de agarosa al 1% teñido con bromuro de etidio y se purificaron siguiendo las instrucciones del kit de QIAGEN para purificar productos de PCR.

Purificación de DNA total de levadura (Burke Dan, 2000)

- Cultivar dos o tres colonias de levadura en 2 ml de medio YPD toda la noche a 30 °C.
- Centrifugar los cultivos 1 min. a 12000 rpm (13200 g) en una centrífuga Eppendorf 5415D, a temperatura ambiente.
- Lavar las células con 500 µl de agua. Centrifugar como en el paso anterior.
- Resuspender el botón celular en 200 µl de amortiguador de lisis.
- Adicionar 200 µl de fenol/cloroformo/alcohol isoamílico (25:24:1).
- Agitar en vortex 3 minutos a velocidad máxima a temperatura ambiente.
- Centrifugar 5 minutos a 12000 rpm (13200 g) en una centrífuga Eppendorf 5415D, a temperatura ambiente.
- Separar la fase acuosa (fase superior) y poner en un tubo nuevo. Precipitar adicionando 1/10 volúmenes de acetato de sodio 3M pH 5.3 y 3 volúmenes de etanol al 100%. Incubar 10 minutos a -20 °C.
- Centrifugar la mezcla anterior 10 minutos a 12000 rpm (13200 g) en una centrífuga Eppendorf 5415D, a 4 °C.
- Lavar el botón de DNA con 1 ml de etanol al 70% previamente enfriado a -20 °C.
- Centrifugar 1 minuto a 12000 rpm (13200 g) en una centrífuga Eppendorf 5415D, a 4 °C. Eliminar completamente el etanol. Dejar secar las muestras al aire.
- Resuspender en 50 µl de agua estéril.

Amortiguador de lisis (20 ml)

Triton X-100	400 µl
SDS 20%	1 ml
NaCl 5M	400 µl
Tris-HCL 2M pH 8.0	100 µl
EDTA-Na 0.5M pH 8.0	40 µl
Agua	18.06 ml

Transformación de levadura (Burke Dan, 2000)

- Cultivar dos o tres colonias de levadura en 2 ml de medio YPD toda la noche a 30 °C.
- A la mañana siguiente adicionar 2 ml más de medio YPD e incubar 2 horas más a 30 °C.
- Centrífugar 600 µl de los cultivos a transformar 1 minuto a 12000 rpm (13200 g) en una centrífuga Eppendorf 5415D, a temperatura ambiente. Eliminar completamente el sobrenadante.
- Resuspender el botón celular en 52.5 µl de amortiguador de transformación.
- Adicionar el DNA (100 ng a varios µg).
- Para transformaciones en donde el DNA debe integrarse al genoma (productos de PCR):
  - Incubar 30 min a 30 °C, posteriormente incubar 30 min a 42 °C. Agitar ligeramente en vortex cada 10 min.
  - Adicionar 1 ml de medio YPD e incubar 2 horas a 30 °C.
  - Centrífugar los tubos 1 minuto a 12000 rpm (13200 g) en una centrífuga Eppendorf 5415D, a temperatura ambiente. Eliminar 900 µl del medio YPD.
- Para transformaciones de plásmidos:
  - Incubar 30 min a 42 °C agitando ligeramente en vortex cada 10 min.
- Plaquear la mezcla en medio de selección necesario.

Amortiguador de transformación (para una transformación)

PEG 3350 50%	40 µl
DTT 1M	5 µl
Acetato de litio 2M	5 µl
DNA de esperma de salmón 10 mg/ml	2.5 µl (antes de usar hervir 10 minutos y mantener en hielo)

Clonación de mutantes de Cox1

Nota: Ver la lista de oligonucleótidos en el Apéndice 3.

El gen mitocondrial *COX1*, flanqueado por 395 nucleótidos del extremo 5´UTR y de 990 nucleótidos del extremo 3´UTR, se clonó en el plásmido pBlueScript SK+, generando al plásmido pXPM57 (Figura 6).
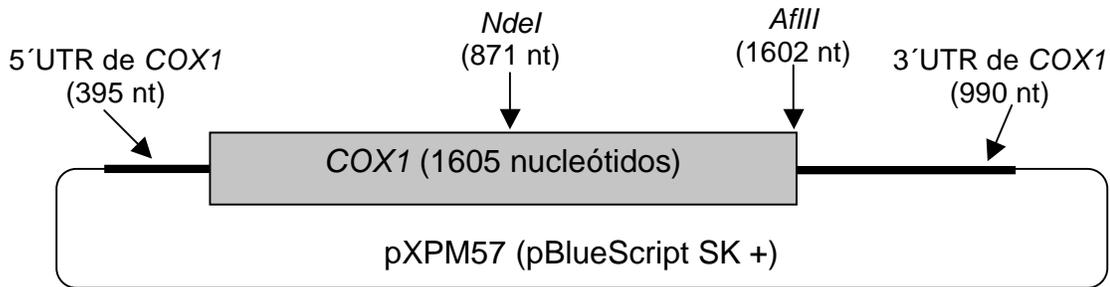
Figura 6. Estructura del plásmido pXPM57 el cual contiene al gen mitocondrial *COX1* flanqueado por sus regiones UTR. Se señalan los sitios de corte por las enzimás de restricción *NdeI* y *AflIII*.

Para eliminar los últimos 5 aminoácidos de Cox1 (PAVQS) se realizó un PCR a partir del DNA purificado de levadura utilizando los oligonucleótidos CO1NdeF y CO1R14-3´. Los oligonucleótidos contienen los sitios de digestión para las enzimas *NdeI* y *AflIII*, respectivamente. El producto de PCR (739 nt) se ligó al plásmido pGEM-T easy (Promega) para generar al plásmido pXPM57.

Posteriormente el plásmido pXPM57 se digirió con las enzimás *NdeI* y *AflIII* para liberar el producto de *COX1*. Este producto se ligó al plásmido pXPM57 previamente digerido con las mismas enzimas, generando de esta manera el plásmido pXPM59.

El mismo procedimiento se siguió para eliminar los últimos 11 (ΔC11, VHSFNTPAVQS) y 15 (ΔC15, SPPAVHSFNTPAVQS) aminoácidos de Cox1. Las reacciones de PCR se realizaron utilizando el oligonucleótido CO1NdeF con los oligonucleótidos CO1R15-3´ para ΔC11 y CO1R16-3´ para ΔC15. Los plásmidos generados son pXPM55 y pXPM56, respectivamente. Estos plásmidos se digirieron con las enzimas *NdeI* y *AflIII* para liberar a los diferentes insertos de *COX1*. Los productos

de la digestión se ligaron en el plásmido pXPM57 previamente digerido con las mismas enzimas. De esta manera se generaron los plásmidos pXPM60 (COX1-ΔC11) y pXPM61 (COX1-ΔC15) (ver tabla de plásmidos en el Apéndice). Estos plásmidos se secuenciaron y se utilizaron para generar las mutantes mitocondriales por biobalística.

Las mutaciones puntuales en el extremo carboxilo terminal de Cox1 se generaron por medio de PCR utilizando el oligonucleótido CO1NdeF con oligonucleótidos diseñados para introducir cada mutación. CO1-521R para introducir el cambio de dos prolinas en la posición 521 y 522 por alaninas (Cox1PP521,522AA), CO1-524R (Cox1V524E), CO1-525R (Cox1H525A), CO1-527R (Cox1F527A) y CO1-530R (Cox1P530A). Cada uno de estos oligonucleótidos contiene un sitio de digestión para *AflII*. Los productos de PCR se ligaron al plásmido pGEM T-easy para generar los plásmidos pXP132 al pXP136. Finalmente el inserto de *COX1* mutante se digirió con las enzimas *NdeI* y *AflII* y se ligó al plásmido pXPM57 digerido con las mismas enzimas. Los plásmidos resultantes se denominaron pXP139 al pXP143 (ver tabla de plásmidos en el Apéndice 3).

Generación de mutantes mitocondriales por biobalística (Bonnefoy and Fox, 2002)

Preparación de las células (cuatro horas antes de bombardear)
- Cultivar la cepa receptora ρ0 (NAB69 rho0) en 5 ml de medio YPR durante dos días a 30 °C con agitación continua.
- Tomar 500 µl del cultivo anterior e inocular 50 ml de medio YPR. Dejar crecer durante dos días a 30 °C con agitación continua. En promedio se usan 50 ml de este cultivo por cada plásmido a bombardear (seis cajas de Petri).
- Centrifugar las células a 4000 rpm (2831 g) por 10 min en el rotor JA10 (Beckman Coulter).
- Resuspender en 600 µl de medio líquido YPD.
- Extender 100 µl de la suspensión de células en cajas de Petri de medio –Ura /Sorbitol. Dejar secar de tres a cuatro horas a temperatura ambiente.

Preparación de los microproyectiles y precipitación del DNA (para seis cajas de Petri)

- Pesar 60 mg de las micropartículas de tungsteno en un tubo Eppendorf de 1.5 ml.
- Esterilizar las micropartículas resuspendiendo vigorosamente en 1.5 ml de etanol al 70 % (p/v). Incubar a temperatura ambiente por 10 minutos.
- Centrifugar 15 min a temperatura ambiente a 12000 rpm (13200 g) en una centrífuga Eppendorf 5415D. Eliminar cuidadosamente el sobrenadante.
- Lavar con 1.5 ml de agua estéril y resuspender a una concentración de 60 mg/ml en glicerol al 50 % (v/v). Mantener en hielo.

Nota: a partir de este paso realizar todo en hielo.

- Mezclar en un tubo Eppendorf de 1.5 ml, 5 µg del plásmido para la selección nuclear (YEp352) con 30 µg del plásmido con la construcción mitocondrial de interés en un volumen total no mayor a 15 µl.
- Adicionar, en este orden, 100 µl de la suspensión de partículas de tungsteno, 4 µl de espermidina 1 M y 100 µl de $CaCl_2$ 2.5 M. Agitar en vortex inmediatamente después de la adición de cada reactivo.
- Incubar de 10 a 15 min en hielo.
- Centrifugar 1 min a 4 °C a 12000 rpm (13200 g) en una centrífuga Eppendorf 5415D. Eliminar cuidadosamente el sobrenadante.
- Adicionar 200 µl de etanol al 100% a -20 °C. Resuspender hasta deshacer los agregados.
- Repetir el paso anterior hasta que las partículas se resuspendan fácilmente.
- Centrifugar 1 min a 4 °C a 12000 rpm (13200 g) en una centrífuga Eppendorf 5415D. Eliminar cuidadosamente el sobrenadante.
- Resuspender las partículas en 60 µl de etanol al 100%. Distribuir la suspensión en el centro de cada uno de los "macrocarriers" previamente colocados en los "holders". Se utilizan 6 "macrocarriers" por construcción.
- Dejar que se evapore el etanol.

Reactivos

Etanol 70%

Etanol 100%

Polvo de tungsteno 4-6 μm, pureza 99.99% (Alfa Aesar/Jonson Matthey, CAS# 7440-33-7).

Glicerol 50%

Plásmido con marcador nuclear (YEp352), concentración mínima 2 μg/μl.

Plásmido para transformación mitocondrial, concentración mínima 2 μg/μl.

Espermidina 1M

Cloruro de calcio 2.5M (esterilizar filtrando)

Bombardeo de células con los microproyectiles recubiertos de DNA.

- Lavar cuidadosamente la cámara del bombardeador humedeciendo completamente con etanol al 70%. Secar completamente la cámara.
- Seguir cuidadosamente las instrucciones del bombardeador (PDS-1000He Biolistic Gun, Biorad). Colocar el disco de ruptura en su lugar y apretar con la llave ligeramente hasta la marca.
- Colocar el "macrocarrier" en su "holder" en el sistema de ensamblaje.
- Colocar la caja Petri a 5 cm del "macrocarrier". La caja se coloca en el segundo nivel de abajo para arriba de la cámara.
- Abrir el tanque de helio a una presión de 1100 psi.
- Permitir que se genere el vacio en la cámara a una presión de 29 a 29.5 pulgadas de mercurio.
- Disparar
- Eliminar los fragmentos de los "macrocarriers" de la caja Petri con pinzas estériles.
- Incubar las placas a 30 °C por cuatro o cinco días hasta que las colonias de levadura aparezcan.

Reactivos y equipo

"Macrocarrier holders" (esterilizar en autoclave)

"Macrocarriers" (Biorad)

Discos de ruptura, 1100 psi

Equipo de bombardeo PDS-1000He (Biorad)

Tanque de helio

Bomba de vacio

Identificación de las transformantes rho- sintéticas (ρ-s) después del bombardeo.

- A los tres días de haber bombardeado, poner un cultivo de la cepa ρ+ (L74) en 3 ml de YPD. Esta cepa contiene una mutación puntual en el exón 4 de *COX1* (G124D) por lo que no respira. Al recombinar con la cepa ρ-s se complementa esta mutación generando cepas diploides que respiran.

- Replicar las placas de la cepa bombardeada ρ- sintética en una caja de – Ura/Sorbitol y en otra caja de YPD en la cual previamente se esparcieron 200 µl del cultivo de la cepa L74.

- Incubar 2 días a 30 °C, para permitir que se lleve a cabo el apareamiento entre la cepa ρ-s y L74 así como la recombinación de sus DNA mitocondriales.

- Replicar la caja de YPD con los diploides en medio YPEG para detectar a las colonias que respiran. Las colonias que respiran son las que portan el plásmido con *COX1* y que complementaron la mutación puntual de la cepa L74.

- Incubar 2 días a 30 °C.

- Resembrar en medio -Ura de 6 a 12 colonias ρ- sintéticas que respiren después de la recombinación con la cepa L74. Repetir los pasos anteriores de réplicas, recombinación y resiembra hasta obtener colonias puras (Figura 7).
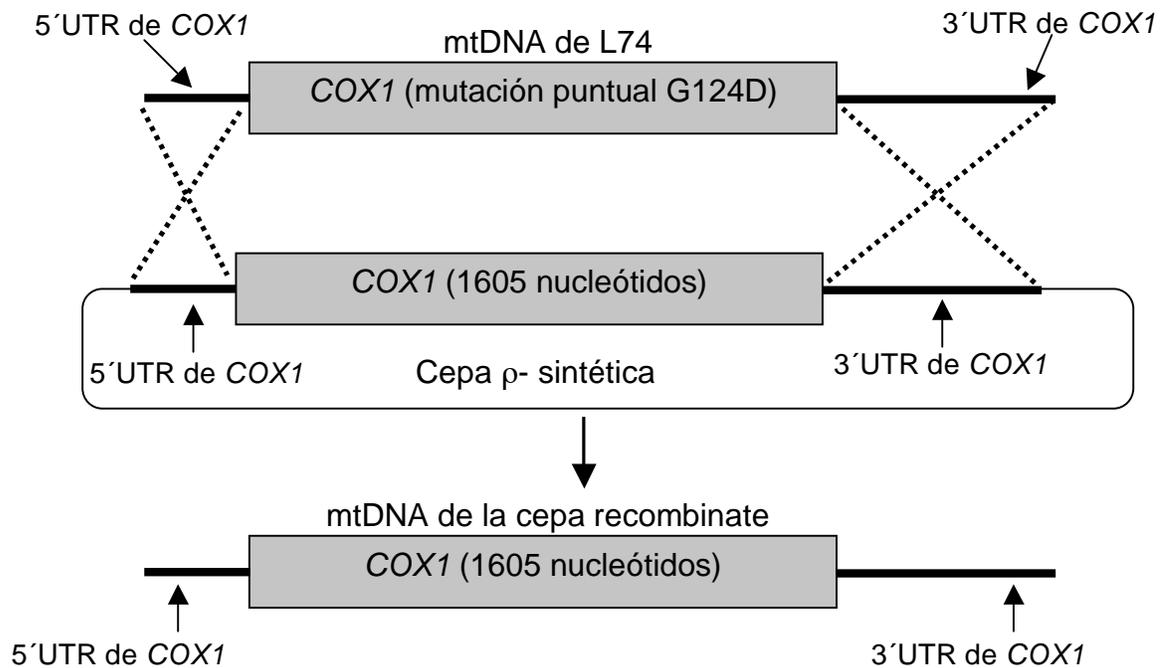
Figura 7. Recombinación de la cepa ρ- sintética (plásmido pXPM57) con la cepa L74, la cual contiene una mutación puntual, por lo que no respira. La cepa obtenida contiene el mtDNA de la cepa L74, en donde se ha eliminado el gen *COX1* silvestre y es reemplazado por el gen COX1 contenido en el plásmido bombardeado; esta cepa es capaz de crecer en medio respiratorio YPEG.

Obtención de la cepa ρ+ con el gen *COX1* mutante.

- Incubar 2 ml de cultivos en YPD de las cepas ρ- sintéticas y ρ+ (XPM10b) toda la noche a 30 °C. La cepas ρ- sintéticas contienen la mutación nuclear *kar1-1*, la cual previene la fusión nuclear durante la recombinación (Conde and Fink, 1976). En la cepa XPM10b se eliminó la región codificante de *COX1* y se reemplazó por el gen reportero ARG8m, el cual está flanqueado por los extremos UTR de *COX1*. Esta cepa no respira y crece en medio sin arginina (Perez-Martinez et al., 2003).

- En un tubo Eppendorf de 1.5 ml mezclar 750 µl de la cepa ρ- con 250 µl de la cepa XPM10b. Centrifugar 1 min a 4 12000 rpm (13200 g) en una centrífuga Eppendorf 5415D a temperatura ambiente. Eliminar la mayor cantidad de sobrenadante.

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- Resuspender las células en el volumen restante. Colocar la suspensión celular en una caja de YPD. Incubar a 30 °C de cuatro a cinco horas para permitir que se lleve a cabo el apareamiento entre la cepa ρ-s y XPM10b así como la recombinación de sus DNAs mitocondriales (Figura 8). Revisar la formación de cigotos al microscopio.

- Tomar un poco de las células e incubarlas durante cuatro horas o toda la noche en 2 ml de medio YPD a 30 °C.

- Diluir el cultivo 1 en 100 con agua estéril el cultivo y plaquear en el medio selectivo para la cepa receptora de acuerdo al genotipo nuclear.

- Incubar 2 días a 30 °C.

- Replicar la caja incubada durante dos días en otra caja de medio selectivo, en una caja con medio selectivo para detectar a los diploides formados, los cuales se descartan, así como en medio YPEG y en otra caja de YPD con el césped de una cepa que complemente la mutación mitocondrial (a Oxi3-7, ver lista de cepas en el apéndice 2).

- Incubar 2 días a 30 °C, para permitir que se lleve acabo la recombinación de sus DNA mitocondriales.

- Replicar la caja de YPD con el césped de la cepa a Oxi3-7 en medio YPEG para detectar a las cepas que respiran.

- Repetir los pasos anteriores de réplicas, recombinación y resiembras hasta obtener colonias puras.

- Una vez que se tengan colonias puras se purifica el DNA y se realiza PCR con los oligonucleótidos CO1NdeF y CO1R10-3´. La reacción de PCR se realiza en varias alícuotas, las cuales posteriormente se mezclan y se secuencia la mezcla, utilizando los mismos oligonucleótidos.
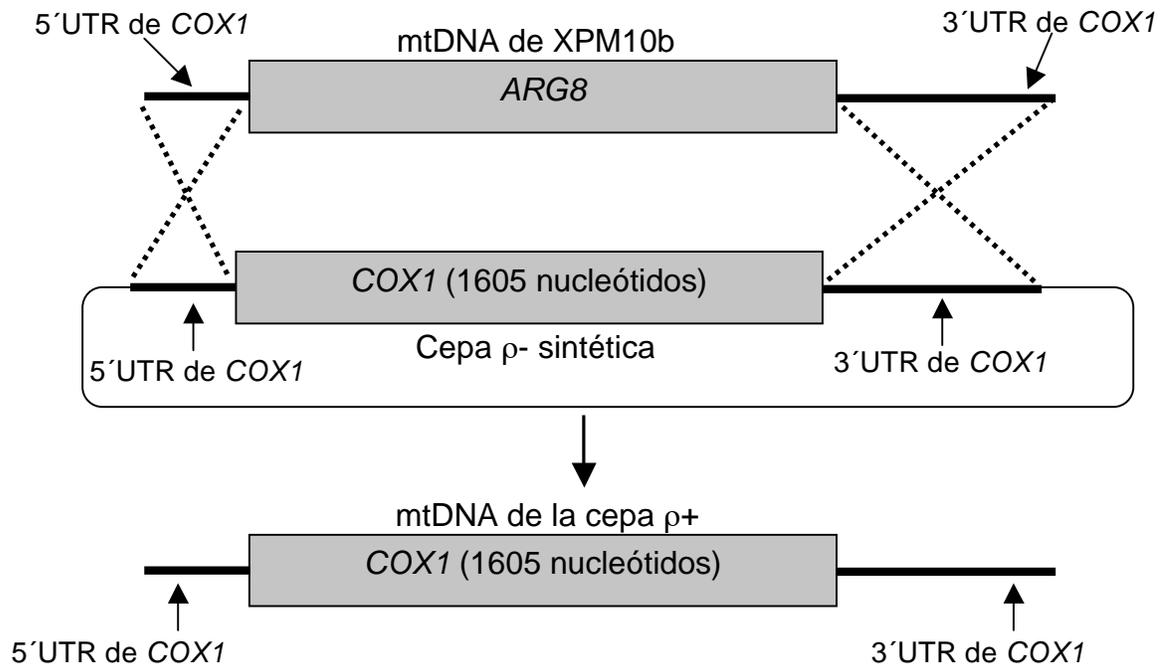
Figura 8. Recombinación de la cepa ρ- sintética (plásmido pXPM57) con la cepa L74, la cual contiene una mutación puntual, por lo que no respira. La cepa obtenida contiene el mtDNA de la cepa L74, en donde se ha eliminado el gen *COX1* silvestre y es reemplazado por el gen *COX1* contenido en el plásmido bombardeado; esta cepa es capaz de crecer en medio respiratorio YPEG.

Purificación de mitocondrias Modificado de (Glick and Pon, 1995)

- Cultivar dos o tres colonias de levadura en 2 ml de medio YPGal toda la noche a 30 °C.

- Resembrar en 25 ml de medio YPGal e incubar a 30 °C hasta una A_{600} entre 1.2 y 1.4 (fase exponencial de crecimiento).

- Del cultivo anterior resembrar en 1 l de medio YPGal e incubar toda la noche a 30 °C hasta una A_{600} entre 1.4 y 1.6 (fase exponencial de crecimiento).

- Centrifugar a 4000 rpm (2831 g) por 10 min en el rotor JA10 (Beckman) a temperatura ambiente.

- Lavar el botón una vez con agua fría, juntar todo el botón en un tubo de centrífuga previamente pesado.

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- Centrifugar a 4000 rpm (2831 g) por 10 min en rotor JA10 (Beckman) a temperatura ambiente. Eliminar el sobrenadante y pesar.
- Resuspender el botón en amortiguador TD (2 ml/g de peso húmedo)
- Agitar 10 min a 30 °C.
- Centrifugar 5 min a 3300 rpm (1927 g) en rotor JA10 (Beckman) a temperatura ambiente.
- Lavar el botón con amortiguador MP2 (7 ml/g de peso húmedo).
- Centrifugar 5 min a 3300 rpm (1927 g) en rotor JA10 (Beckman).
- Resuspender el botón en amortiguador MP2 (7 ml/g de peso), adicionar zimoliasa-20T (3 mg/g de peso húmedo).
- Agitar de 30 a 60 min a 30 °C, hasta que los esferoplastos se formen (Revisar la formación de esferoplastos a los 30, 45 y 60 min). Para revisar la formación de esferoplastos:

Comparar 2 ml de agua contra 2 ml de sorbitol 1.2 M adicionando a cada uno 50 µl de células, agitar en vortex. Cuando se hayan formado los esferoplastos el tubo con agua se verá claro comparado con el tubo con sorbitol que se observará turbio debido a que con la diferencia de osmolaridad se romperán los esferoplastos.

A partir de este punto todo se hace a 4 °C y sin usar vortex

- Centrifugar a 2500 rpm (1106 g) por 5 min a 4 °C en rotor JA10 (Beckman).
- Resuspender en 1/4 del volumen requerido de amortiguador de homogeneización (70 ml por cada cepa).
- Homogeneizar 10 veces usando un homogenizador y pistilo de vidrio.
- Centrifugar 5 min a 3300 rpm (1106 g) en rotor JA10 (Beckman) a 4 °C. Recuperar el sobrenadante.
- Centrifugar 5 min a 3300 rpm (1106 g) en rotor JA10 (Beckman) a 4 °C. Recuperar el sobrenadante.
- Centrifugar 12 min a 12000 rpm (17418 g) en rotor JA25.5 (Beckman) a 4 °C (en el botón se encuentran las mitocondrias crudas).
- Resuspender el botón en 10 ml de amortiguador SEH (Primero resuspender en 2 ml, con la punta de la micropipeta cortada).
- Centrifugar 5 min a 3300 rpm (1106 g) en rotor JA25.5 (Beckman) a 4 °C. Recuperar el sobrenadante.

- Centrifugar el sobrenadante a 12 min a 12000 rpm (17418 g) en rotor JA25.5 (Beckman) a 4 °C.
- Resuspender el botón en 300-500 µl de amortiguador SEH (con la punta de la micropipeta cortada).
- Hacer alícuotas de 25 o 30 µl de solución de mitocondrias, congelar en nitrógeno liquido y guardar a -70 °C hasta su uso.

Amortiguador TD (Tris-DTT)

Tris-SO$_4$ pH 9.4	0.1 M
DTT	10 mM

Amortiguador MP2

Sorbitol	1.2 M
Amortiguador de fosfatos (pH 7.4)	20 mM
Zimoliasa-20T (Seikagaku Biobusiness Cat. No. 120491)	3 mg/g

Amortiguador de homogeneización

Sorbitol	0.6 M
Tris pH 7.4	10 mM
EDTA	1 mM
Albúmina sérica bovina (libre de ácidos grasos)	0.2%
PMSF (preparar al momento en etanol)	1 mM
TLCK	50 µg/ml

Amortiguador SEH (Sorbitol/EDTA/HEPES) 1X

HEPES pH 7.4	20 mM
Sorbitol	0.6 M
EDTA	1 mM

Cuantificación de proteínas por Lowry modificado (Markwell et al., 1978)

Curva de calibración

Usar una solución de albúmina de 1 mg/ml.

Preparar soluciones de albúmina con 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 y 100 µl de la solución de albúmina. Llevar a 1 ml con agua como indica el paso 1.

1. Ajustar la muestra con agua a 1 ml.
2. Adicionar 3 ml de solución C y mezclar en vortex. Incubar 10 min a temperatura ambiente.
3. Adicionar 300 µl de solución D y mezclar en vortex e incubar 30 min a temperatura ambiente.
4. Medir absorbancia a 740 nm.

Solución A

Na_2CO_3	2 % (p/v)
NaOH	0.4 % (p/v)
Na_2tartrato	0.16 % (p/v)
SDS	1 % (p/v)

Solución B

$Cu_2SO_4·5H_2O$	4% (p/v)

Solución C

Mezclar 100 volúmenes de solución A con 1 volumen de solución B (preparar al momento).

Solución D

Mezclar 1 volumen de reactivo de Folin con 1 volumen de agua (preparar al momento).

Traducción *in organello* (ioTL) y co-inmuno precipitación (coIP) partir de mitocondrias puras (Herrmann et al., 2001; Westermann et al., 2001)

- Preparar 1 ml de amortiguador 1.5X ioTL con 0.2% (v/v) de piruvato cinasa.
- Descongelar en hielo 250 µg de proteína mitocondrial y ajustar a un volumen de 25 µl con SH en un tubo Eppendorf de 1.5 ml.
- Mezclar como sigue: 55 µl de amortiguador 1.5X ioTL con los 25 µl de mitocondrias (10 µg/µl).
- Incubar 5 min a 30 °C.
- Adicionar 25 µCi de [35S]-Met.
- Incubar 20 min a 30 °C.
- Tomar 4 µl de cada reacción y pasar a otro tubo (fracción total). Agregar 1 ml de SH y centrífugar 10 min a 12000 rpm (13200 g) en una centrífuga Eppendorf 5415D a 4 °C, resuspender en 20 µl de amortiguador de Laemmli 1X y guardar en hielo.
- Al resto de la reacción de traducción agregar 1 ml de SH y centrífugar 10 min a 4 °C, eliminar el sobrenadante.
- Resuspender el botón de mitocondrias en 1 ml de amortiguador de lisis con la punta de la micro pipeta cortada. Incubar 10 min en hielo (**NO** agitar en vortex).
- Centrífugar 10 min a 4 °C a 12000 rpm (13200 g) en una centrífuga Eppendorf 5415D, pasar el sobrenadante a un tubo nuevo etiquetado.
- A cada sobrenadante adicionar 40 µl de anticuerpo acoplado a proteína A sefarosa (ver más adelante, anticuerpos).
- Agitar en un agitador circular (Barnstead Internacional, Model 400110) 2 horas a 4 °C.
- Lavar dos veces con 500 µl de amortiguador de lisis.
- Lavar 1 vez con 1 ml de Tris 20 mM, pH 7.4.

NOTA: Entre cada lavado agitar 5 min en rosticero a 4 °C y centrífugar a 2400 rpm 1 min a 4 °C

- Después del último lavado centrífugar 1 min a 12000 rpm a 4 °C, eliminar completamente el sobrenadante.
- Agregar al anticuerpo-proteína A sefarosa 20 µl de amortiguador de Laemmli 1X. Agitar en vortex 15 min a temperatura ambiente.

- Centrífugar 15 segundos a 12000 rpm (13200 g) en una centrífuga Eppendorf 5415D. Cargar con microjeringa en gel de SDS-acrilamida/Bis-acrilamida al 16%/0.15%. Correr el gel a 27 mA a 7 cm de la interfase formada por los geles.
- Transferir a membrana de PVDF 1.5 horas a 1.5 mA/cm^2. Dejar secar la membrana y exponer para obtener autorradiografía o realizar western blot.

Amortiguador 1.5X para traducción (1.5X ioTL)[♥] con 0.2% (v/v) de piruvato cinasa[&].

Amortiguador	Solución concentrada	Adicionar para 1 ml
Sorbitol	2.4 M	375 µl
KCl	1 M	225 µl
Amortiguador de fosfatos, pH 7.2	1 M	22.5 µl
Tris-HCl, pH 7.4	1 M	30 µl
MgSO$_4$	1 M	19 µl
Albúmina	100 mg/ml	45 µl
ATP	200 mM[§]	30 µl
GTP	50 mM[§]	15 µl
Aminoácidos (sin Met, Tyr y Cys)	2 mg/ml	9.1 µl
Cisteína	10 mM	10 µl
Tirosina	1 mg/ml	18.2 µl
Ácido α-ceto glutárico[+]		1.7 mg
Fosfoenol piruvato[+]		3.5 mg

Notas:
[♥]   Preparar en hielo.
[&]   Adicionar 2 µl justo antes de mezclar con las mitocondrias.
[§]   Adicionar al final.
[+]   Pesar el ácido α-ceto glutárico y disolver en 200 µl de H$_2$O, adicionar al fosfoenol piruvato y disolver. Adicionar a la mezcla para tener un volumen final de 1 ml.

SH

Sorbitol   0.6 M

Hepes, pH 7.4   20 mM

Amortiguador de lisis

Digitonina   1 %

NaCl   100 mM

Tris-HCl, pH 7.4   20 mM

Amortiguador de Laemmli 2X

Tris-HCl, pH 6.8   0.06 M

Glicerol   5 %

SDS   2%

β-Mercaptoetanol   4%

Azul de bromofenol   0.0025%

Anticuerpos

Anti c-MYC (Santa Cruz Biotechnology) dilución 1:400.

Anti-HA affinity Matriz (Roche) dilución 1:2000.

Traducción *in vivo* (ivTL) a partir de células completas (Bonnefoy et al., 2001)

- Cultivar una colonia en 2 ml de YPGal a 30°C toda la noche.

- Inocular 0.1 ml del cultivo anterior en 10 ml de YPGal, incubar a 30°C toda la noche.

- A la mañana siguiente adicionar 10 ml de YPGal incubar 3 horas a 30°C.

- Centrifugar el volumen necesario para tener 0.12 g de células en tubos de con tapa de rosca previamente pesados a 3000 g por 5 min a TA.

- Lavar dos veces con 1 ml de agua estéril. En el último lavado pesar los tubos y eliminar la cantidad de agua/células necesaria para tener 0.12 g de células en todos los tubos.

- Resuspender en 500 µl de medio Gal/-Met. Incubar 30 min a 30°C con agitación.

- Adicionar 5 µl de cicloheximida (10 mg/ml, disuelta en etanol). Incubar 5 min a 30°C con agitación.

- Adicionar 5 µCi de $^{[35S]}$-Met. Incubar 10 min a 30°C con agitación.

- Inmediatamente enfriar en hielo/agua 5 min.

- Centrifugar a velocidad máxima a 4°C por 1 min) en una centrífuga Eppendorf 5415D. Eliminar sobrenadante.
- Lavar una vez con 500 µl de Gal/-Met con 2.5 mM de metionina no radioactiva.
- Centrifugar a velocidad máxima a 4 °C por 1 min) en una centrífuga Eppendorf 5415D. Eliminar sobrenadante.

A partir de 0.12 g de células

- Lavar el pellet con 200 µl de SHP frio, con inhibidor de proteasas de SIGMA (3.3 µl/10ml)
- Resuspender el pellet en 200 µl de SHP frio, con inhibidor de proteasas de SIGMA.
- Adicionar un volumen de perlas de vidrio, previamente enfriadas a -20 °C.
- Agitar en vortex 30 seg a velocidad máxima e incubar 30 seg en hielo.
- Repetir el paso anterior.
- Centrifugar a 6000 g por 5 min a 4 °C en una centrífuga Eppendorf 5415D.
- Tomar el sobrenadante y poner en tubo eppendor etiquetado en hielo.
- Adicionar 200 µl de SHP frio. Repetir el ciclo vortex/hielo dos veces.
- Combinar los sobrenadantes y centrifugar a velocidad máxima 10 min a 4 °C en una centrífuga Eppendorf 5415D.
- Eliminar el sobrenadante y adicionar 40 µl de amortiguador de Laemmli 1X. Calentar a 65°C por 5 min.
- Cargar 10 µl en gel de acrilamida/bis-acrilamida al 16%/0.15%, el resto guardar a -70 °C. Correr a 27 mA a 7 cm de la interfase formada por los geles.
- Transferir a membrana de PVDF 1.5 horas a 1.5 mA/cm^2. Dejar secar y exponer.

SHP

Sorbitol	0.6 M
Hepes, pH 7.4	20 mM
Inhibidor de proteasas de SIGMA	3.3 µl por cada 10 ml

Geles de poliacrilamida desnaturalizantes (SDS-PAGE) (Laemmli, 1970)

	Gel separador 16 %	Gel apilador 4 %
Acrilamida 30% Bisacrilamida 0.8%	9.0 ml	0.83 ml
Tris-HCl 2M, pH 8.8	3.28 ml	-
Tris-HCl 2M, pH 6.8	-	150 µl
SDS 20 %	85 µl	50 µl
Agua	4.5 ml	4 ml
APS 10%	100 µl	25 µl
TEMED	20 µl	10 µl

Amortiguador de corrida 5X

Tris-HCl, pH 8.2   0.125 M

Glicina   0.96 M

SDS   0.5 %

Correr a 7 cm de la interfase formada por los geles a 27 mA constantes con amortiguador de corrida 1X.

Replica en Western Blot

1. Las proteínas separadas por SDS-PAGE se transfirieron a membranas de PVDF (Millipore) mediante un método semi-seco, de la siguiente manera:

3 hojas de papel filtro prehumedecidas en amortiguador de transferencia

Membrana de PVDF prehumedecida en metanol y amortiguador de transferencia

Gel prehumedecido en amortiguador de transferencia

3 hojas de papel filtro prehumedecidas en amortiguador de transferencia
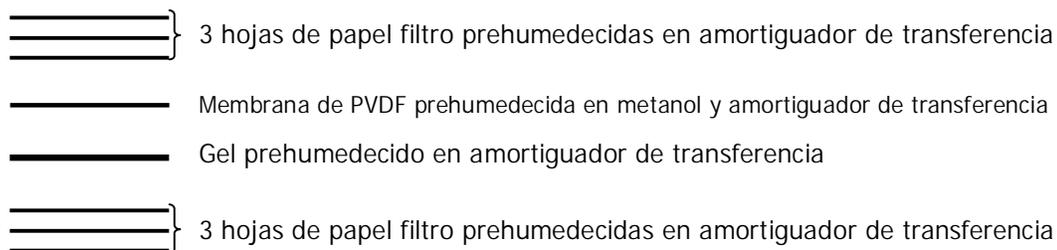
Transferir de 1 a 2 horas a 1.5 mA/cm^2, cuidando que no pase de 10 V.

2. La membrana con las proteínas se bloquea toda la noche a 4 °C o 2 hr a temperatura ambiente con solución de bloqueo.

3. Incubar la membrana con solución de réplica, la cual contiene el anticuerpo adecuado, durante 1 h a temperatura ambiente.
4. Lavar la membrana con solución de lavado por 10 min, repetir el lavado 2 veces más.
5. Revelar la presencia del anticuerpo unido a la proteína siguiendo las indicaciones del kit ECL plus (Amersham).
6. Tomar autorradiografía con la película BioMAx XAR Film (Kodak).
7. Eliminar el anticuerpo unido a las proteínas de la membrana incubando con la solución de eliminación de anticuerpo por 30 min a 50 °C.
8. Repetir desde el paso número dos con los anticuerpos necesarios.

Solución de bloqueo (50 ml)

Leche en polvo libre de grasas (Svelty)	5 g
Tris-HCl 1M, pH 7.6	2.5 ml
NaCl 5M	1.5 ml

Solución de replica (10 ml)

Leche en polvo	0.1 g
Tris-HCl 1M, pH 7.6	100 µl
NaCl 5M	200 µl
EDTA 500mM	20 µl
Tween-20 al 5%	200 µl

• Adicionar el anticuerpo indicado a la dilución apropiada.

Solución de lavado (1000 ml)

Tris-HCl 1M, pH 7.6	10 ml
NaCl 5M	20 ml
EDTA 500mM	2 ml
Tween-20	1 ml

Solución de eliminación del anticuerpo (100 ml)

β-mercaptoetanol   700 µl

Tris HCl 1M, pH 6.8   6.25 ml

SDS 10%   20 ml

Diluciones usadas para los anticuerpos

Anti-Arg8	1:10000	(Donado por el Dr. Thomas Fox)
Anti-ATPasa	1:10000	(Donado por el Dr. Diego Gonzalez Halphen)
Anti-citrato sintasa	1:1000	(Donado por el Dr. Thomas Fox)
Anti HA/peroxidasa (clona 3F10)	1:500	(Roche)
Anti cMYC	1:400	(Roche)
Anti conejo/peroxidasa	1:5000	(Sigma)
Anti ratón/peroxidasa	1:3000	(BioRad)

Electroforesis azul-nativa de mitocondrias puras (Wittig et al., 2006)

Preparación de la muestra

- Descongelar en hielo 50 µg de proteína mitocondrial. (Opcional, hacer traducción *in organello*).
- Adicionar 1 ml de SH y centrífugar 10 min a 4 °C, a 12,000 rpm.
- Adicionar 10 µl de amortiguador A, homogeneizar subiendo y bajando con la punta de la micropipeta.
- Adicionar 1 µl de laurilmaltósido al 10%, subir y bajar con la micropipeta 3 veces. Incubar en hielo 10 min.
- Centrífugar 15 min a 20,000 rpm (16979 g) en el rotor TLA 100.3, Beckman a 4 °C. Separar el sobrenadante en un tubo nuevo.
- Agregar al sobrenadante 1.5 µl de glicerol al 5%. Adicionar 1 µl de Serva G al 5%.
- Cargar todo (15 µl aprox) en gel de gradiente del 5 al 13% de acrilamida. Correr el gel a 30 mA a 4 °C hasta que el amortiguador del cátodo B esté a 1/3 del gel (aprox 1 hr).
- Cambiar el amortiguador del cátodo B por el B/10 y correr a 30 mA hasta que el

colorante azul fuerte se salga (aprox 3.5 hrs) a 4 °C.

Nota: En este paso el gel puede usarse para teñir, determinar actividad de super complejos, transferir a membrana para western blot, etc.

- Transferir a membrana de PVDF a 1 mA/cm^2 por 1.5 hrs.
- Inmediatamente después de la transferencia desteñir la membrana lavando con metanol al 100% durante 15 segundos. Secar completamente la membrana.

SH

Sorbitol	0.6 M
HEPES, pH 7.4	20 mM

Amortiguador A (1 ml)

NaCl	50 mM
Imidazol-HCl, pH 7.0	50 mM
Ácido 6-aminohexanoico	5 mM
EDTA	1 mM

Lauril maltósido   10 % (p/v)

Azul de Coomásie G-250   5 % (p/v)

Glicerol   5 % (v/v)

Preparación del gel

	Acrilamida 3.5%	Acrilamida al 5%	Acrilamida al 13%
AB-3	660 µl	1.9 ml	4.9 ml
Amortiguador de gel 3X	3 ml	6.2 ml	6.2 ml
Glicerol (80%)	-	1.2 ml	4.73 ml
Agua	5.1 ml	9.2 ml	2.7 ml
APS 10%	75 µl	100 µl	75 µl
TEMED	7.5 µl	10 µl	7.5 µl
Volumen total	9 ml	18.6 ml	18.6 ml

AB-3 (100 ml)

Acrilamida   48 g

Bis-acrilamida   1.5 g

    Filtrar y guardar en un frasco oscuro a temperatura ambiente.

Amortiguador del gel 3X

Imidazol-HCl, pH 7.0   75 mM

Ácido 6-aminohexanoíco   1.5 M

Amortiguador del cátodo B

Tricina   50 mM

Imidazol-HCl, pH 7.0   7.5 mM

Azul Coomásie G-250   0.02 %

Amortiguador del cátodo B/10

Tricina   50 mM

Imidazol-HCl, pH 7.0   7.5 mM

Azul de Coomasie G-250   0.002 %

Amortiguador del ánodo

Bis-Tris-HCl, pH 7.0   50 mM

Resultados

La traducción de Cox1 disminuye al afectar el ensamblaje de la CcO

En diversos estudios se ha observado que Cox1 marcada radioactivamente con [^{35}S]-Met disminuye en mutantes nucleares o mitocondriales que afectan el ensamblaje de la CcO, sin que estas mutaciones estén en el gen mitocondrial *COX1* (Figura 5) (Barrientos et al., 2004; Cabral and Schatz, 1978; Perez-Martinez et al., 2003; Rak et al., 2007). Aún no se entiende el mecanismo por el cual disminuye este marcaje de Cox1 en mutantes que no ensamblan a la CcO y que por lo tanto no respiran. Para determinar si esta reducción en el marcaje se debe a una disminución en la traducción del mRNA de *COX1* o a una rápida degradación proteolítica de la proteína Cox1 recién sintetizada, se utilizó al gen reportero *ARG8^m*.

El gen *ARG8* es de origen nuclear y codifica a la acetilornitina aminotransferasa, un enzima localizada en la matriz mitocondrial que participa en la biosíntesis de la arginina. Contiene un péptido señal que al importarse a la mitocondria se procesa por las proteasas mitocondriales, generando a la proteína madura (Heimberg et al., 1990). Este gen se modificó e insertó en el mtDNA para que pudiera ser leído y expresado por el sistema genético mitocondrial (*ARG8^m*) (Steele et al., 1996). En la cepas utilizadas en este trabajo *ARG8^m* está fusionado a la región carboxilo terminal del gen *COX1* y porta su sitio de corte para las proteasas mitocondriales, por lo que cuando se sintetiza la proteína de fusión Cox1-Arg8, ésta última es separada de Cox1 y se comporta como una proteína soluble de matriz mitocondrial. Esta construcción permite distinguir entre traducción del mRNA o degradación proteolítica, así como estudiar el nivel de traducción de la proteína quimérica, ya que el estado de ensamblaje de la CcO y por lo tanto de degradación de Cox1 no afecta a la proteína Arg8 (Perez-Martinez et al., 2003) (Figura 9).
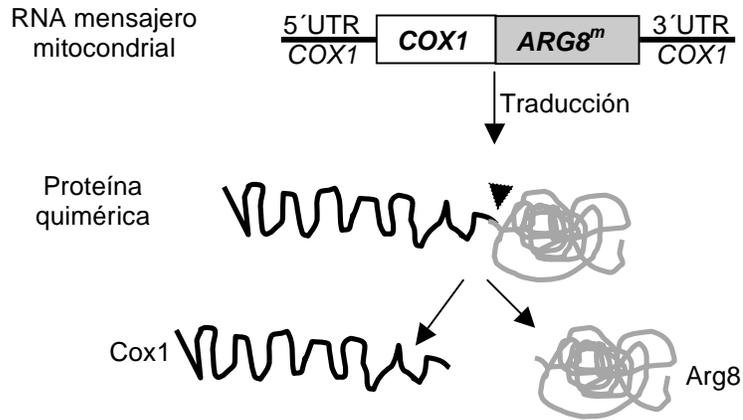
Figura 9. Esquema del gen reportero *COX1(1-512)::ARG8^m*. El triángulo negro indica el sitio de corte por las proteasas mitocondriales MPP en la proteína quimérica.

Para analizar si las mutantes de ensamblaje de la CcO afectaban la síntesis o la estabilidad del gen reportero se realizaron experimentos de dilución seriada en medios con o sin arginina (+Arg o − Arg, respectivamente) en diferentes cepas con la construcción del gen quimérico mitocondrial (*COX1(1-512)::ARG8^m*). Se introdujo una mutación que afectó el ensamblaje de la CcO debido a que el gen mitocondrial *COX2* se eliminó (*cox2-62*) (Figura 10A).
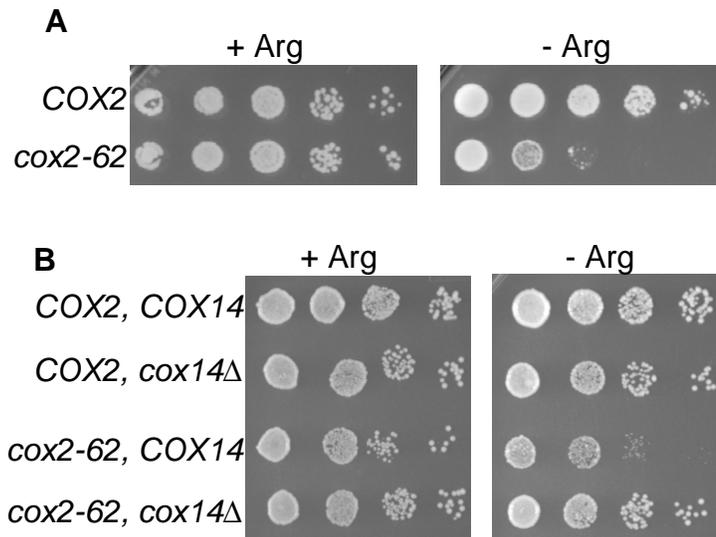
Figura 10. Diluciones seriadas (1/10) de cepas con el gen quimérico mitocondrial *COX1(1-512)::ARG8^m* mostrado en la Figura 9. **A)** Los niveles de traducción del gen reportero disminuyen en las cepas con el defecto en el ensamblaje de la CcO (*cox2-62*). **B)** Los niveles de síntesis del gen reportero *ARG8^m* se reestablecen en la doble mutante *cox2-64, cox14Δ*. Las placas se crecieron en medio completo (+Arg) o en medio sin arginina (-Arg) y se incubaron a 30 °C por tres (+Arg) o cuatro días (-Arg).

En la Figura 10A se observa que el crecimiento en –Arg de las cepas en donde la CcO no se ensambla (*cox2-62*) se ve severamente reducido. Esto sugiere que esta disminución en el crecimiento se debe a una menor traducción del gen reportero, ya que por codificar a una proteína soluble de matriz mitocondrial, ésta no es susceptible a la degradación proteolítica responsable de la degradación de Cox1. Por otro lado, en la Figura 10B se observa que el menor crecimiento en –Arg de la cepa *cox2-62* se restablece al eliminar a Cox14. Esto es consistente con la idea de que Cox14 es un regulador negativo de la traducción, por lo que al eliminarla se reestablece la síntesis a niveles semejantes a los de una cepa silvestre (Barrientos et al., 2004).

Diferentes mutantes que afectan el ensamblaje de la CcO disminuyen la síntesis de Cox1

Se analizó si la disminución en la traducción de Cox1 es general, es decir, cuando el ensamblaje de la CcO se afecta debido a mutaciones diferentes a *cox2-62*. Se eliminaron subunidades estructurales o chaperonas de ensamblaje codificadas en el núcleo (Tabla 1) en cepas con el gen reportero *COX1(1-512)::ARG8^m* descrito en la Figura 9. Para estudiar la síntesis del reportero se realizaron experimentos de dilución seriada en un medio con o sin arginina (Figura 11).

Tabla 1. Proteínas cuyo gen se interrumpió en el núcleo y que afectan el ensamblaje de la CcO.

Proteína	Función	Referencia
Mss2	Participa en el correcto ensamblaje de Cox2 en la membrana interna mitocondrial.	(Broadley et al., 2001)
Pet54	Activador traduccional del mRNA de *COX3*. Facilita el procesamiento del RNA de *COX1*. Mutantes nulas no respiran.	(Costanzo et al., 1986; Kaspar et al., 2008; Valencik et al., 1989; Valencik and McEwen, 1991)
Pet122	Activador traduccional de *COX3*. Mutantes nulas no respiran.	(Haffter et al., 1990; Kloeckener-Gruissem et al., 1988)
Cox4	Subunidad estructural de la CcO. Participa en la estabilidad del complejo. Mutantes nulas no respiran. Es de las últimás subunidades en unirse a Cox1.	(Glerum and Tzagoloff, 1997; Glerum and Tzagoloff, 1998; Tsukihara et al., 1996)
Cox6	Subunidad estructural de la CcO. Participa en la estabilidad del complejo. Mutantes nulas no respiran. Es de las primeras subunidades en unirse a Cox1.	(Glerum and Tzagoloff, 1997; Glerum and Tzagoloff, 1998; Mick et al., 2007; Tsukihara et al., 1996)

Cox7	Subunidad estructural de la CcO. Participa en la estabilidad del complejo. Mutantes nulas no respiran.	(Calder and McEwen, 1991; Tsukihara et al., 1996)
Cox11	Participa en la inserción del grupo Cu_B en Cox1. Mutantes nulas no respiran. Esta inserción se da después de la unión de Mss51 y Cox14 a Cox1.	(Carr et al., 2002; Horng et al., 2004; Khalimonchuk et al., 2009)
Cox15	Involucrada en la maduración de los grupos hemo a y a_3 de Cox1. Mutantes nulas no respiran. Esta inserción se da después de la unión de Mss51 y Cox14 a Cox1	(Barros et al., 2001; Khalimonchuk et al., 2009; Petruzzella et al., 1998)
Coa1	Participa en pasos postraduccionales de Cox1. Facilita la inserción del grupo hemo. Mutantes nulas respiran parcialmente.	(Mick et al., 2007; Pierrel et al., 2007)
Pet100	Chaperona requerida para los últimos pasos de ensamblaje de la CcO. Mutantes nulas acumulan intermediarios de ensamblaje y no respiran.	(Church et al., 1996; Church et al., 2005; Forsha et al., 2001)
Pet191	Chaperona requerida para el ensamblaje de la CcO. Mutantes nulas no respiran.	(Khalimonchuk et al., 2008; Tay et al., 2004)
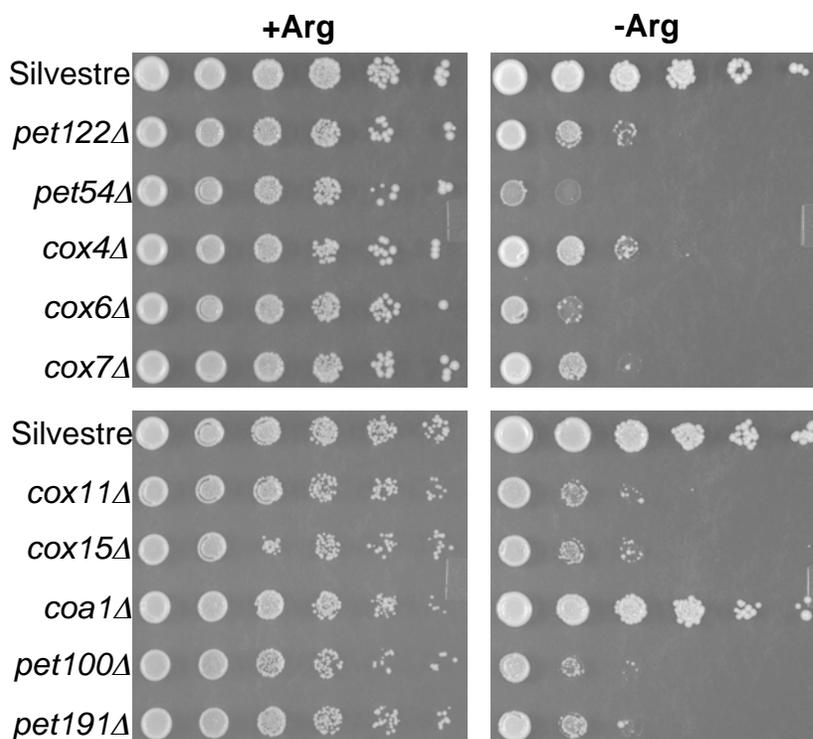
Figura 11. Diluciones seriadas (1/10) de cepas con el gen quimérico mitocondrial *COX1(1-512)::ARG8^m*. Las cepas con diferentes proteínas eliminadas se crecieron en medio completo (+Arg) o en medio sin arginina (-Arg) y se incubaron a 30 °C por tres o cuatro días.

En la Figura 11 se observa que la eliminación de diferentes proteínas, ya sean subunidades estructurales (panel superior) o chaperonas de ensamblaje de la CcO (panel inferior), afecta en diferentes grados el crecimiento en ausencia de arginina (–Arg). Se observa que la síntesis de Arg8 en la mutante nula de *cox6* es una de las más afectadas. Creemos que esto tiene relación con que Cox6 es una de las primeras subunidades en ser ensamblada (ver discusión) (Glerum and Tzagoloff, 1997; Mick et al., 2007). Otra mutante en la que se afecta dramáticamente el crecimiento en –Arg es *pet54D*, activador traduccional de *COX3*. Esto pudiera deberse a que Pet54 también está involucrada en el procesamiento de intrones del mRNA de *COX1*, por lo que al eliminarla no se acumule el RNA maduro. Finalmente, la mutante *coa1Δ* no se ve afectada, lo cual es consistente con reportes previos (Mick et al., 2007; Pierrel et al., 2007) que indican que Coa1 no es esencial para la traducción del mRNA de *COX1* y no responde al control negativo ejercido por Cox1.

Este resultado sugiere que el mecanismo (o mecanismos) que disminuye la traducción del mRNA de *COX1* es general. Como se observa en las diferentes mutantes, este mecanismo detecta el ensamblaje de Cox1 desde su inicio hasta los pasos finales.

Los extremos UTR del mRNA de *COX1* portan señales de regulación negativa de la expresión de este gen

La traducción del mRNA de *COX1* en la mitocondria está fuertemente controlada por los activadores traduccionales específicos de *COX1*, Pet309 y Mss51. Estas dos proteínas tienen sus sitios de acción en el extremo 5´UTR del mRNA de *COX1* (Manthey and McEwen, 1995; Perez-Martinez et al., 2009; Siep et al., 2000; Tavares-Carreon et al., 2008).

Para analizar si en la regulación negativa de la síntesis de Cox1 participan los extremos UTR de *COX1* se realizó un experimento de traducción de proteínas mitocondriales en células completas (*in vivo*) en presencia de cicloheximida, un inhibidor de la síntesis protéica citoplásmica. Se emplearon dos fondos genéticos mitocondriales diferentes. Una cepa con el mtDNA silvestre, en donde el gen *COX1* está flanqueado por sus extremos UTR normales (Figura 12A). Por otro lado, se utilizó otra cepa en donde la región codificante del gen *COX1* fue reemplazado por el gen

reportero *ARG8^m* flanqueado por los extremos UTR de *COX1*. En esta misma cepa el gen *COX1* se insertó en un sitio del mtDNA ectópico y se codifica a partir de los extremos UTR del gen mitocondrial *COX2* (Figura 12B). A esta última construcción se le dio el nombre de DNA mitocondrial quimérico (Perez-Martinez et al., 2003). En la cepa quimérica la expresión de *ARG8^m* depende de Pet309 y Mss51 y la expresión de *COX1* depende de Pet111, el activador traduccional de *COX2* (Mulero and Fox, 1993b). Este experimento se realizó en cepas con o sin la eliminación de *MSS2*, un gen involucrado en la translocación de Cox2 y que por lo tanto afecta el ensamblaje de la CcO (Broadley et al., 2001) (Figura 13).

A) DNA mitocondrial silvestre
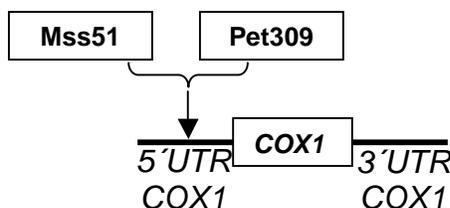
B) DNA mitocondrial quimérico
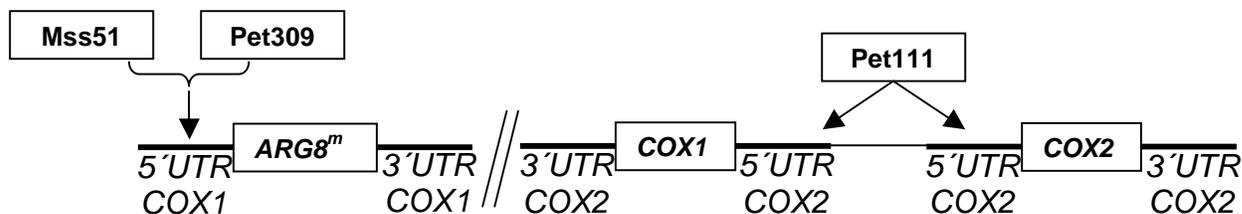
Figura 12. Construcciones utilizadas para estudiar por traducción *in vivo* el papel de los extremos UTR de *COX1* en la regulación negativa de la síntesis de Cox1 Modificado de (Perez-Martinez et al., 2003). Con flechas se señala el sitio donde actúan Pet309 y Mss51, activadores traduccionales específicos de *COX1* y Pet111, el activador traduccional específico de *COX2*.

A)
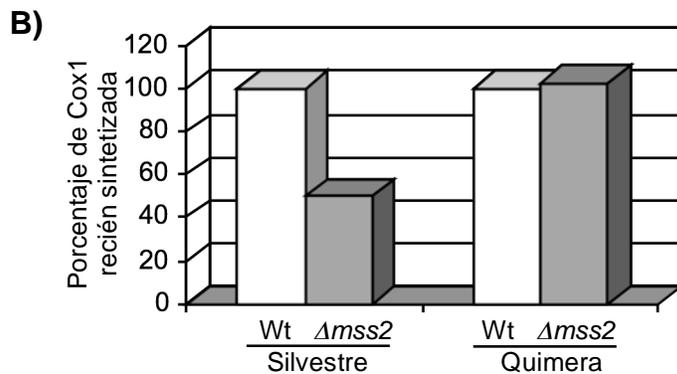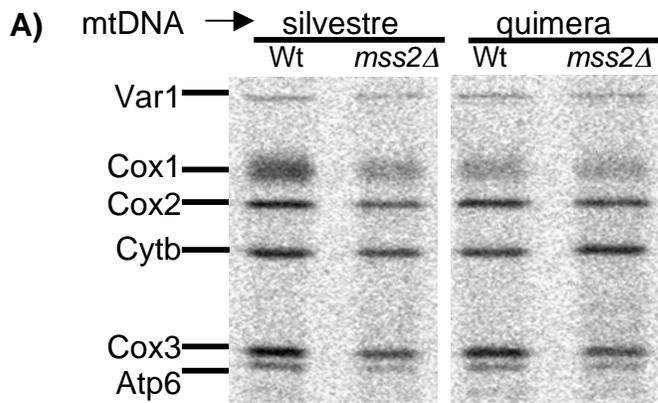
Figura 13. Traducción de proteínas mitocondriales *in vivo* con [³⁵S]-Met en presencia de cicloheximida. **A)** Las proteínas se separaron por SDS-PAGE al 16%/0.15% y se revelaron utilizando una pantalla sensible a radioactividad. Se indican los mtDNAs utilizados, así como la interrupción del gen *MSS2*. **B)** Cuantificación de la intensidad de la señal correspondiente a Cox1 normalizada con respecto al "doblete" de Cox3/Atp6, graficada en porcentaje. Wt = silvestre (sin eliminación de *MSS2*). La señal de la cepa silvestre se tomó como el 100% en cada caso.

El la Figura 13A se observa que en las cepas con el mtDNA silvestre la síntesis de Cox1 disminuye cuando se afecta el ensamblaje de la CcO (*mss2Δ*), comparado con la cepa en donde la CcO se ensambla correctamente (Wt). Por otro lado, en las cepas con el mtDNA quimera la síntesis de Cox1 disminuye respecto a la cepa con el mtDNA silvestre, y esta síntesis es similar a la de la cepa mutante (*mss2Δ*). Hasta la fecha no sabemos a qué se debe que en la mutante ectópica quimérica se sintetice menor cantidad de Cox1, sin embargo creemos que esto se debe a que los extremos UTR y la región codificante del mRNA de *COX1* deben estar en *cis* para una regulación y síntesis de Cox1 más eficiente.

Los últimos 15 aminoácidos de Cox1 contienen señales de regulación negativa de la traducción

Como se mencionó en la introducción, Cox1 es una proteína hidrofóbica. La única región de la proteína expuesta a la matriz mitocondrial es el extremo carboxilo terminal (Figura 2), el cual puede ser el sitio de unión de proteínas como Mss51. Con el fin de analizar el papel que juega la región carboxilo terminal de Cox1 en su regulación negativa se crearon mutantes en donde se eliminaron los últimos 5 (ΔC5, PAVQS), 11 (ΔC11, VHSFNTPAVQS) y 15 (ΔC15, SPPAVHSFNTPAVQS) aminoácidos del extremo carboxilo terminal de Cox1 en el mtDNA (Figura 14, residuos subrayados).

```
Sb    FFVVVAITSSSG----------------KNKRCAESPWAVEQNPTTLEWLVQSPPAFHTFGELPTIK ⎤
Zm    FFVVVAITSSSG----------------KNKRCAESPWAVEQNPTTLEWLVQSPPAFHTFGELPTIK ⎥ Plantas
Os    FFVVVAITSSSG----------------KNKRCAESPWAVEQNPTTLEWLVQSPPAFHTFGELPAIK ⎥
Bj    FFVVVTITISSG----------------NNKRCAPSPWALESNSTTPEWMVQSPPAFHTFGELPAIK ⎦
Bt    MVFIIWEA FAS ----------------KREVLT---VDLTTTNLEWLNGCPPPYHTFEEPTYVN ⎤ Mamíferos
Mm    MIFMIWEAFAS-----------------KREVMS---VSYASTNLEWLHGCPPPYHTFEEPTYVK ⎦
Sc    FIYILYDQLVNGLNNKVNNKSVIYNKAPDFVESNTIFNLNTVKSSSIEFLLTSPPAVHSFNTPAVQS ⎤
Sd    FIYILYDQLVNGLNNKVNNKSVIYTKAPDFVESNTIFNLNTVKSSSIEFLLTSPPAVHSFNTPAVQS ⎥
Cg    FIYILYDQFVNGLTNKANNKSVLYTKSPDFVESNEIFNLNTIKTSSIEFLLTSPPAVHSFNTPAVQS ⎥ Hongos
Kl    FIYILYDQLVNGLENKVNNKSVIYNKGPDFVESNQIFATNKIKSSSIEFLLTSPPAVHTFNTPAVQS ⎥
Yl    FIYVVYDQLTNGLHQ--GNKALDSQFKPSFMGTN--LNVEGYTGPTLEWTVSTPPSLHAFNTPAVLY ⎥
Um    FGYIIYDILVNG-----KPVDANPWAVPAFFQSTPEFWMESHTASSLEWALESPPPFHSFNMLPVQS ⎦
Cn    FLYVIYDMLVSQ-----PVASMNPWGTPGYFMSTPSYLTESSYSTSLEWTIPSPPPYHAFLMMPVQS ⎤ Bacterias
Rs    FLGVIFYTLTRG-----------------ARVTANNYWNEHADTLEWTLTSPPPEHTFEQLPKRE ⎦
                                                    **   * *
```

Figura 14. Alineamiento de los últimos 67 aminoácidos del extremo carboxilo terminal de Cox1 de *S. cerevisiae* (en negritas) con los últimos aminoácidos de la subunidad 1 de la citocromo *c* oxidasa de otros organismos. Se utilizó el software ClustalW (www.ebi.ac.uk/clustalw/). Los asteriscos indican los residuos conservados. Los residuos subrayados indican los aminoácidos eliminados. El recuadro en la secuencia de bovino (*Bt*) indica los tres últimos aminoácidos del ultimo cruce transmembranal de Cox1. *Sb: Sorghum bicolor; Zm: Zea mays; Os: Oryza sativa; Bj: Brassica juncea; Bt: Bos taurus; Mm: Mus musculus; Sc: Saccharomyces cerevisiae; Sd: Saccharomyces douglassi; Cg: Candida glabrata; Kl: Kluyveromyces lactis; Yl: Yarrowia lipolytica; Um: Ustilago maydis; Cn: Cryptococcus neoformans; Rs: Rhodobacter sphaeroides*

Con el fin de analizar el efecto de la eliminación de aminoácidos en la respiración celular se realizaron ensayos de dilución seriada de las mutantes ΔC15, ΔC11 y ΔC5 en medio respiratorio YPEG (Figura 15).
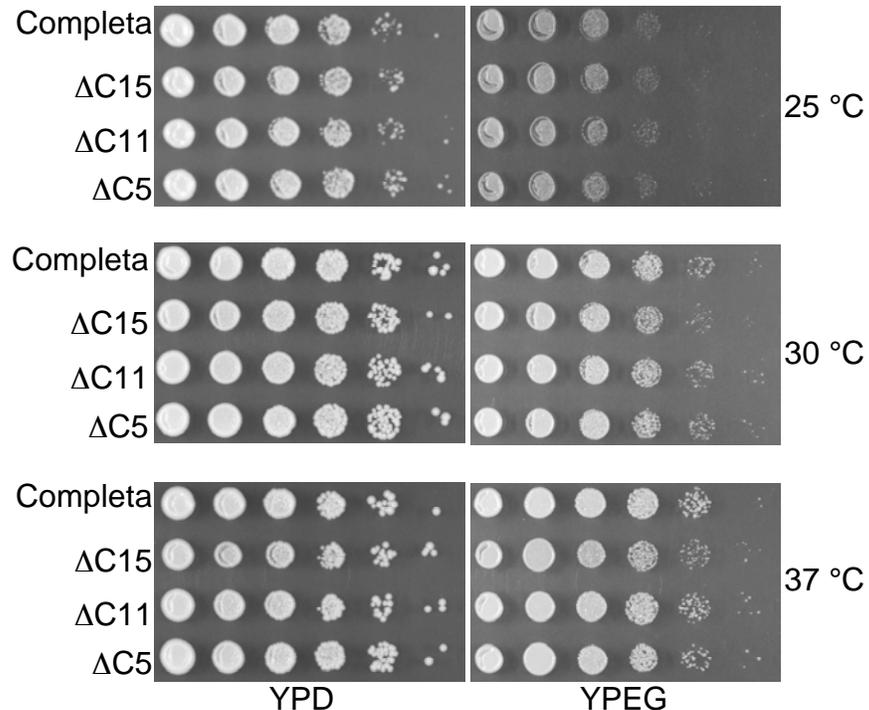
Figura 15. Diluciones seriadas (1/10) de las cepas de levadura que portan la eliminación de 5 (ΔC5), 11 (ΔC11) y 15 (ΔC15) aminoácidos en el extremo carboxilo terminal de Cox1, así como de Cox1 completa. Las diluciones se incubaron a diferentes temperaturas en medio fermentable (YPD) por dos días o en medio no fermentable (YPEG) por tres días.

Se observó que la eliminación de estos aminoácidos en el extremo carboxilo terminal de Cox1 no afecta el crecimiento respiratorio de las células, lo que indica que estas mutaciones no afectan el ensamblaje de Cox1 y que la enzima es funcional. Sin embargo, en la mutante ΔC15 las colonias se observan ligeramente más pequeñas a 37 °C comparadas con las otras cepas.

Para determinar si el extremo carboxilo terminal de Cox1 participa en la señal de regulación negativa de la síntesis de la proteína se realizaron ensayos de traducción de proteínas mitocondriales marcadas radiactivamente con [35S]-Met en células completas en presencia de cicloheximida. En la Figura 16 se observa que la síntesis de Cox1

completa disminuye cuando se ha eliminado al gen mitocondrial *COX2* (*cox2-62*), comparado con la cepa sin defectos en el ensamblaje (Wt). Inversamente, en la cepas Cox1ΔC15 y Cox1ΔC11 se observó que la síntesis de Cox1 se restablece a niveles comparados a los de la cepa con Cox1 completa (Figura 16B), aún cuando no hay ensamblaje de la CcO. Al eliminar los últimos 5 aminoácidos de Cox1 (CoxΔC5) se observa que el marcado de Cox1 baja en *cox2-62*, indicando que en esta construcción persiste la regulación negativa de la síntesis de Cox1 aunque no fue tan marcada como en la cepa con Cox1 completa.
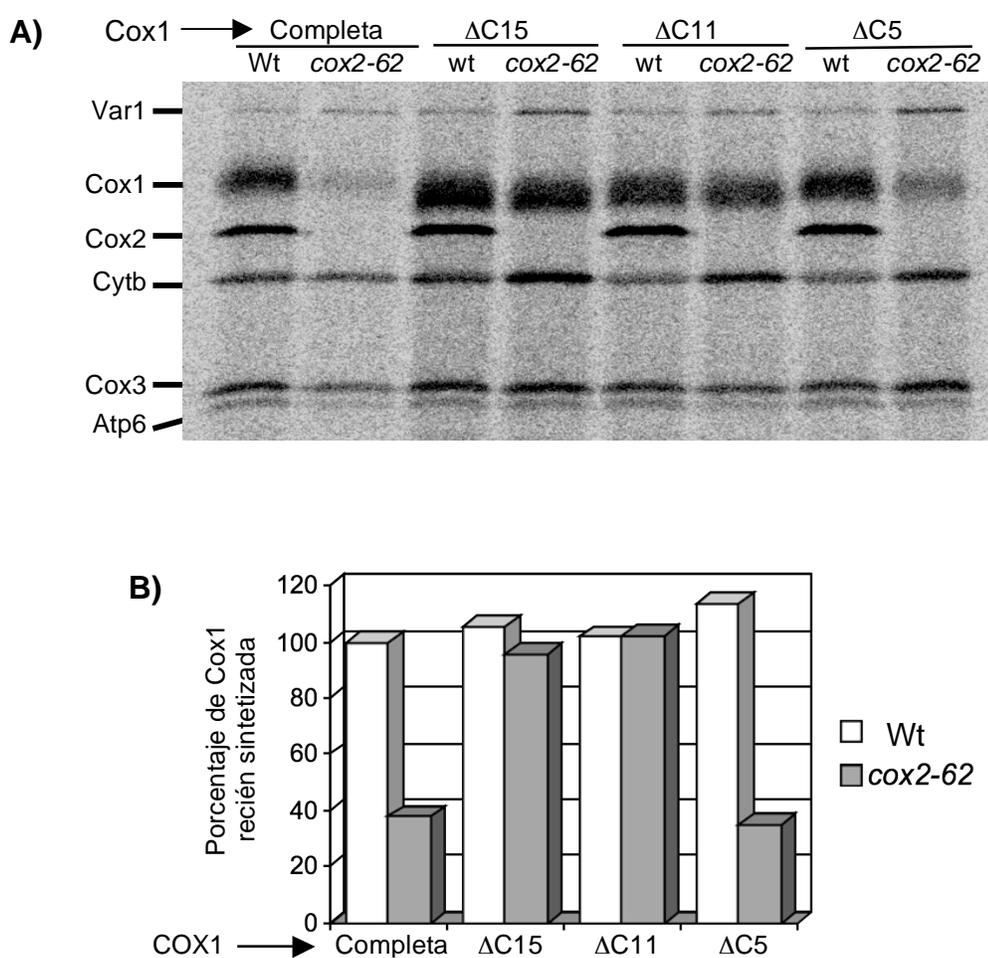
Figura 16. A) Síntesis de proteínas mitocondriales *in vivo* con [35S]-Met en presencia de cicloheximida. Las proteínas se separaron por SDS-PAGE al 16%/0.15% y se revelaron utilizando una pantalla sensible a radioactividad. Wt = cepa silvestre sin defectos en el ensamblaje, *cox2-62* = cepa sin Cox2. **B)** Cuantificación de la intensidad de la banda correspondiente a Cox1 normalizada con respecto al "doblete" de Cox3/Atp6, graficada en porcentaje de la síntesis de Cox1. Se tomó como 100% a Cox1 completa en una cepa silvestre.

Estos resultados demuestran que los últimos 11 y 15 aminoácidos de Cox1 portan señales de regulación negativa de la síntesis de Cox1 cuando esta no se ensambla correctamente.

El efecto de la desregulación en la síntesis de Cox1 al eliminar los últimos 15 aminoácidos es general para Cox1, excepto para Pet54

Para analizar si el efecto de desregulación de la síntesis de Cox1ΔC15 observado en la Figura 16 se presenta cuando se eliminan otras proteínas que afectan el ensamblaje de la CcO (Tabla 1), se generaron diversas mutaciones nucleares que afectaban diversos pasos del ensamblaje de la CcO. Posteriormente se realizaron ensayos de traducción de proteínas mitocondriales marcadas radioactivamente con [^{35}S]-Met en células completas (traducción *in vivo*) en presencia de cicloheximida (Figura 17).

B)

C)
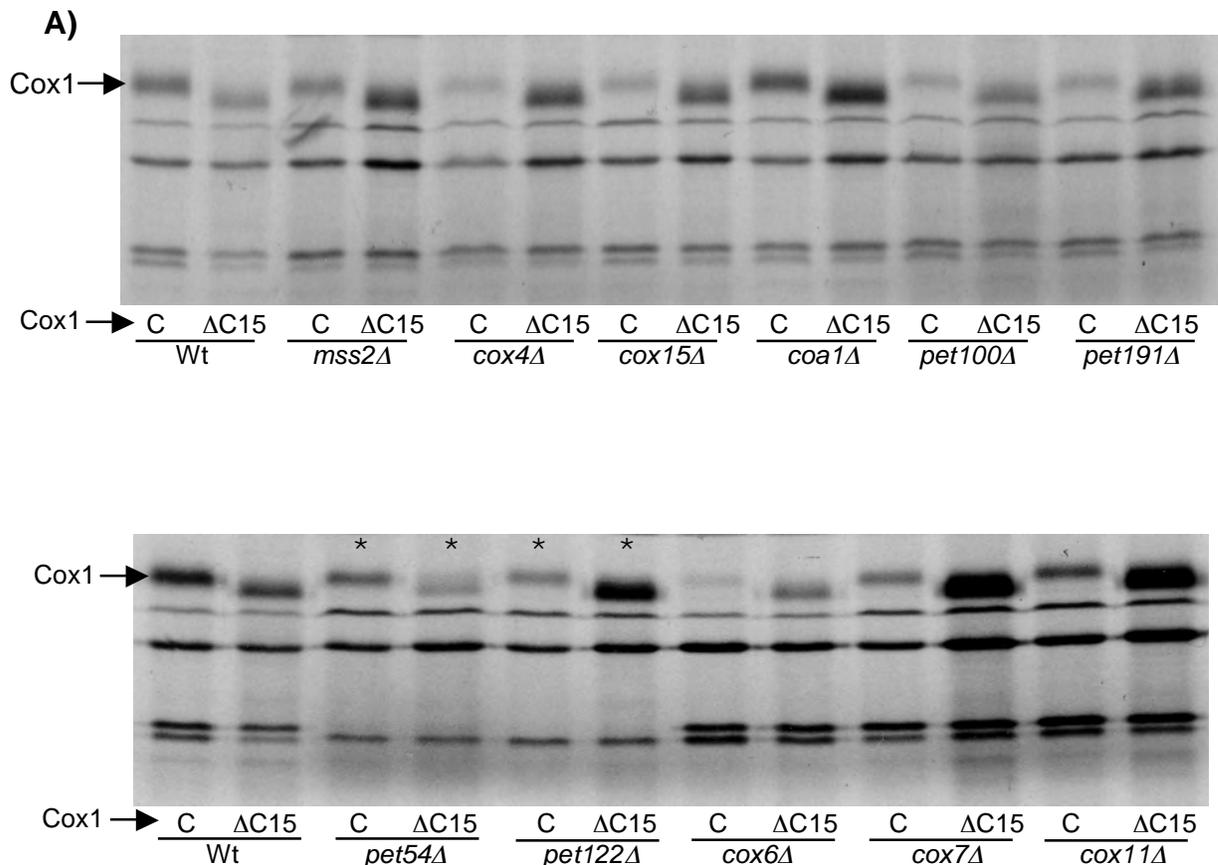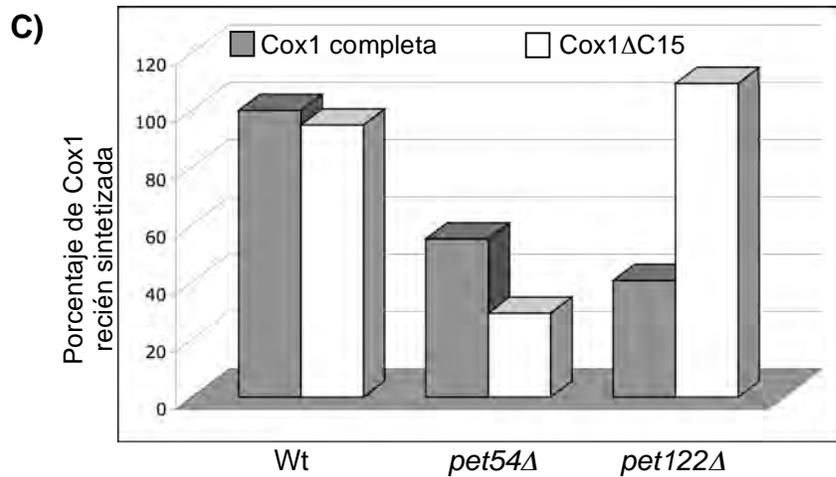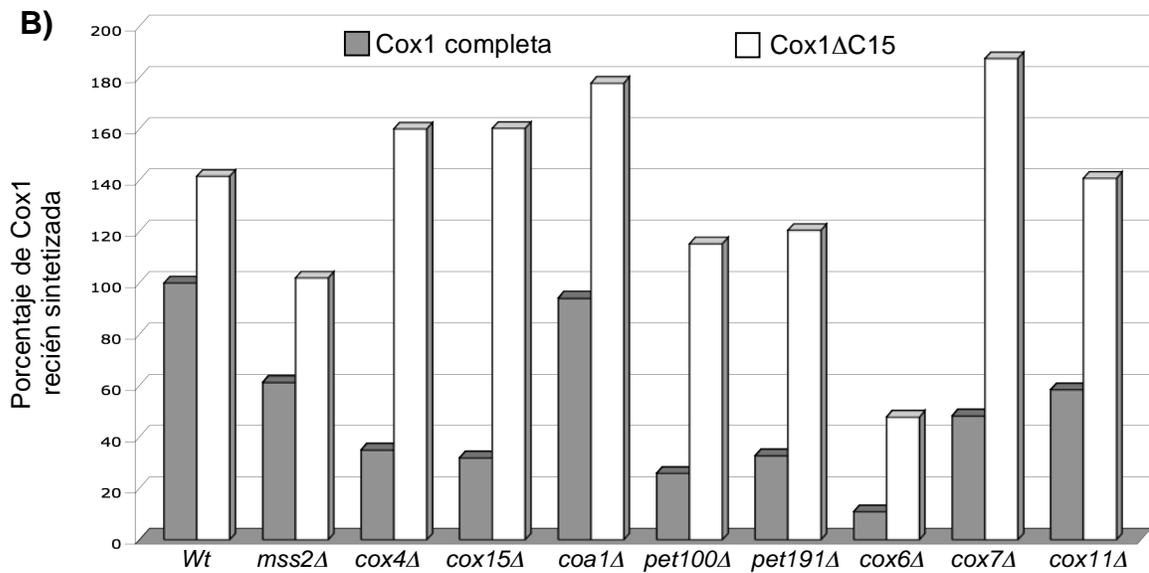
Figura 17 (Página anterior y ésta página). A Traducción de proteínas mitocondriales *in vivo* con [35S]-Met en presencia de cicloheximida. Las proteínas se separaron por SDS-PAGE al 16%/0.15% y se revelaron utilizando una pantalla sensible a radioactividad. **B)** Cuantificación de la intensidad de la banda correspondiente a Cox1 normalizada con respecto al "doblete" de Cox3/Atp6, graficada en porcentaje. Se consideró la síntesis de Cox1 completa en una cepa silvestre como el 100%. C = Cox1 completa; Wt = cepa silvestre que ensambla a la CcO. **C)** Cuantificación de la intensidad de la banda correspondiente a Cox1, graficada en porcentaje. La eliminación de *pet54* no solo disminuye la síntesis de Cox1, sino que esta disminución no se reestablece al eliminar la región reguladora de Cox1 (los últimos 15 aminoácidos). Este efecto es específico de Pet54 ya que no se observa al eliminar a Pet122.

50

La Figura 17 muestra que al eliminar las diferentes proteínas mencionadas en la Tabla 1, la síntesis de Cox1 completa disminuye (Figura 17A y B). Sin embargo, al eliminar los últimos 15 aminoácidos de Cox1 la síntesis se restablece en la mayoría de estas mutantes, en algunos casos la cantidad de Cox1 sintetizada es aún mayor que en la cepa silvestre (Figura 17C). Este resultado apoya al observado en la Figura 11, en donde, por medio del gen reportero de la traducción *ARG8^m*, se observa una disminución en la síntesis de este reportero al afectar el ensamblaje de la CcO, lo cual se refleja en un crecimiento diferencial en medio –Arg. La eliminación de Coa1 no mostró ninguna reducción en la síntesis de Cox1, lo cual es consistente con reportes previos en donde se demuestra que el marcaje de Cox1 con [^{35}S]-Met en ausencia de Coa1 no se ve afectado (Mick et al., 2007; Pierrel et al., 2007).

Las mutantes de *cox6Δ* en Cox1 completa o Cox1ΔC15 muestran una fuerte reducción en la síntesis de Cox1. Sin embargo, la mutante de Cox1 sin los últimos 15 aminoácidos recupera ligeramente la síntesis aunque no a los niveles observados en otras mutantes. Esto sugiere que la eliminación de Cox6 genera un arresto en la síntesis mucho más fuerte que el resto de las mutantes, lo que confirma lo observado en la Figura 11.

La eliminación de Pet54, uno de los tres activadores traduccionales de *COX3*, generó un arresto en la síntesis de Cox1 completa. Sin embargo, y contrario a lo esperado, la síntesis de Cox1DC15 en ausencia de Pet54 no se restableció como en el resto de las mutantes, incluso se observa que la cantidad de Cox1DC15 recién sintetizada es menor (Figura 17B). Ésta es la única proteína donde la regulación negativa de la síntesis de Cox1 no se rompe al eliminar los últimos 15 aminoácidos del extremo carboxilo terminal de Cox1. Este efecto es específico de la eliminación de Pet54, ya que la eliminación de Pet122, otro activador traduccional del mRNA de *COX3*, mostró un patrón similar al de las otras mutantes (Figura 17D).

La eliminación de Cox14 no restablece la síntesis de Cox1 generada por la falta de Pet54

Como se mencionó en la introducción, Cox14 es una proteína sensora del ensamblaje y reguladora negativa de la síntesis de Cox1. Se sabe que en mutantes que afectan el ensamblaje de la CcO y que por lo tanto disminuyen la síntesis de Cox1, al eliminar a Cox14 en estas mutantes se restablece la síntesis de Cox1. Se propone que esto sucede porque la ausencia de Cox14 desestabiliza la interacción de Cox1 con Mss51, permitiendo que esta última quede disponible para activar la traducción de más mRNAs de *COX1* (Barrientos et al., 2004). Para analizar si la eliminación de Cox14 en la mutante sin Pet54 restablece la síntesis de Cox1 se realizaron experimentos de marcaje radioactivo con [^{35}S]-Met en cepas con la doble mutación y con Cox1 completa o Cox1ΔC15 (Figura 18).
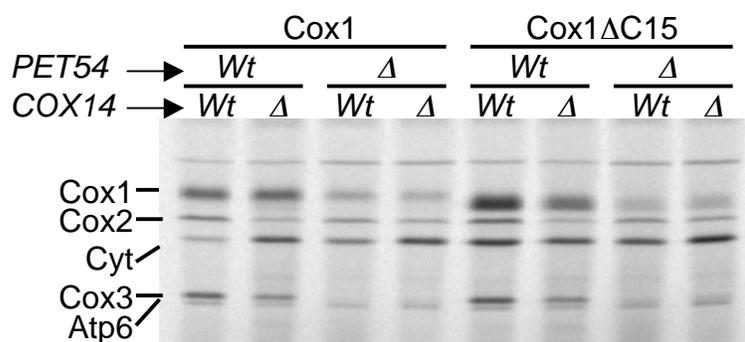
Figura 18. Traducción de proteínas mitocondriales *in vivo* con [^{35}S]-Met en presencia de cicloheximida. Las proteínas se separaron por SDS-PAGE al 16%/0.15% y se revelaron utilizando una pantalla sensible a radioactividad.

En la Figura 18 se observa que la eliminación de Cox14 no restablece la síntesis de Cox1 cuando se ha eliminado a Pet54. Éste es un fenotipo diferente al observado para otras mutantes del ensamblaje de la CcO (Barrientos et al., 2004).

La sobreexpresión de Mss51 restablece ligeramente la síntesis de Cox1 generada por la falta de Pet54

Pet309 y Mss51 son los activadores traduccionales del mRNA de *COX1* y se sabe que ambos son limitantes para la expresión de este gen (Perez-Martinez et al., 2009). Para analizar si la sobreexpresión de Pet309 y Mss51 compensan la síntesis de Cox1 cuando se ha eliminado a Pet54, se realizaron ensayos de dilución seriada en donde diferentes cepas con el gen reportero *ARG8^m* descrito en la Figura 9 sobreexpresan a dichos activadores traduccionales (Figura 19).
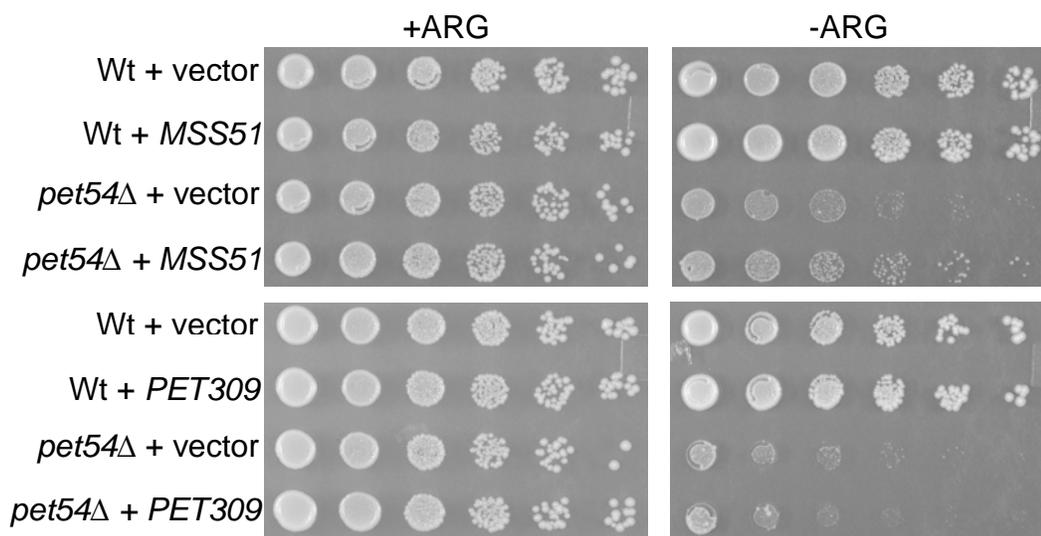
Figura 19. Diluciones seriadas (1/10) de cepas con el gen reportero *COX1-ARG8^m*. Las cepas se transformaron con vectores que sobreexpresan a los activadores traduccionales del mRNA de *COX1* Mss51 y a Pet309 (*MSS51, PET309*) o con el vector vacío (Vector). Las placas se crecieron en medio completo (+Arg) o en medio sin arginina (-Arg) y se incubaron a 30 °C por tres o cuatro días.

Como se observa en la Figura 19, las cepas sin Pet54 que sobreexpresan a Mss51 recuperan muy ligeramente la síntesis del gen reportero *ARG8^m*. Sin embargo, la sobreexpresión de Pet309 no genera ningún efecto.

En conjunto, los resultados mostrados en las Figuras 18 y 19 sugieren que Pet54 es un facilitador de la síntesis de Cox1, ya que no es estrictamente necesario para la síntesis de *COX1*, debido a que se observan niveles basales de traducción. Sin

embargo, la eliminacion de los últimos 15 aminoácidos de Cox1 no rompe con la regulación negativa debido a la ausencia de Pet54 éste mismo efecto es observado al eliminar a Cox14, lo anterior sugiere que Pet54 es un nuevo componente que participa en la biogénesis de Cox1.

Los últimos 15 aminoácidos de Cox1 son necesarios para estabilizar la interacción entre Mss51 y Cox14

En 2003 Pérez-Martínez y colaboradores (Perez-Martinez et al., 2003) determinaron por ensayos de coinmunoprecipitación que Cox1 interactua físicamente con Mss51 marcada con un triple epítope de hemaglutinina (Mss51-3xHA). Para analizar si Mss51 sigue unida a Cox1ΔC15 en procesos posteriores a la síntesis de Cox1 se realizaron ensayos de pulso y caza en cepas con Mss51-3xHA. Se realizó traducción *in organillo* dando un pulso radioactivo de 20 minutos, se detuvo el marcaje y posteriormente inmunoprecipitando con un anticuerpo anti-HA a las 0, 1, 3 y 5 horas posteriores al pulso. Las proteínas se separaron por SDS-PAGE y se transfirieron a una membrana de PVDF. Posteriormente se tomó la autorradiografía (Figura 20).
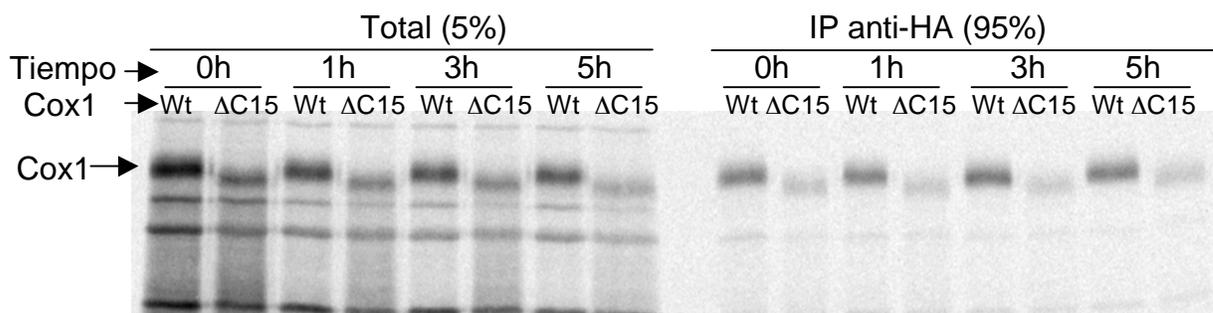
Figura 20. Traducción *in organello* de proteínas mitocondriales con [35S]-Met y co-inmuno precipitación (IP). Las proteínas se separaron por SDS-PAGE al 16%/0.15% y se revelaron utilizando una pantalla sensible a radioactividad. Cox1 wt = Cox1 completa.

En la Figura 20 se observa que la asociación entre Mss51 y Cox1ΔC15 es estable durante cinco horas, lo que apoya el hallazgo de que existen dos poblaciones de Cox1, una parte ensamblada, y otra no ensamblada que permanece unida a Mss51 (Perez-Martinez et al., 2009).

Recientemente, por ensayos de coinmunoprecipitación en cepas con Mss51-3xHA y Cox14-3xMYC, determinamos que Cox14 estabiliza la unión de Mss51 a Cox1 recién sintetizada, por lo que cuando se elimina a Cox14, Mss51 ya no se une a Cox1. Adicionalmente, reportamos que la asociación de Mss51 y Cox14 depende de la presencia de Cox1 (Perez-Martinez et al., 2009).

Para estudiar si Cox1ΔC15 aún interactua con Mss51 y Cox14 y para analizar de manera simultanea si Mss51 y Cox14 interactuan entre ellas cuando se han eliminado los últimos 15 aminoácidos de Cox1, se realizaron experimentos de traducción de proteínas mitocondriales marcadas radioactivamente con [^{35}S]-Met en mitocondrias purificadas (traducción *in organello*) en una cepa que contiene a Mss51 marcada con un triple epítope de hemaglutinina (Mss51-3xHA) y a Cox14 etiquetada con un triple epítope de MYC (Cox14-3xMYC). Cabe mencionar que la adición de epítopes no afectó el crecimiento respiratorio de las células (datos no mostrados). Después de la traducción las mitocondrias se solubilizaron con digitonina al 1% (3.0 g/g), se eliminó a las proteínas no solubilizadas y se inmuno precipitó con un anticuerpo anti-HA. Las proteínas se separaron en un gel de acrilamida/bis-acrilamida desnaturalizante (16%/0.15%), posteriormente se transfirieron a una membrana de PVDF. La asociación de Mss51 con Cox1 completa o Cox1ΔC15 se determinó por autorradiografía. Adicionalmente, la interacción de Cox14 y Mss51 se analizó por western blot con un anticuerpo anti-MYC (Figura 21).
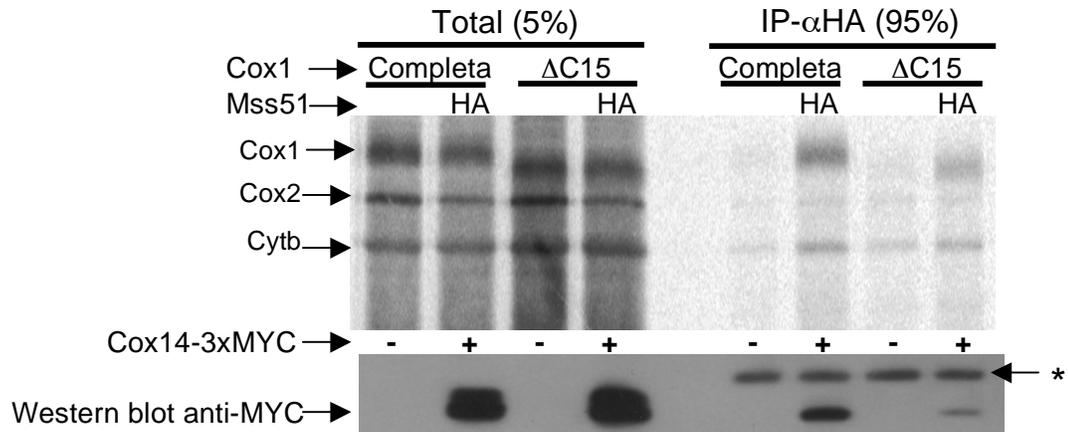
Figura 21. Traducción *in organello* de proteínas mitocondriales con [^{35}S]-Met y co-inmuno precipitación (IP-αHA). Las proteínas se separaron por SDS-PAGE al 16%/0.15% y se transfirieron a membrana de PVDF. Se reveló utilizando una pantalla sensible a radioactividad y western blot. La banda señalada con el asterisco corresponde a la subunidad pequeña del anticuerpo con el que se realizó la inmunoprecipitación. La fracción total corresponde al 5% de la reacción de traducción.

Como se observa en la Figura 21, Mss51-3xHA continuó interactuando con Cox1ΔC15 recién sintetizada de manera similar a la interacción con Cox1 completa antes reportada (Perez-Martinez et al., 2003), lo que indica que la interacción de Mss51 con Cox1 no depende únicamente de los últimos 15 aminoácidos del extremo carboxilo terminal de Cox1. Sin embargo, al realizar el western blot con un anticuerpo anti-MYC en esta misma membrana se observa que la interacción entre Mss51-3xHA y Cox14-3xMYC, cuando Cox1 carece de los últimos 15 aminoácidos, disminuye dramáticamente con respecto a la cepa con Cox1 completa.

Para estudiar si Cox1ΔC15 interactuaba con Cox14 se realizó el mismo experimento descrito en la Figura 20 pero ahora inmunoprecipitando con anticuerpo anti MYC para bajar a Cox14-3xMYC (Figura 22).
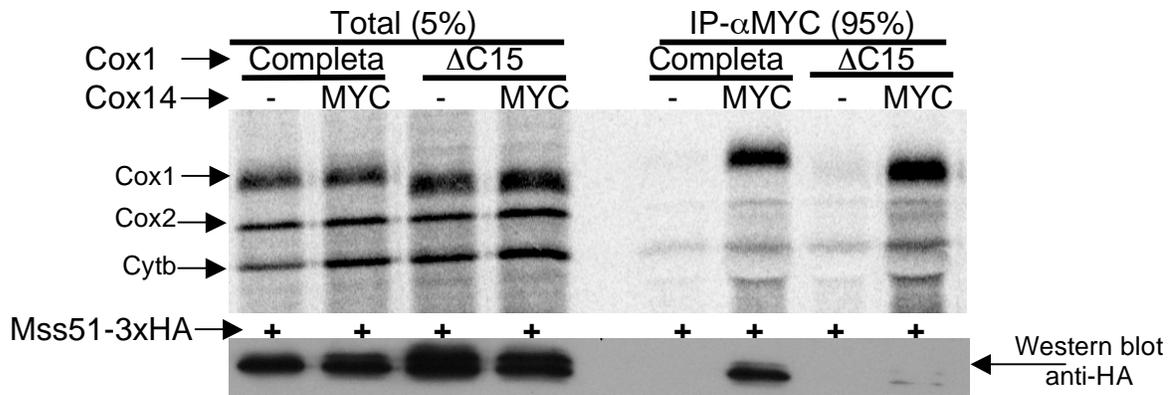
Figura 22. Traducción *in organello* de proteínas mitocondriales con [^{35}S]-Met y coinmuno precipitación (IP-αMYC). Las proteínas se separaron por SDS-PAGE al 16%/0.15% y se transfirieron a membrana de PVDF. Se reveló utilizando una pantalla sensible a radioactividad y western blot. La fracción total corresponde al 5% de la reacción de traducción.

En la Figura 22 se observa que Cox1ΔC15 recién sintetizada interactua con Cox14-3xMYC con igual eficiencia que con Cox1 completa, indicando nuevamente que la asociación entre estas proteínas no depende exclusivamente de los últimos 15 aminoácidos de Cox1. Al igual que en la Figura 21, la interacción entre Mss51-3xHA y Cox14-3xMYC cuando Cox1 carece de los últimos 15 aminoácidos disminuyó dramáticamente con respecto a la cepa con Cox1 completa (western blot, Figura 22).

A continuación se estudió si la asociación de Cox1ΔC15 recién sintetizada con Cox14-3xMYC, así como con Mss51-Cox14, se alteraba al aumentar la fuerza del detergente utilizado al solubilizar a las mitocondrias. Para esto se realizó traducción *in organello* con [^{35}S]-Met y posteriormente, las mitocondrias se solubilizaron con digitonina al 0.5%, con dodecil maltósido al 1% o con tritón-X100 al 0.1%. Se inmunoprecipitó con un anticuerpo anti-MYC y las proteínas se separaron por gel de acrilamida/bis-acrilamida desnaturalizante (16%/0.15%); posteriormente las proteinas se transfirieron a una membrana de PVDF. La asociación de Cox14 con Cox1 completa o Cox1DC15 se determinó por autorradiografía. Adicionalmente, para estudiar la unión de Mss51 a Cox14 se incubó a la membrana con un anticuerpo anti-HA. También se incubó con anticuerpo anti-MYC como control de inmunoprecipitación (Figura 23).
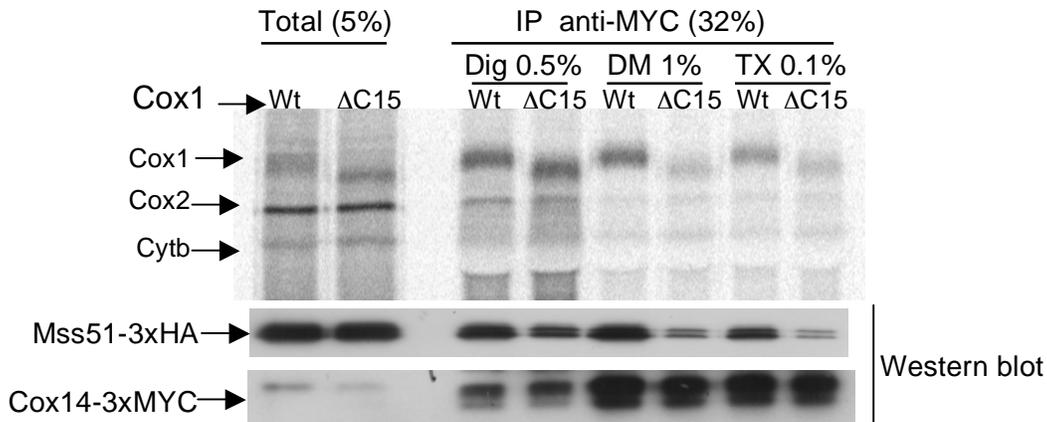
Figura 23. Traducción *in organello* de proteínas mitocondriales con [^{35}S]-Met y coinmunoprecipitación (IP). Las proteínas se separaron por SDS-PAGE al 16%/0.15% y se transfirieron a membrana de PVDF. Se reveló utilizando una pantalla sensible a radioactividad y western blot. Dig = Digitonina; DM = Dodecil maltosido; TX = Triton-X100.

En la Figura 23 se observa que al solubilizar las mitocondrias con digitonina al 0.5% la asociación de Cox1DC15 con Cox14 es similar a la asociación de Cox1 completa. La unión de Mss51 a Cox14 tampoco se ve afectada. Sin embargo, al solubilizar con dodecil maltósido, la asociación de Cox1DC15 con Cox14 se ve ligeramente afectada. Lo mismo se observa para la unión de Mss51 con Cox14. Este efecto es aún más marcado al solubilizar con Triton-X100, en donde incluso la interacción de Cox1 completa con Cox14 se ve disminuída. Estos datos indican que la formación del complejo Mss51-Cox14-Cox1DC15 recién sintetizada aún existe, sin embargo se debilita.

Finalmente, se piensa que la asociación de Mss51 con Cox14, cuando existen defectos en el ensamblaje de la CcO, se hace más fuerte, lo que estabiliza al complejo Mss51-Cox14-Cox1 recién sintetizada y de esta manera, Mss51 es secuestrada en este complejo. En este trabajo estudiamos si ésta idea al eliminar a Cox4 (*cox4Δ*), una subunidad de origen nuclear y por lo tanto bloquear el ensamblaje de la CcO. Esta mutación se generó en cepas con Cox1 completa o Cox1ΔC15 y en presencia de Mss51-3xHA y Cox14-3xMYC. Se realizó traducción de proteínas mitocondriales con [^{35}S]-Met y las mitocondrias se solubilizaron con 1% de dodecil maltósido. Se inmunoprecipitó con un anticuerpo anti-MYC y las proteínas se separaron en un gel de acrilamida/bis-acrilamida desnaturalizante (16%/0.15%). Posteriormente las

proteínas se transfirieron a membrana de PVDF. Adicionalmente, la unión de Mss51 a Cox14 se analizó por western blot con un anticuerpo anti-HA (Figura 24).
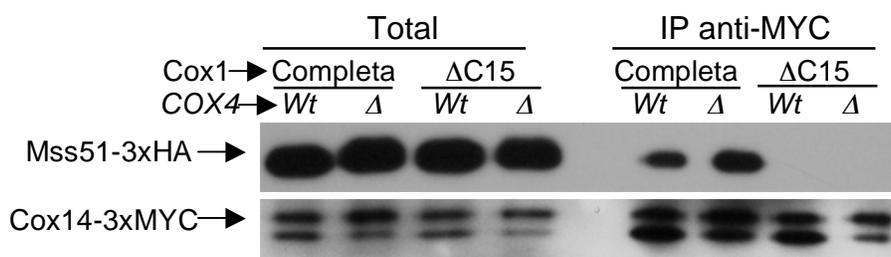
Figura 24. Traducción *in organello* de proteínas mitocondriales con [^{35}S]-Met y co-inmuno precipitación (IP). Las proteínas se separaron por SDS-PAGE al 16%/0.15% y se transfirieron a una membrana de PVDF. Se reveló realizando un western blot con los anticuerpos anti-MYC y anti-HA. Con (Wt) o sin (Δ) Cox4. No se muestra la autorradiografía por razones de simplicidad.

En la Figura 24 se observa que en la mutante que no ensambla a la CcO, con Cox1 completa, Mss51 se queda unida a Cox14 de manera semejante a la cepa que sí ensambla a la CcO. Por el contrario, en la inmunoprecitación de la cepa con Cox1ΔC15, Mss51 se disocia de Cox14 aún cuando existen defectos en el ensamblaje de la CcO.

En conjunto, las Figuras 21, 22, 23 demuestran que la asociación de Cox1 recién sintetizada con Mss51 y Cox14 no se altera. Sin embargo, los últimos 15 aminoácidos de Cox1 son necesarios para estabilizar la asociación de Mss51 con Cox14. Esto concuerda con la idea de que la eliminación de estos residuos de Cox1 genera inestabilidad de los complejos que contienen a Mss51.

Los últimos 15 aminoácidos de Cox1 estabilizan los complejos que forma Mss51

Se ha observado que Mss51 y Cox14 forman complejos de alto peso molecular, los cuales podrían regular la síntesis de Cox1. Estos complejos se han observado mediante geles de acrilamida azul nativos (Mick et al., 2007; Pierrel et al., 2007) o por centrífugación en gradientes de sacarosa (Barrientos et al., 2004; Fontanesi et al., 2009; Mick et al., 2007; Pierrel et al., 2007). Recientemente se describió que la asociación de Mss51 con Cox14 depende de la presencia de Cox1 (Perez-Martinez et al., 2009). Por lo que decidimos estudiar esta asociación en la mutante de Cox1ΔC15 para analizar si esta región de la proteína participa en la estabilidad de los complejos de

alto peso molecular que forma Mss51. Se hicieron geles azules nativos para analizar los complejos en los que Mss51-3xHA está presente en cepas silvestres o con defectos en el ensamblaje de la CcO (*cox4Δ*, Figura 25). Las mitocondrias se solubilizarón con dodecil maltosido al 1% y se separaron en un gel no desnaturalizante de acrilamida/bis-acrilamida del 5 al 13%. Las proteínas se transfirieron a una membrana de PVDF. Finalmente se hizó western blot anti-HA para ver la localización de Mss51. Se utilizó un anticuerpo policlonal que reconoce al complejo ATP sintasa como control de carga.
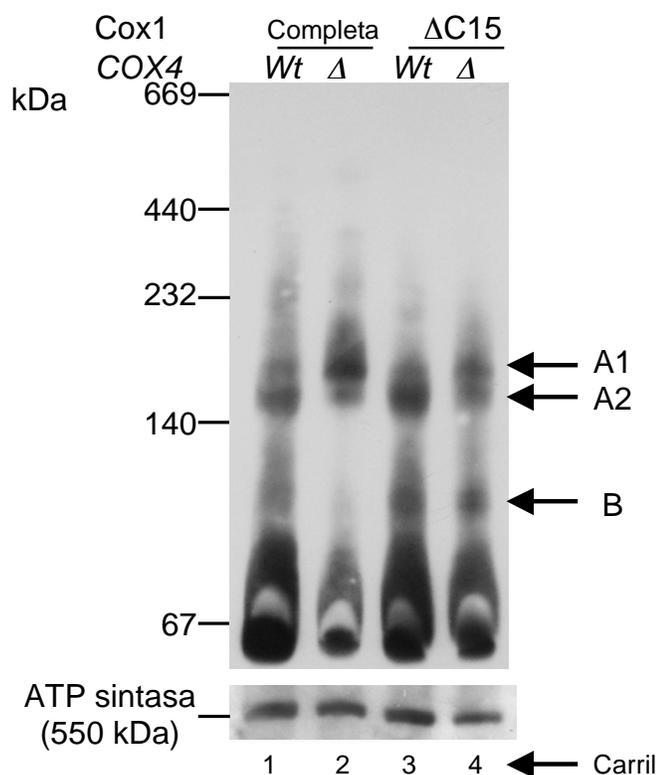
Figura 25. Gel azul nativo. 50 μg de proteínas mitocondriales se solubilizarón en 1% de dodecil maltósido y se separaron en un gel azul nativo de gradiente del 5 al 13% de acrilamida/bis-acrilamida. Posteriormente las proteínas se transfirieron a una membrana de PVDF y se realizo western blot con anticuerpo anti-HA (Mss51). Las cepas son silvestres (Wt) o con defectos en el ensamblaje de la CcO (*cox4Δ*). A la izquierda se indican los pesos moleculares estimados y a la derecha se señala la presencia de los complejos de alto peso molecular observados.

En la Figura 25 se observan los complejos que Mss51 forma en una cepa silvestre (carril 1). Éstos son designados A1, A2 (de aproximadamente 200 kDa) y B (aproximadamente 100 kDa). Además se observa una banda de aproximadamente 67 kDa que corresponde a Mss51 libre. En la cepa con defecto en el ensamblaje de la CcO se observa que el complejo B desaparece y se enriquece el complejo A1 de alto peso molecular (carril 2). En la cepa con Cox1ΔC15 se observan los mismos complejos que en la cepa silvestre (A1, A2 y B) (carril 3). En la cepa Cox1ΔC15 sin Cox4, esta distribución de complejos no cambia, a diferencia de su contraparte con Cox1 completa: el complejo B no desaparece, y en general la distribución de los complejos A1 y A2 es muy similar a las cepas que ensamblan a la CcO correctamente. Hasta el momento no ha sido posible observar a Cox14 por western blot en este tipo de geles, probablemente debido a que por ser geles nativos el epítope MYC de Cox14-3xMYC esté inaccesible al anticuerpo.

Los complejos observados en la Figura 25 difieren en tamaño de los reportados previamente. Se ha reportado que al solubilizar con digitonina al 1.5%, Mss51 forma complejos en una cepa silvestre de aproximadamente 200 y 400 kDa (Mick et al., 2007; Pierrel et al., 2007). Sin embargo, en este trabajo se utilizó el detergente dodecil maltosido al 1%, ya que en nuestras manos no se observaban diferencias en los geles nativos al solubilizar con digitonina, ver por ejemplo la Figura 23, en donde se observa como cambia la solubilización de los complejos con los diferentes detergentes.

El resultado de la Figura 25 confirma el obtenido en las Figuras 19 y 20, en donde se observa que los últimos 15 aminoácidos de Cox1 son necesarios para estabilizar los complejos en los que se encuentra Mss51.

Datos preliminares

Generación de mutantes puntuales en el extremo carboxilo terminal de Cox1

Con el fin de analizar detalladamente el papel del extremo carboxilo terminal de Cox1 en la regulación negativa de su traducción se crearon mutantes puntuales en el extremo carboxilo terminal de Cox1. Para generar dichas mutaciones se realizo un alineamiento del extremo carboxilo terminal de Cox1 de diferentes organismos, se utilizo el software Clustal W (www.ebi.ac.uk/clustalw/). Se identificaron residuos conservados desde bacterias hasta mamíferos, todos estos residuos se cambiaron por alaninas (Figura 26).
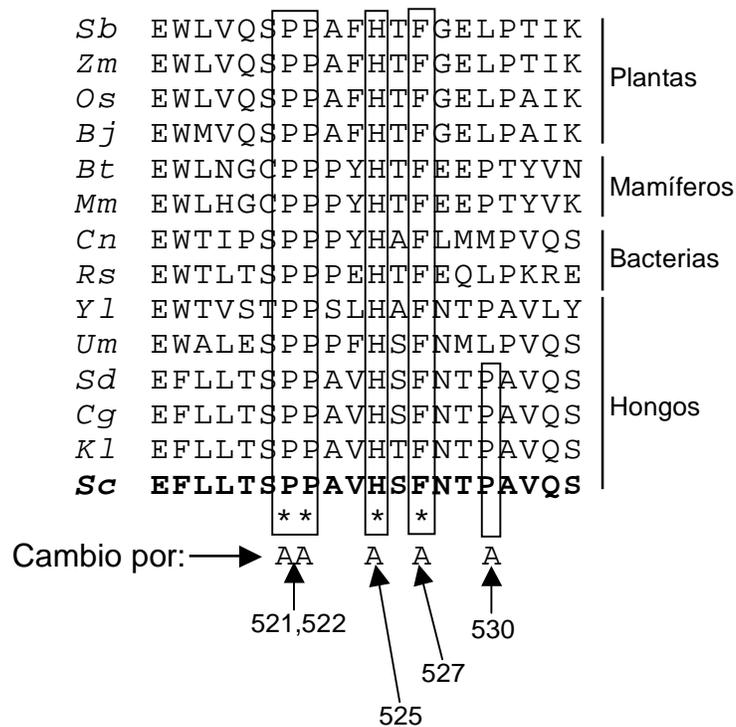
Figura 26. Alineamiento de los últimos 20 aminoácidos del extremo carboxilo terminal de Cox1. La secuencia *S. cerevisiae* se presenta en negritas. Se utilizó el software ClustalW (www.ebi.ac.uk/clustalw/). Los asteriscos indican los residuos conservados. La numeración corresponde a Cox1 de *S. cerevisiae.* Los aminoácidos dentro de los rectángulos se cambiarón por los aminoácidos señalados en la parte inferior. *Sb: Sorghum bicolor; Zm: Zea mays; Os: Oryza sativa; Bj: Brassica juncea; Bt: Bos taurus; Mm: Mus musculus; Sc: Saccharomyces cerevisiae; Sd: Saccharomyces douglassi; Cg: Candida glabrata; Kl: Kluyveromyces lactis; Yl: Yarrowia lipolytica; Um: Ustilago maydis; Cn: Cryptococcus neoformans; Rs: Rhodobacter sphaeroides.*

Con el fin de determinar si los cambios de aminoácidos generados en el extremo carboxilo terminal de Cox1 afectaban el crecimiento respiratorio de las células, se realizaron ensayos de dilución seriada (1/10) en medio respiratorio. Las diferentes cepas se cultivaron en medio YPD (medio fermentable) o en medio YPEG (medio respiratorio) y se incubaron por tres o cuatro días a 30ºC (Figura 27). Éstas mutaciones no afectan el crecimiento respiratorio.
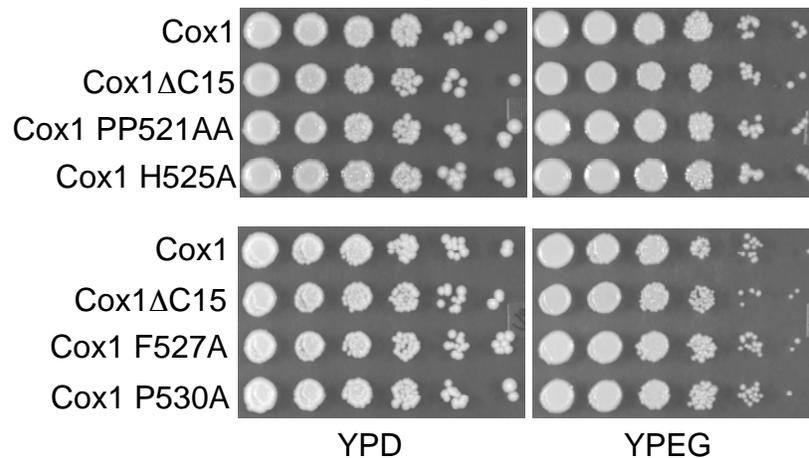
Figura 27. Diluciones seriadas de las cepas de levadura que portan diferentes mutaciones en el carboxilo terminal del gen mitocondrial *COX1*. Las diluciones se incubaron a 30 °C en medio fermentable (YPD) por tres días o en medio no fermentable (YPEG) por cuatro días.

Las mutaciones puntuales generadas en el extremo carboxilo terminal no restablecen la síntesis de Cox1

Para determinar si la síntesis de Cox1 se restablecía en las diferentes mutantes descritas en la Figura 26, se realizaron ensayos de traducción de proteínas mitocondriales marcadas radioactivamente con [35S]-Met en células completas (traducción *in vivo*) en presencia de cicloheximida. Las cepas portan defectos en el ensamblaje de la CcO debido a que se eliminó el gen mitocondrial *COX2* (*cox2-62*) (Figura 28).
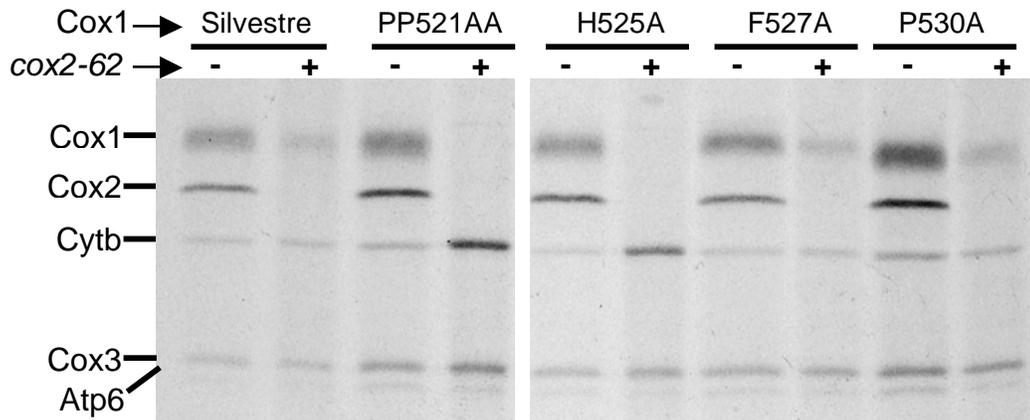
Figura 28. Traducción de proteínas mitocondriales in vivo con [^{35}S]-Met en presencia de cicloheximida. Las proteínas se separaron por SDS-PAGE al 16% y se transfirieron a una membrana de PVDF. Se reveló utilizando una pantalla sensible a radioactividad.

En la Figura 28 se observa que los cambios de aminoácidos F527A y P530A no reestablecen la síntesis de Cox1 cuando la CcO no se ensambla. Contrario a lo esperado, se observa que tanto en la doble mutante PP521,522AA como en la mutante H525A no sólo no reestablecen la síntesis de Cox1, sino que parece que la síntesis de Cox1 se abate por completo cuando existen defectos en el ensamblaje de la CcO (Figura 28). Sin embargo, ésta observación requiere más estudios para entender el fenotipo que encontramos.

Discusión

Regulación traduccional de Cox1

Como se mencionó en la introducción, Cox1 es la subunidad más grande e hidrofóbica de la CcO, y aunque el extremo carboxilo terminal es la región menos conservada, presenta algunos aminoácidos conservados (Gawryluk and Gray, 2009) (Figura 26). La biogénesis de está subunidad esta fuertemente regulada. En diferentes trabajos se ha observado que cualquier defecto en el ensamblaje de la CcO disminuye el marcaje radioactivo de Cox1. Hasta el momento no se había estudiado el mecanismo por el cual se da esta disminución y no se podía establecer si era reducción en síntesis o una mayor degradación de la proteína recién sintetizada. En este trabajo se utilizó el gen reportero *ARG8^m* (Steele et al., 1996) (Figura 6) como un reportero de la traducción de Cox1 (Perez-Martinez et al., 2003). Determinamos que la síntesis de Cox1 disminuye cuando existen defectos en el ensamblaje de la CcO. Se utilizaron mutantes que afectan la expresión o el ensamblaje de Cox2, sin embargo, el efecto de disminución de la síntesis de Cox1 es general, ya que al eliminar otras subunidades de la CcO o chaperonas que participan en el ensamblaje (Tabla 1) se observó una disminución en el crecimiento en medio sin arginina, lo que refleja una disminución en la síntesis de Arg8 (Figuras 10 y 11). Esto se confirmó por marcaje radioactivo de proteínas mitocondriales en células completas (Figura 17). Esto implica que la regulación en la traducción se da en diferentes etapas del ensamblaje de Cox1. Por ejemplo, Cox6 es una de las primeras o la primera subunidad en ser ensamblada con Cox1 (Horan et al., 2005), por lo que la observación de que la síntesis Cox1 se ve severamente afectada al eliminar a esta subunidad sugiere que el mecanismo sensor del ensamblaje de Cox1 se activa desde el inicio de la traducción. Por otro lado, Coa1, una chaperona del ensamblaje que se piensa que participa en pasos tardíos no genera un arresto en la síntesis del reportero.

Para que esta regulación negativa sea eficiente es necesario que los extremos UTR del mRNA de *COX1* estén en *cis* con la región codificante (Figura 13). Se sabe que Mss51 es limitante para la expresión de Cox1 (Perez-Martinez et al., 2009), por lo que cuando Cox1 no se encuentra en su contexto genético natural, flanqueada por sus extremos UTR (Figura 12), Mss51 no limita la síntesis de Cox1, ya que *COX1* se expresa bajo el control de Pet111, el activador traduccional de *COX2*. Esto explica por

qué cuando existen defectos en el ensamblaje de la CcO en el mtDNA quimérico se observa un aumento en la síntesis de Cox1 (Figura 13). Aunque Mss51 esté secuestrada por el complejo regulador, no se necesita en el extremo 5′-UTR del mRNA de *COX1* para activar la traducción de éste (Figura 29).

Nuestros resultados muestran que la formación del complejo Cox1-Mss51-Cox14 es estabilizada por los últimos 15 aminoácidos de Cox1 (SPPAVHSFNTPAVQS, Figura 14). Cuando Cox1 está recién sintetizada, Mss51 y Cox14 interactúan con la proteína completa, como lo demuestran las ensayos de inmunoprecipitación (Figuras 21 a 23). Proponemos que el correcto ensamblaje de Cox1 genera un cambio estructural en el extremo carboxilo terminal, lo que hace que al avanzar el ensamblaje, los últimos 15 aminoácidos ya no sean accesibles a la unión de Mss51 y Cox14. Este complejo regulador es inestable y Mss51 se libera de la interacción con Cox14 y Cox1 para seguir actuando en el extremo 5′-UTR de *COX1* y promover la traducción de la proteína. Si Cox1 no se ensambla, los últimos 15 aminoácidos de esta proteína seguirán asociados a Mss51 y Cox14, por lo que Mss51 quedará atrapada en este complejo y no podrá actuar como activador traduccional de *COX1* (Figura 29).

En la Figura 17 se observa que la mayoría de la proteínas eliminadas, a excepción de Coa1, afectan la síntesis de Cox1, siendo la falta Cox6 una de las que más afectan. En estas cepas el gen reportero *ARG8^m* está fusionado a *COX1* y porta una secuencia de procesamiento por proteasas mitocondriales (Figura 9), así que cuando se sintetiza la proteína quimérica, Arg8 ese separa de Cox1 y se comporta como una proteína soluble de matriz mitocondrial sin verse afectada por el estado de ensamblaje de Cox1 y por lo tanto de su degradación. Así la expresión de Arg8 sólo se afecta a nivel de traducción. Como se mencionó anteriormente, Cox6 es una de las primeras subunidades en ser ensamblada con Cox1 (Horan et al., 2005), por lo que la observación de que la síntesis de Arg8^m se ve severamente afectada al eliminar a esta subunidad, sugiere que el mecanismo sensor del ensamblaje de Cox1 se activa desde el inicio de la traducción.
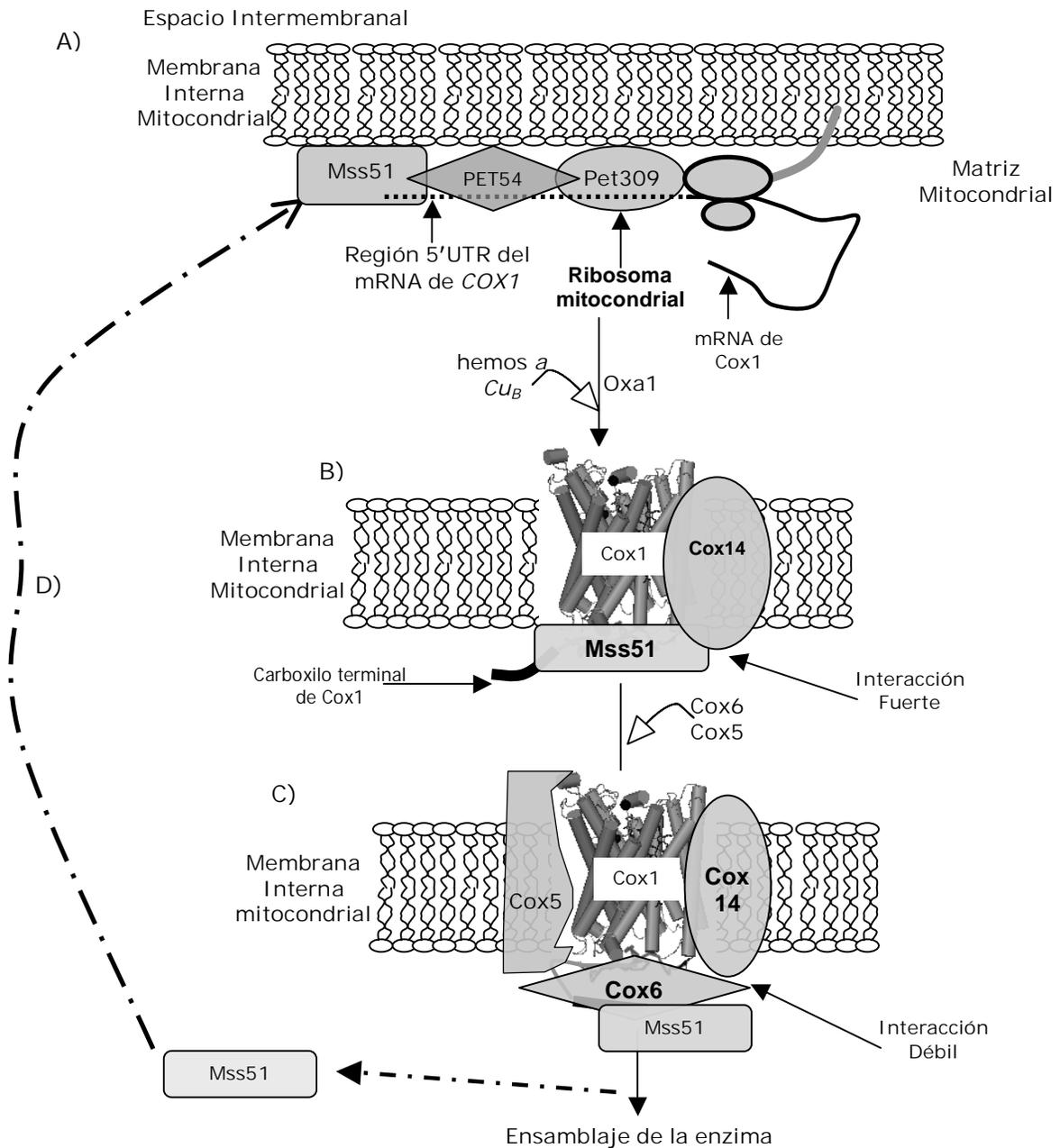
Figura 29. Modelo propuesto de regulación de la síntesis de Cox1. **A)** Los activadores traduccionales de *COX1* se unen al extremo 5'-UTR del mRNA para localizar al ribosoma en el sitio correcto de inicio de la traducción. Además de Pet309 y Mss51, Pet54 es una proteína que podría tener un sitio de acción en esta región de *COX1*. Cox1 recién sintetizada sa inserta a la membrana vía Oxa1, una translocasa general de inserción.Se cree que la inserción de grupos hemo y átomos de cobre se da de manera cotraduccional. **B)** Mss51 y Cox14 se asocian entre sí a través de los últimos 15 aminoácidos de Cox1. **C)** La inserción de otras subunidades de la CcO (Cox6, Cox5, etc.) y cofactores genera un cambio estructural en el extremo carboxilo terminal de Cox1 lo que debilita la asociación entre Mss51 y Cox14 liberando a Mss51 de este complejo ternario así Mss51 esta disponible para actuar en el 5-UTR de *COX1* y sintetizar más proteína. El extremo carboxilo terminal queda protegido por Cox6. No se muestran Cox24 y Coa1 por simplicidad del modelo.

El hecho de que Cox1 regula su síntesis mediante la interacción con sus proteínas reguladoras Mss51 y Cox14 puede ser un mecanismo de ahorro energético para evitar ensamblar una CcO no funcional, ya que este defecto produce especies reactivas de oxígeno, las cuales son dañinas para la célula. Estas especies reactivas de oxígeno se producen debido a que el sitio catalitico de Cox1 queda expuesto al medio acuoso de la matriz mitocondrial o del espacio intermembranal. (Khalimonchuk et al., 2009; Khalimonchuk et al., 2007; Poyton, 1998).

La cepas utilizadas en este trabajo para estudiar la expresión del gen *ARG8^m* son cepas que contienen intrones en *COX1*. Por lo que al eliminar a Pet54 el intrón al5β del RNA de *COX::ARG8^m* no puede ser procesado lo que podría verse reflejado en un menor crecimiento en medio −Arg (Figura11). Sin embargo, las cepas utilizadas para realizar el marcaje radioactivo de proteínas mitocondriales son cepas que no tienen intrones en *COX1*, por lo que al eliminar a Pet54 (*pet54Δ*) no tendría por qué verse afectada la expresión de Cox1 tan dramáticamente (Figura 17). Sin embargo, se observa que la eliminación de Pet54 no sólo disminuye la síntesis de Cox1 completa, sino que a excepción de todas las otras proteínas eliminadas, al eliminar los ultimos 15 aminoácidos de Cox1 (Cox1ΔC15) no se reestablece la síntesis de esta última proteína, por lo que no se rompe la regulación negativa en la doble mutante Cox1ΔC15 /pet54Δ (Figura 17D).

Pet54, un nuevo componente en la regulación de la síntesis de Cox1

La expresión del gen mitocondrial *COX3* depende de tres activadores traduccionales, los cuales actúan sobre el extremo 5' UTR del mRNA (Costanzo and Fox, 1988; Costanzo et al., 1986). Estos activadores son Pet122, Pet494 y Pet54, los dos primeros son proteínas integrales de membrana (Costanzo et al., 1989) y datos genéticos indican que por lo menos Pet122 interactua con la subunidad chica del ribosoma mitocondrial (Haffter et al., 1990; McMullin and Fox, 1993; McMullin et al., 1990).

Pet54 es una proteína periférica de membrana interna mitocondrial, de 293 aminoácidos, con una mása molecular de aproximadamente 34.6 kDa (Costanzo et al., 1989). Recientemente se ha reportado como una proteína que pertenece a la familia de las proteínas RRM (RNA Recognition Motif) (Kaspar et al., 2008). El motivo RRM

consiste de 80 a 100 aminoácidos, los cuales adoptan un plegamiento globular de cuatro hojas β antiparalelas empacadas entre dos α-hélices (Messias and Sattler, 2004). Se sabe que Pet54 participa en la activación traduccional del mRNA de *COX3* al unirse a una región de 69 nucleótidos que comprenden del -585 al -516, respecto al codón de inicio de la traducción (Costanzo and Fox, 1988; Costanzo et al., 1989; Kaspar et al., 2008); otro papel adicional es facilitar el procesamiento del intrón al5β del RNA de *COX1*. Este efecto es específico para este intrón, ya que en mutantes nulas de Pet54 en cepas con *COX1* con varios intrones se observa una acumulación del pre-mRNA de *COX1*, el cual no es traducido. Por otro lado, en esta misma cepa, pero con *COX1* sin intrones o sin el intrón al5β, se observa una acumulación tanto del mRNA maduro como de la proteína Cox1 a niveles semejantes a los de la cepa silvestre (Costanzo et al., 1989).

En 2008, Kaspar y colaboradores {Kaspar, 2008 #62} identificaron, por experimentos *in vitro* de protección a la degradación de RNA por RNAsa A, que existe una secuencia conservada en el intrón al5β de *COX1* y la región 5´-UTR del mRNA de *COX3* (Figura 30) y que por lo tanto es la región donde se une Pet54.

```
al5β:            AATTTAATAa-----gtgtcgtgcttaaaattcactaAAATAATATataataaattataata
COX3:    ttatatctatcttAATATAATAatatttatttattaaataaaaaaaaaatAAATAATATtaattaatataagattct
                 *** ******     *  *  *  *****  *  *********  *  ***  **  **
                       a)                                              b)
```

Figura 30. Alineamiento de las secuencias del intrón de *COX1* (al5β) y de la region 5´UTR de *COX3* que son protegidas de la degradación por RNAsa A Modificado de (Kaspar et al., 2008). Las región en mayúsculas y negritas es la más conservada (a y b), mientras que la región en mayúsculas y subrayada es la que se protege a la degradación por RNAsa A, probable sitio de unión de Pet54 (a).

Analizando la secuencia de las cepas con las que trabajamos, las cuales carecen de intrones en *COX1*, se encontró que existen dos regiones en el extremo 5´UTR de *COX1* (-257 y a -52 nucleótidos respecto al codón de inicio) que contienen una secuencia semejante a la que se une a Pet54 en el RNA de *COX3* y en el intrón al5β (Figura 31). Esta región se sobrelapa con los nucleótidos -245 a +23, que se ha propuesto como el sitio de unión de Mss51 al mRNA de *COX1* (Zambrano et al., 2007).

```
COX3:            ctatcttAATATAATAatatttatttattaaataaaaaaaaaaatAAATAATATtaattaa
COX1 -257:       actttatAATATAATAatatttatttataaagatataaaagaattgtttaaagttataa
COX1 -52:        ttttttaatgAATATAATAataataatattattaaaattaatatataaaaaaaaagtaaaaATGg
                        *********** * ** *    *    ** * *     * * * *
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Figura 31. Alineamiento de las dos regiones donde presumiblemente se une Pet54 al extremo 5'-UTR del mRNA de *COX1* (nucleótidos en negritas y mayúsculas). Las negritas en minúsculas demuestran que esta región comprende más nucleótidos y que está altamente conservada (recuadro). El sitio marcado con la flecha es el codón de inicio de la traducción de *COX1*.

Creemos que la región identificada en la Figura 31 es un sitio donde probablemente Pet54 se une al mRNA de *COX1* para facilitar la traducción de *COX1*, ya que como se observa en la Figura 17-B, Pet54 es necesaria para sintetizar a Cox1; sin embargo, es la única proteína que al ser eliminada no se reestablece la síntesis de Cox1ΔC15. Esto sugiere que es una facilitadora de la expresión de *COX1*. Además de que los experimentos de las Figuras 18 y 19 apoyan esta idea, se ha observado por experimentos de doble híbrido que Pet54 interactúa con Pet309, uno de los activadores traduccional del mRNA de *COX1* (Naithani et al., 2003).

Finalmente, éste es el primer ejemplo en mitocondrias en que una proteína autorregula su síntesis por medio de la secuencia de aminoácidos. Sin embargo recientemente se reportó que Atp6 no se sintetiza cuando existen defectos en el ensamblaje de la región F1 de la ATP sintasa, sin embargo la regulación en la expresión de este gen no depende de la región codificante sino de los extremos UTR (Rak and Tzagoloff, 2009), por lo que el mecanismo, aún no estudiado, es diferente al que estudiamos en este trabajo.

Existen varios ejemplos de autoregulación negativa de la traducción; por ejemplo, en *Chamydomonas reinhardtii* el citocromo *f* (cyt *f*), codificado por el gen del cloroplasto *petA*, disminuye su síntesis cuando existe una deficiencia en alguna de las subunidades estructurales del complejo b_6f (Chen et al., 1993; Kuras, 1994). Se han propuesto dos mecanismos por los cuales se pueda estar dando esta regulación negativa. En uno se propone que el carboxilo terminal de cyt *f* no ensamblado interactua con su mRNA, haciéndolo inaccesible a su activador traduccional (Tca1) o al ribosoma para inhibir la traducción (Choquet et al., 1998). En el otro modelo se propone que el extremo

carboxilo terminal de cyt *f* no ensamblado interactua con su propio activador traduccional, la proteína nuclear Tca1. De esta manera cyt *f* secuestra a su propio activador traduccional, el cual ya no está disponible para activar la traducción de cyt f (Choquet et al., 1998; Wostrikoff et al., 2001). El mecanismo descrito en el presente trabajo se asemeja a este último modelo.

Otro ejemplo es el de las proteínas L11, L20 y L4 de la subunidad grande del ribosoma de *Escherichia coli*. Cuando no se ensamblan correctamente en el ribosoma, éstas se unen a su propio RNA mensajero inhibiendo la traducción y de esta manera se detienen la síntesis de más proteína (Allemand et al., 2007; Dean and Nomura, 1980). En mamíferos se han identificado a las proteínas "death associated protein kinase" (DAPK) y "zipper-interacting protein kinase" (ZIPK), las cuales forman complejos de cascadas de señalización. Cuando éstas no se ensamblan correctamente generan una cascada de señalización que termina en la fosforilación de la proteína ribosomal L13a, la cual está involucrada en la traducción de los mRNA de estas proteínas (Morley and Willett, 2008; Mukhopadhyay et al., 2008).

Un ejemplo extensamente estudiado de regulación negativa de la traducción es la proteína de *E. coli* SecM, la cual se secreta al periplasma por el sistema de translocación "SRP-Sec". Cuando SecM no se secreta correctamente debido a defectos en el sistema SRP-Sec, SecM detiene su traducción por medio de señales presentes en su extremo carboxilo terminal (Nakatogawa and Ito, 2002). Se sabe que la señal para detener la traducción y fijar al péptido saliente en el ribosoma es una región rica en prolinas ubicada en el extremo carboxilo terminal. Cuando estas prolinas se cambian por alaninas, la proteína SecM se sintetiza como en una cepa silvestre aún cuando no se excrete correctamente (Tanner et al., 2009). Para entender mejor el proceso por el cual Cox1 regula su síntesis se realizaron mutaciones puntuales en las que se cambiaron por alanina varios aminoácidos, incluidas las prolinas, que se encuentran en el extremo carboxilo teminal. Estas mutantes se analizaron por ensayos de traducción de proteínas mitcondriales, sin embargo éstos son aún resultados preliminares (Figuras 26 a 28).

Recientemente se ha descrito que existen microorganismos como *Acanthamoeba castellanii* en los que el gen mitocondrial *COX1* está dividido en dos partes, la región del gen que codifica la parte hidrofóbica de Cox1 se encuentra en el genoma mitocondrial (llamado *COX1(-)*). De manera interesante, la región que codifica la parte soluble del

extremo carboxilo terminal de Cox1 se encuentra codificado en el genoma nuclear (*COX1-c*) (Gawryluk and Gray, 2009). Por análisis bioinformático se determinó que en los supergrupos filogenéticos Amoebozoa, Chromalveolata y Excavata existen los genes *COX1(-)* mitocondrial y *COX1-c* nuclear. En todos estos organismos la región carboxilo terminal de Cox1, de aproximadamente 25 aminoácidos, muestra una gran similitud a una región altamente conservada desde bacterias hasta mamíferos, la cual corresponde a la región carboxilo terminal que en este trabajo eliminamos (Gawryluk and Gray, 2009).

El hecho de que la región codificante del extremo carboxilo terminal de Cox1 haya sido retenida en el genoma nuclear después de su pérdida en el genoma mitocondrial sugiere que esta región tiene una función muy importante. En este trabajo demostramos que esta región es necesaria para sensar el correcto ensamblaje y síntesis de Cox1. Estudios cristalográficos de la CcO de bovino demuestran que la histidina 525 (Figura 23), la cual esta ampliamente desde bacterias hasta mamíferos, es una parte importante del sitio de entrada de los protones que la CcO usa para reducir el oxígeno a agua (Muramoto et al., 2007). Esta histidina se encuentra dentro de los 15 aminoácidos que se eliminaron en este trabajo, sin embargo su eliminacion no afecto el crecimiento respiratorio en *S. cerevisiae*.

Conclusiones

- La disminución en el marcaje radioactivo de Cox1 en presencia de defectos en el ensamblaje de la CcO se debe a una menor traducción del mRNA de *COX1*.

- Es necesario que los extremos UTR y la región codificante del mRNA de *COX1* se encuentren en *cis* para regular la traducción de este mensajero cuando existen defectos en el ensamblaje de la CcO.

- El extremo carboxilo terminal de Cox1 porta señales de regulación negativa de la traducción del mRNA de *COX1*. Cuando esta región está ausente, entonces la regulación negativa de la síntesis de Cox1 se rompe aunque no se ensamble la CcO.

- Los últimos 15 aminoácidos de Cox1 estabilizan la asociación de Mss51 con Cox14.

- Se identificó por primera vez a Pet54 como una proteína facilitadora de la síntesis de Cox1.

Perspectivas

1. Analizar el efecto de mutaciones puntuales en el extremo carboxilo terminal de Cox1 descritas en la Figura 26, realizando ensayos de traducción de proteínas mitocondriales y ensayos de inmunoprecipitación para determinar la asociación de Cox14 y Mss51.

2. Eliminar todo el extremo carboxilo terminal de Cox1 (56 aminoácidos), así como una región que parece ser una extensión de aminoácidos que forman un asa que sólo se encuentra en hongos y analizar dichas mutantes.

3. Determinar si existe asociación de Pet54 con la región identificada del mRNA de *COX1* descrita en la Figura 29, a través de geles de retardo o de protección a la digestión pro RNAsa A.

Apéndice 1. Artículos publicados durante el doctorado

1 **Miguel Shingú-Vázquez**, Yolanda Camacho-Villasana, Luisa Sandoval-Romero, Christine Butler, Thomás D. Fox and Xochitl Pérez-Martínez. (2010) The carboxyl-terminal end of Cox1 is involved in the feedback-assembly regulation of Cox1 synthesis in yeast mitochondria. *J. Biol. Chem.* JBC/2010/161976

2 Perez-Martinez X, Butler CA, **Shingu-Vazquez M**, Fox TD. (2009) Dual functions of Mss51 couple synthesis of Cox1 to assembly of cytochrome c oxidase in *Saccharomyces cervisiae* mitochondria. *Mol. Biol. Cell.* Oct;20(20):4371-80.

3 Pérez-Martínez X, Funes S, Camacho-Villasana Y, Marjavaara S, Tavares-Carreón F, **Shingú-Vázquez M.** (2008) Protein synthesis and assembly in mitochondrial disorders. *Curr Top Med Chem.* Vol. 8(15). Pp. 1335-50.

4 Tavares-Carreón F, Camacho-Villasana Y, Zamudio-Ochoa A, **Shingú-Vázquez M**, Torres-Larios A, Pérez-Martínez X. (2008) The pentatricopeptide repeats present in Pet309 are necessary for translation but not for stability of the mitochondrial COX1 mRNA in yeast. *J Biol Chem.* Vol. 18;283(3). Pp. 1472-9.

THE CARBOXYL-TERMINAL END OF Cox1 IS REQUIRED FOR FEEDBACK-ASSEMBLY REGULATION OF Cox1 SYNTHESIS IN *Saccharomyces cerevisiae* MITOCHONDRIA

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Synthesis of the largest cytochrome *c* oxidase (CcO) subunit, Cox1, on yeast mitochondrial ribosomes is coupled to assembly of CcO. The translational activator Mss51 is sequestered in early assembly intermediate complexes by an interaction with Cox14 that depends on the presence of newly synthesized Cox1. If CcO assembly is prevented, the level of Mss51 available for translational activation is reduced. We deleted the C-terminal 11 or 15 residues of Cox1 by site-directed mutagenesis of mtDNA. While these deletions did not prevent respiratory growth of yeast, they eliminated the assembly feedback control of Cox1 synthesis. Furthermore, these deletions reduced the strength of the Mss51-Cox14 interaction as detected by co-immune precipitation, confirming the importance of the Cox1 C-terminal residues for Mss51 sequestration. We surveyed a panel of mutations that block CcO assembly for the strength of their effect on Cox1 synthesis, both by pulse-labeling and by expression of the *ARG8^m* reporter fused to *COX1*. Deletion of the nuclear gene encoding Cox6, one of the first subunits to be added to assembling CcO, caused the most severe reduction in Cox1 synthesis. Deletion of the C-terminal 15 amino acids of Cox1 increased Cox1 synthesis in the presence of each of these mutations, except *pet54*. Our data suggest a novel activity of Pet54 required for normal synthesis of Cox1 that is independent of the Cox1 C-terminal end.

Introduction

Cytochrome *c* oxidase (CcO) is the terminal electron acceptor of the mitochondrial respiratory chain. It transfers electrons from cytochrome *c* to oxygen, with a coupled translocation of protons from the matrix to the intermembrane space. In the yeast *Saccharomyces cerevisiae*, this enzyme is composed of eleven subunits, three of which, Cox1, Cox2 and Cox3 are encoded by the mitochondrial genome, synthesized by organellar ribosomes and integrated into the inner membrane from the matrix side. Assembly of this enzyme is very complex. It involves not only coordinated assembly of nuclear and mitochondrial encoded subunits, but the addition of metallic prosthetic groups like heme *a*, and Cu centers. For this process, more than 30 factors are necessary, although the functions of these proteins are just starting to be elucidated (1,2).

The yeast model is widely used to study the mechanisms of CcO biogenesis, as several pathogenic mutations affecting CcO assembly have been described in human genes having yeast homologues. The majority of these encephalomyopathies are associated with mutations in nuclear genes encoding CcO assembly factors (3). In recent years some mutations associated with Leigh syndrome have been found in genes affecting expression of mitochondrial genes. This is the case for LRPPRC, a human protein distantly related to the yeast translational activator Pet309 (4-6), and *TACO1*, a gene specifically required for Cox1 synthesis (7).

Cox1 is the largest subunit of the CcO and spans the mitochondrial inner membrane 12 times (8). Cox1 contains the heme *a* and heme a_3-Cu_B centers for oxygen reduction. Cox1 is present from the first assembly intermediate, and the rest of subunits and cofactors are thought to be added in a sequential order (9,10). Partial assembly of Cox1 is associated with peroxide sensitivity due to pro-

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oxidant intermediates containing unassembled heme a_3 (11). Hence, stoichiometry of Cox1 in the inner membrane has to be highly regulated. For this, many factors have been identified that control Cox1 biogenesis. Pet309 and Mss51 are specific translational activators that function through the *COX1* mRNA 5'-UTR (untranslated region) (4,12). In addition, Mss51 physically interacts with Cox1, suggesting that it has a central role in coordinating the synthesis and assembly of this subunit (13,14). Cox1 and Mss51 form a high-molecular complex with Cox14. Next, Coa1 could insert into this complex (15). While Mss51 and Coa1 are proposed to be liberated from this complex at early steps (16,17), Cox14 might remain associated to the assembling CcO until the formation of supercomplexes (16).

The current model proposes that Mss51 limits translational activation of the *COX1* mRNA, and is sequestered from this function by its incorporation into assembly intermediate complexes containing newly synthesized Cox1 and Cox14. In CcO assembly mutants, Mss51 is trapped in these complexes and thus unavailable for efficient *COX1* mRNA translational activation (12,14).

Pulse-labeling of Cox1 *in vivo* with [^{35}S]methionine is specifically reduced in several mutations affecting CcO assembly (for examples see (14,18,19)). Lower levels of Cox1 labeling have even been documented for mutations affecting the ATP synthase (20,21) and the CcO substrate cytochrome *c* (22). This reduction in Cox1 labeling is presumed to be due to decreased *COX1* mRNA translation. Here, through mutagenesis of the mitochondrial *COX1* gene we have found that the C-terminal domain of Cox1 is necessary for assembly-coupled translational down-regulation. Absence of Cox6, one of the first subunits to be added to the CcO, showed one of the most dramatic C-terminal end dependent reductions of Cox1 synthesis. In addition, we report that Pet54 is a new component required for normal *COX1* mRNA translation. A mutation in Pet54 seems to reduce Cox1 synthesis by a mechanism that is independent of the Cox1 C-terminal end.

Experimental Procedures

Strains and genetic methods

Saccharomyces cerevisiae strains used in this study, all congenic or isogenic to D273-10B (ATCC 24657), are listed in the Supplemental Table 1. Genetic methods and media were as previously described (23,24). Complete fermentable media were YPD or YPGal (containing 2% glucose or 2% galactose). Non-fermentable medium was YPEG (3% glycerol, 3% ethanol). Minimal medium was synthetic complete (0.67% yeast nitrogen base, 2% glucose) lacking the indicated amino acids. The nuclear deletion constructs with *KanMX4*, *LEU2* or *URA3* cassettes were obtained by PCR. Plasmids carrying the *cox1* mutations were transformed into the rho0 strain NAB69 by high-velocity microprojectile bombardment (25). Transformants were selected by their ability to rescue respiratory growth when mated with a strain carrying a Cox1 D369N mutation, L45 (26). Transformants were mated with XPM10b (containing the *cox1Δ::ARG8^m* construct) or XPM13a (containing the *cox2-62* and the *cox1Δ::ARG8^m* construct) (13). Cytoductants were selected for their ability to grow on YPEG as haploids or after mating to a strain with the mutation G253D (27). In all cases, correct integration of the different constructs into the mtDNA was confirmed by PCR and DNA sequencing.

Construction of the cox1 mutant genes.

Plasmid pXPM57, containing the full length, intronless *COX1* gene was used as template for PCR amplifications. This plasmid contains 395 and 990 nucleotides of the *COX1* 5'-UTR and 3'-UTR, respectively, and was cloned in the *XbaI-XhoI* sites from pBluescript (Stratagene). All *cox1* mutant plasmids were generated by the fusion PCR technique (28) using Accuzyme (Bioline). The 3' half of the *COX1* coding region was amplified with primers that incorporated the mutations. These products were digested with *NdeI* and *AflII* and ligated into pXPM57 equally digested. Plasmids were sequenced to confirm the presence of the desired mutations in *COX1*.

Analysis of mitochondrial proteins

Yeast cells were grown in complete or minimal galactose media until late log phase. Crude mitochondria were obtained by disruption of cells with glass beads or by zymoliase 20T treatment as described (29). Proteins were

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separated by SDS-PAGE on a 16% gel (30), and western blots were probed with antibodies to HA (Roche), cMyc (Roche) or citrate synthase. Secondary goat anti-mouse or anti-rabbit (Sigma) conjugated to horseradish peroxidase was detected with the ECL kit (GE Healthcare).

In vivo pulse labeling of cells with [^{35}S]methionine was performed as previously described (31). After pulse labeling, cells were chilled on ice and disrupted by vortexing with glass beads to obtain mitochondria (29). The radiolabeled proteins were separated on a 16% polyacrylamide gel and transferred to a PVDF membrane before they were analyzed with a Typhoon 8600 Phosphoimager (Molecular Dynamics).

Translation in isolated mitochondria (3 mg protein/ml) in the presence of [^{35}S]methionine was performed as previously described (32). After translation, mitochondria were washed with 0.6 M sorbitol, 20 mM HEPES pH 7.4, and lysed with a buffer containing 100 mM NaCl, 20 mM Tris pH 7.4, and either 1% digitonin (wt/vol) or 1% dodecyl maltoside (wt/vol). Immunoprecipitation of labeled mitochondrial products with an HA-specific antibody coupled to protein A agarose (Invitrogen) or Myc-specific antibody coupled to protein A agarose (Santa Cruz) was performed according to the provider instructions. Proteins were analyzed as described for *in vivo* labeling experiments.

Results

The carboxyl-terminal end of Cox1 is required for assembly-mediated reduction of Cox1 synthesis.

Synthesis of Cox1 is reduced in several mutants affecting CcO assembly. Mss51 has a central role in this process by interacting with the Cox1 protein (13,14).

We explored which regions of Cox1 might be involved in regulating synthesis. Based on the bovine CcO crystallographic structure (8), a model for the yeast Cox1 structure was generated using the program SWISS MODEL (33). The largest hydrophilic portion of Cox1 was the C-terminal region, comprising 59 amino acids, exposed on the matrix side of the inner membrane. This domain has an extended secondary structure that turns and covers the bottom of Cox1 (Fig. 1A). We reasoned that this portion of Cox1 could contain sites for

interaction of peripheral membrane proteins like Mss51.

By site-directed mutagenesis and mitochondrial transformation we deleted codons encoding the last 5 (Cox1ΔC5; PAVQS), 11 (Cox1ΔC11; VHSFNTPAVQS) or 15 (Cox1ΔC15; SPPAVHSFNTPAVQS) residues from the C-terminal portion of Cox1. The three mutant strains retained the ability to grow on non-fermentable YPEG medium at levels comparable to wild-type (data not shown), demonstrating that these Cox1 variants are assembled into active CcO.

To disrupt assembly, we first introduced a mitochondrial *cox2* deletion (*cox2-62*) (34) into mtDNA encoding the truncated forms of Cox1. Mitochondrial translation products were pulse-labeled in cells incubated with cycloheximide and [^{35}S]methionine. As expected, in cells synthesizing wild-type Cox1, the *cox2Δ* mutation induced a dramatic reduction of Cox1 labeling (Fig. 1B). Similarly, the synthesis of the Cox1ΔC5 protein was reduced by the *cox2Δ* mutation. In contrast, the synthesis of Cox1ΔC15 and Cox1ΔC11 was not reduced by the *cox2Δ* mutation. These data indicate that residues between -5 and -11 from the Cox1 C-terminus, VHSFNT, are required to down-regulate Cox1 synthesis in a *cox2* mutant.

The last 15 residues of Cox1 facilitate the interaction between Mss51 and Cox14.

Assembly-mediated control of Cox1 synthesis involves sequestration of Mss51 in complexes containing Cox14 and newly synthesized Cox1. Interaction of Mss51 and Cox14 from wild-type mitochondrial extracts has been previously observed (14), and is known to be dependent upon synthesis of Cox1 (12). We therefore tested whether interactions among these components were affected by deletion of the last 15 residues of Cox1. We attached a triple Myc epitope to the C-terminus of Cox14, and a triple hemagglutinin (HA) epitope to the C-terminus of Mss51. The respiratory competence of the Mss51-HA, Cox14-Myc strains were comparable to wild-type levels, indicating that the tagged proteins were functional (data not shown). We first asked whether immuneprecipitation of Cox14-Myc would co-precipitate newly synthesized Cox1ΔC15. Mitochondria isolated from strains containing Cox14-Myc, Mss51-HA and either

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wild-type Cox1 or Cox1ΔC15, were allowed to synthesize proteins in the presence of [^{35}S]methionine. After solubilization in 1% digitonin, the mitochondrial extracts were immunoprecipitated with a Myc-specific antibody. The immunoprecipitated proteins were separated by SDS-PAGE and transferred to a PVDF membrane. Co-precipitation of newly synthesized Cox1 and Cox1ΔC15 with Cox14-Myc was equally efficient (Fig. 2A). However, probing the PVDF membrane with HA-specific antibody to detect Mss51-HA revealed that almost no Mss51-HA was co-precipitated with Cox14-Myc in the presence of the truncated Cox1ΔC15, in contrast to wild-type Cox1. We were unable to analyze the interaction of unlabeled Cox1ΔC15 with Cox14-Myc by western blotting since the truncation apparently removed the epitope recognized by the Cox1-specific antibodies we tested (data not shown).

We also immunoprecipitated these mitochondrial extracts with HA-specific antibody. As previously reported, newly made Cox1 co-precipitates with Mss51-HA (13). This interaction was similarly efficient in Cox1ΔC15 mitochondria (Fig. 2B). However, co-immune precipitation of Cox14-Myc with Mss51-HA was dramatically reduced in the presence of Cox1ΔC15, compared to wild-type Cox1. Taken together, these results indicate that the ability of the truncated Cox1ΔC15 protein to bridge the interaction between Cox14-Myc and Mss51-HA is compromised.

Taken together, these data indicate that the C-terminal 15 residues of Cox1 are required for normal stability of a complex (or complexes) containing Mss51, Cox14, and newly synthesized Cox1. Instability of this complex could account for the robust synthesis of Cox1ΔC15 in a mutant that is unable to assemble CcO, since Mss51 would not be efficiently sequestered and therefore available to activate *COX1* mRNA translation. To test this, we next examined the Mss51-Cox14 interaction in strains unable to assemble CcO due to a *cox4Δ* mutation that contained either wild-type Cox1 or Cox1ΔC15 (Cox1 synthesis in a *cox4Δ* mutant was also regulated by the Cox1 C-terminal end, see below). Mitochondria were isolated, allowed to synthesize unlabeled proteins, and solubilized with 1% dodecyl maltoside. Cox14-Myc was precipitated with a Myc-specific antibody, and the

precipitates were analyzed by western blotting. We observed an increased efficiency of Mss51-HA co-immunoprecipitation in *cox4Δ* as compared to *COX4* mitochondria in the presence of Cox1. However, the Cox14-Mss51 interaction was not detected in the presence of Cox1ΔC15, regardless of whether the *cox4Δ* mutation prevented CcO assembly (Fig. 3).

The carboxyl-terminal end of Cox1 regulates Cox1 synthesis in several mutants affecting CcO assembly.

A wide range of mutations that affect CcO assembly show reduced levels of Cox1 labeling in the presence of [^{35}S]methionine (14,18,19). We tested whether labeling of the truncated variant Cox1ΔC15 would also remain unaffected when CcO assembly was disrupted by mutations other than *cox2Δ*. Two groups of CcO mutants were created. In the first group, synthesis of CcO subunits was prevented by *cox4Δ*, *cox6Δ* and *cox7Δ* mutations, or by *COX3* mRNA translational activator mutations *pet122Δ* and *pet54Δ*. Cox6 is added to the first assembly intermediate containing Cox1, while Cox3, Cox4 and Cox7 are assembled later (35). In the second group, assembly chaperones were eliminated: *mss2Δ* (necessary for assembly of Cox2 (36)), *cox11Δ*, *cox15Δ* (involved in formation of Cu$_B$ and heme *a* centers in Cox1, respectively (37,38)), *coa1Δ* (participates in Cox1 assembly (16,17)), *pet100Δ* (involved in formation of intermediates containing Cox7, Cox7a and Cox8 (39), and *pet191Δ* (twin Cx$_9$C protein necessary for full assembly of CcO (40)). Cox11, Cox15 and Coa1 seem to participate in early stages of CcO assembly (1,15).

We constructed strains carrying each nuclear mutation with either wild-type mtDNA or the mtDNA encoding Cox1ΔC15, and carried out [^{35}S]methionine labeling (Fig. 4A and 4B). With the exception of the *coa1Δ* strain, which was previously demonstrated to have normal levels of Cox1 [^{35}S]methionine labeling (16,17), and in our hands had no respiratory growth defect in the D273-10B nuclear genetic background used here, the CcO mutants showed reduced labeling of wild-type Cox1 by 42 to 85%. In contrast, labeling of the truncated Cox1ΔC15 protein was not reduced by most of these mutations, indicating that the C-terminal end of Cox1 regulates Cox1 synthesis

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Running title: The Cox1 C-terminal end regulates COX1 translation

independently of the stage where CcO assembly is interrupted Two mutants consistently showed the most dramatic reduction of both Cox1 and Cox1ΔC15 [³⁵S]methionine labeling: cox6Δ and pet54Δ.

The cox6Δ mutation reduced labeling of wild-type Cox1 by 85%, and also reduced labeling of Cox1ΔC15 by 63%. Thus, the cox6Δ mutation strongly reduced Cox1 labeling, and this effect is only slightly ameliorated by the C-terminal truncation of Cox1. The pet54Δ mutation reduced labeling of wild-type Cox1 by 60%. In contrast to other CcO assembly mutants, the pet54Δ mutation similarly reduced labeling of truncated Cox1ΔC15. This was the only mutant analyzed whose Cox1 labeling was not increased by C-terminal truncation of Cox1, suggesting that this effect might not be due simply to the lack of CcO assembly. Indeed, deletion of another COX3 mRNA translational activator, Pet122, resulted in a labeling pattern similar to those of the majority of CcO assembly mutants.

The more dramatic reduction of Cox1 labeling in cox6Δ and pet54Δ could be due to decreased synthesis or a more rapid degradation of newly made Cox1. To distinguish these possibilities, we analyzed expression of the mitochondrial reporter gene ARG8^m, which codes for a soluble biosynthetic enzyme in the matrix, and whose activity does not depend on the presence of CcO (41). ARG8^m was fused in frame to the end of the COX1 coding region to create the construct COX1(1-512)::ARG8^m (13). This ARG8^m sequence specifies the cleavage site for the pre-Arg8 mitochondrial targeting signal, such that accumulation of mature Arg8 should not be affected by the stability of Cox1. Thus, expression of ARG8^m from this construct provides a readout of COX1 mRNA translation. Furthermore, the Cox1 moiety encoded by COX1(1-512)::ARG8^m is assembled into active CcO complexes, supporting normal respiratory growth (13).

We combined some of the nuclear mutations described in Fig. 4A with the COX1(1-512)::ARG8^m construct. All the CcO mutants analyzed showed reduced growth in medium lacking arginine as compared to a wild-type strain with the COX1(1-512)::ARG8^m construct (Fig. 4C). However absence of Cox6 and Pet54 consistently showed the most dramatic reduction

in Arg⁺ growth. [³⁵S]methionine labeling of the Cox1-Arg8 fusion protein in these cells was reduced in all the CcO mutants, but most dramatically reduced in the cox6Δ and pet54Δ mutants (supplemental Fig. S1). These data confirm that the CcO assembly defect caused by the loss of Cox6 or Pet54 reduced synthesis of the reporter fused to full-length Cox1 more than other CcO mutants.

Cox6, together with Cox5a, are the first subunits to assemble with Cox1 (35). It has been suggested that Cox5a and Cox6 confer stability to newly synthesized Cox1 (42). To further test whether deletion of Cox6 confers a strong decrease in Cox1 synthesis we first asked whether conditions that alter Cox1 pulse labeling in CcO assembly defective mutants similarly alter expression of the COX1(1-512)::ARG8^m reporter. In cox1ΔC15 cells, [³⁵S]methionine labeling of Cox1 is restored to wild-type levels even in the presence of mutations affecting CcO assembly (14). Consistent with this finding, double mutant cox6Δ cox1Δ cells grew on medium lacking arginine as well as wild-type COX6, COX14 cells (Fig. 5A). Mitochondrial [³⁵S]methionine labeling of these strains showed a band corresponding to the Cox1-Arg8 fusion protein Labeling of the Cox1-Arg8 precursor, as well as the small amount of processed Cox1 was reduced by cox6Δ. However Cox1 labeling was largely restored in the cox6Δ cox1Δ double mutant (Fig. 5B). Together these data confirm that translation of the reporter Arg8 reporter is decreased in the absence of Cox6.

We next performed [³⁵S]methionine labeling in the presence of cycloheximide of Cox1 or Cox1ΔC15 cells. The cox6Δ mutation reduced labeling of Cox1 and Cox1ΔC15. However labeling was restored to almost wild-type levels in the cox6Δ cox14Δ double mutant (Fig. 5C). Cox1 pulse-labeling in CcO assembly mutants can also be increased by over-expression of Mss51 (14). We found that over-expression of MSS51 partially restored labeling of Cox1 and Cox1ΔC15 in a cox6Δ mutant (supplemental Fig. S2).

Taken together, these data indicate that the assembly defect caused by the cox6Δ reduces Cox1 synthesis more strongly than the other assembly defects tested. It apparently acts by reducing the level of Mss51 available for translational activation, since Cox1 synthesis is

partially restored by the *cox14Δ* and C-terminal truncation of Cox1, both of which weaken the Cox1-Mss51 interaction.

Discussion

Assembly of the CcO is a multi-step process that involves the coordinated incorporation of mitochondrially and nuclearly encoded subunits, as well as prosthetic groups. It is now recognized that mitochondrially encoded Cox1 is the foundation upon which further assembly occurs. A common observation is that pulse-labeling of Cox1 is reduced in the majority of mutants with defects in CcO assembly, reflecting decreased synthesis of Cox1. The mechanisms by which the CcO assembly state is sensed to regulate Cox1 synthesis are not completely understood. The proteins Mss51 (13,14), Cox14 (14) and Coa1 (16,17) form complexes with newly synthesized Cox1, coupling regulation of Cox1 synthesis to CcO assembly.

One question that remains is the mechanism by which the assembly state of Cox1 is sensed. To map the portions of Cox1 involved in this regulation we analyzed the C-terminal domain of Cox1 by site-directed mutation. This 59-amino acid region is exposed on the matrix side of the inner membrane in the assembled enzyme, and could interact with peripheral proteins like Mss51. We found that deletion of the last 11 or 15 residues of Cox1 disrupted the assembly-feedback control of Cox1 synthesis without inactivating CcO. These mutants showed wild-type levels of Cox1 synthesis when the CcO assembly was impaired. These C-terminal deletions of Cox1 also reduced the interaction between Mss51 and Cox14 during otherwise normal assembly. It is unknown whether Mss51 and Cox14 interact directly or via intermediate proteins in early assembly complexes. In any event, Mss51 and Cox14 do not interact normally even in the absence of CcO assembly when Cox1 lacks its last 15 residues. This weakened interaction could reduce sequestration of Mss51 in CcO assembly intermediates, making more Mss51 available for activation of *COX1* mRNA translation.

Interestingly, during the analysis of a wide range of CcO deficient mutants, we found that Pet54 is required for normal levels of Cox1 synthesis. Pet54 is required both for *COX3*

translation (43) and for splicing of the aI5β intron present in the *COX1* gene of many yeast strains (44), and these activities are genetically separable (45). However, the *COX1* gene in the strains employed here lacks introns. This novel Pet54 activity is independent of the C-terminal end of Cox1.

The hydrophilic Cox1 C-terminal end could adopt different conformations during assembly of the newly synthesized protein in response to its association with assembly factors and other subunits. These conformations could differ in their ability to sequester Mss51. We observed that among the CcO mutants analyzed in the present study, absence of Cox6 caused one of the most dramatic reductions in Cox1 synthesis, as analyzed by [^{35}S]methionine labeling experiments or expression of the *ARG8^m* reporter fused to *COX1*. We propose that early in assembly the C-terminal end of Cox1 has a conformation that strongly stabilizes the association of Mss51 with a high molecular weight complex containing Cox14 and Cox1 (Fig. 7, step 2). When Cox6 and possibly Cox5a are added to the complex, and/or when hemes are inserted (15), the C-terminal end of Cox1 could change. The crystal structure of assembled bovine CcO shows that these subunits are in close proximity to the C-terminal end of Cox1 (8). This conformational change could weaken the Cox14-dependent interaction of Mss51 with the assembly complex. It was previously proposed that Mss51 is liberated from the assembling enzyme early: at (16) or before (15) the point where Cox5a and Cox6 are added. However it is possible that some Mss51 remains weakly bound to the complex until further steps of assembly are completed. This would help explain how mutations blocking assembly downstream in the pathway, such as the *cox4Δ* mutation also elicit reduced Cox1 synthesis by sequestration of Mss51, but less strongly than the *cox6Δ* (Fig. 7, step 3). At the latest, once Cox1 is assembled within the CcO and it's C-terminal end acquires the final or close to final conformation, Mss51 must be liberated from the complex (Fig. 7, step 4) and available for further rounds of translational activation of the *COX1* mRNA (Fig. 7, steps 5 & 1).

It remains unclear whether assembly-feedback regulated synthesis of Cox1 in mitochondria occurs in other species. A few

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examples in mammals suggest that translation of the *COX1* mRNA might be reduced by defects associated with CcO assembly. It has been documented that a 15-base pair deletion in the human mitochondrial *COX3* gene (46), as well as lack of cytochrome *c* in mouse fibroblasts (47) leads to a modest reduction of [^{35}S]methionine labeling of Cox1. However, these studies did not clearly distinguish whether decreased Cox1 labeling was due to reduced synthesis or increased turnover.

Regulated synthesis of Cox1 in *S. cerevisiae* is the first identified example in mitochondria where an organelle-encoded protein has amino acid sequences that couple regulation of its own synthesis to assembly. However a similar mechanism has been demonstrated in the chloroplast of *Chlamydomonas reinhardtii*. Synthesis of some organelle-encoded subunits is strongly reduced when other subunits from the photosynthetic complexes are missing (48-50). For the *b_6f* complex, a C-terminal extension of 11 residues within cytochrome *f* is necessary for this regulation, possibly by stabilizing an interaction with the translational activator Tca1 when the

enzyme is not assembled (51). This extension showed no obvious similarity with the Cox1 C-terminus.

The C-terminal end of Cox1 contains the consensus motif S-P-P-P/A-X-H, where His503 (as numbered in the bovine sequence) is necessary for the tunneling of protons trough the D channel to the heme a_3-Cu$_B$ center (52). Interestingly, this histidine is part of the VHSFNT motif and is removed by our deletion of the last 15 residues of Cox1, demonstrating that it is not essential for oxidative phosphorylation in yeast.

The hydrophilic carboxyl terminal domain of Cox1 is less conserved overall than the transmembrane domains. However this region seems to be crucial for CcO activity. In the protist *Acanthamoeba castellanii* the mitochondrial *COX1* gene lacks the region coding for the C-terminal end. Interestingly, a nucleus-encoded protein homologous to this domain is imported into mitochondria, suggesting that the two peptides interact in trans to fulfill the same role as the intact protein (53).

Acknowledgements

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Figure legends

Figure 1. The Cox1 carboxyl terminal end is required to down-regulate Cox1 synthesis in a *cox2Δ* mutant. (A) Model of the *S. cerevisiae* Cox1 protein, based on the crystal structure of the bovine CcO. The model was constructed using SWISS MODEL, and visualized with MacPymol (http://www.pymol.org). Alignment of the yeast and bovine Cox1 sequences revealed that *S. cerevisiae* has approximately 23 additional residues in the C-terminal region, from Lys483 to Asn505, which are located at -52 to -29 with respect to the C-terminal end of Cox1 (Loop). Arrows indicate residues on the Cox1 C-terminal end where deletions start. The number in parenthesis indicates the position of these residues with respect to the last amino acid of Cox1. (B) Mitochondrial translation products were labeled with [^{35}S]methionine in the presence of cycloheximide, and proteins were analyzed as described in Experimental Procedures. Cells carried either the wild-type Cox1 protein (Cox1), or the Cox1 protein lacking 15 (Cox1ΔC15), 11 (Cox1ΔC11) or 5 (Cox1ΔC5) amino acids of the carboxyl terminal end. The *cox2Δ* mutation (Δ) was introduced as indicated. Abbreviations are as follows: Cytochrome c oxidase subunit 1, Cox1; subunit 2, Cox2; subunit 3, Cox3; cytochrome b, Cytb; subunit 6 of ATPase, Atp6; and the ribosomal protein, Var1.

Figure 2. The C-terminal end of Cox1 is necessary for stable interaction of Mss51 and Cox14. (A) Translation in isolated mitochondria from wild-type Cox1 or Cox1ΔC15 strains was performed in the presence of [^{35}S]methionine. Mitochondria were washed and solubilized with 1% digitonin. Mitochondrial extracts were immunoprecipitated with a Myc-specific antibody to precipitate Cox14-Myc or (B) HA-specific antibody to precipitate Mss51-HA. As control, strains lacking the Myc or HA epitopes in Cox14 and Mss51, respectively, were included as indicated. Translation products were separated by SDS-PAGE, and transferred to PVDF membrane before autoradiography. The membranes were incubated with HA-specific antibody to detect Mss51-HA or Myc-specific antibody to detect Cox14-Myc as indicated. The immunoprecipitated fractions in the western blot with anti-Myc antibody showed an additional, unspecific band, which is probably due to the IgG light chain of the antibody used for co-IP (*). Total samples represent 5% of the aliquots used for immunoprecipitation.

Figure 3. Interaction of Mss51 with Cox14 is not stable in CcO assembly mutants lacking the C-terminal end of Cox1. Mitochondria from Cox1 or Cox1ΔC15 in the presence of either wild-type *COX4* (*WT*) or *cox4Δ* (Δ) mutation were solubilized with 1% dodecyl maltoside and immunoprecipitated with a Myc-specific antibody. The immunoprecipitated was analyzed by western blot with an antibody to HA, the membrane was stripped and then reprobed with an antibody to Myc. The total fractions represent 5% of mitochondria before solubilization. Western blot with anti-Myc antibody showed a doublet, which is probably due to partial cleavage of the triple Myc epitope.

Figure 4. The Cox1 C-terminal end regulates Cox1 synthesis in many CcO mutants. (A) Cox1 (-) or Cox1ΔC15 (+) cells with a deletion in the indicated genes were pulse-labeled with [^{35}S]methionine in the presence of cycloheximide, and proteins were analyzed as described in Experimental Procedures. (B) Quantification of the Cox1 signals from (A). The level of Cox1 labeling was normalized to the Cox3/Atp6 signal, and was expressed as a percentage of the wild-type, Cox1 signal (except for signals from the *pet54Δ* and *pet122Δ* mutants, which were normalized with respect to Cytb). Error bars indicate standard deviations from 3 independent experiments. We also compared the signal of the cytochrome b to the Cox3/Atp6 signal (or the signal from Cox2 to the cytochrome b in the *pet54Δ* and *pet122Δ* mutants), and in those cases no significant difference was observed (data not shown). (C) Translation of the mitochondrial reporter gene *COX1(1-512)::ARG8^m* was analyzed by growth of the indicated mutants on media lacking (-Arg) or containing arginine (+Arg). In this construct the precursor of Arg8 was fused to the C-terminal end of the complete Cox1. Cells were spotted as serial dilutions and grown for 3 days at 30°C.

Figure 5. Synthesis of Cox1 is reduced in a *cox6Δ* mutant. (A) Translation of *COX1(1-512)::ARG8^m* in the indicated mutants was analyzed as in figure 4C. (B) Mitochondrial translation products of cells from (A) were obtained in the presence of cycloheximide and [^{35}S]methionine. In addition, a strain with wild type *COX1* was included as control. The Cox1-Arg8 precursor protein is indicated with an arrow. For unknown reasons this fusion is detected as a doublet. (C) Cox1 or Cox1ΔC15 cells with a deletion in the indicated genes were pulse-labeled with [^{35}S]methionine in the presence of cycloheximide, and proteins were analyzed as described in Experimental Procedures.

Figure 6. Model for assembly-feedback translational regulation of the *COX1* mRNA. See text for details. The C-terminal 15 residues of Cox1 are indicated with a thick black line. A gray thick line represents the rest of the Cox1 protein.

A

V (-11)

P (-5)

S (-15)

Loop

B

Cox1   Cox1 Cox1   Cox1
ΔC15 ΔC11   ΔC5

COX2→ WT Δ   WT Δ WT Δ   WT Δ

Var1
Cox1
Cox2
Cytb
Cox3
Atp6

Figure 1

11

Figure 2

Figure 3

88

13

Figure 4

A

COX1(1-512)::ARG8^m

	+Arg	-Arg
WT		
cox6Δ		
cox14Δ		
cox6Δ,cox14Δ		

B

COX1 → WT COX1(1-512)::ARG8^m
COX14 → WT WT Δ
COX6 → WT WT Δ WT Δ

Cox1-Arg8 →

Var1
Cox1
Cox2
Cytb
Cox3
Atp6

C

	Cox1				Cox1ΔC15			
COX14 →	WT		Δ		WT		Δ	
COX6 →	WT	Δ	WT	Δ	WT	Δ	WT	Δ

Cox1
Cox2
Cytb

Cox3
Atp6

Figure 5

15

90

1

COX1 mRNA

Mss51

Pet309

Oxa1

Mss51

2

Matrix

Cox1

Cox14

Cox6

5

Mss51

Cox6

Mss51

3

Matrix

Cox1

Cox14

Other
Subunits

Cox6

Mss51

4   Matrix

Cox1

Cox14

Assembly and
supercomplexes

16

Figure 6

Dual Functions of Mss51 Couple Synthesis of Cox1 to Assembly of Cytochrome *c* Oxidase in *Saccharomyces cerevisiae* Mitochondria

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Functional interactions of the translational activator Mss51 with both the mitochondrially encoded *COX1* mRNA 5'-untranslated region and with newly synthesized unassembled Cox1 protein suggest that it has a key role in coupling Cox1 synthesis with assembly of cytochrome *c* oxidase. Mss51 is present at levels that are near rate limiting for expression of a reporter gene inserted at *COX1* in mitochondrial DNA, and a substantial fraction of Mss51 is associated with Cox1 protein in assembly intermediates. Thus, sequestration of Mss51 in assembly intermediates could limit Cox1 synthesis in wild type, and account for the reduced Cox1 synthesis caused by most yeast mutations that block assembly. Mss51 does not stably interact with newly synthesized Cox1 in a mutant lacking Cox14, suggesting that the failure of nuclear *cox14* mutants to decrease Cox1 synthesis, despite their inability to assemble cytochrome *c* oxidase, is due to a failure to sequester Mss51. The physical interaction between Mss51 and Cox14 is dependent upon Cox1 synthesis, indicating dynamic assembly of early cytochrome *c* oxidase intermediates nucleated by Cox1. Regulation of *COX1* mRNA translation by Mss51 seems to be an example of a homeostatic mechanism in which a positive effector of gene expression interacts with the product it regulates in a posttranslational assembly process.

INTRODUCTION

The largest subunit of mitochondrial cytochrome *c* oxidase, Cox1, is encoded in the mitochondrial DNA (mtDNA) of all eukaryotic species that have been examined (Gray *et al.*, 2004), and it is synthesized by their organellar genetic systems. Cox1 is highly hydrophobic, spanning the inner mitochondrial membrane 12 times, and it is complexed with several metal ions and two heme A moieties that participate directly in electron transport (Tsukihara *et al.*, 1996). It is assembled into the core of cytochrome *c* oxidase, largely surrounded by subunits encoded by nuclear genes. The processes by which Cox1 is assembled with the other subunits and cofactors into an active enzyme are highly complex, requiring at least 30 genes in *Saccharomyces cerevisiae* (Herrmann and Funes, 2005; Khalimonchuk and Rodel, 2005; Cobine *et al.*, 2006; Fontanesi *et al.*, 2006; Barrientos *et al.*, 2009). The assembly pathway is not understood in detail. In mammals, analysis of mutant and drug-treated cell lines indicates that Cox1 is a component of the earliest assembly intermediates (Nijtmans *et al.*, 1998; Williams *et al.*, 2004), and similar analysis in yeast is consistent with this idea (Horan *et al.*, 2005).

An important function of this assembly process may be to prevent incompletely assembled components of cytochrome

c oxidase from generating damaging reactive oxygen species, before they are contained by the holoenzyme. Indeed, mutations in several yeast genes required for cytochrome *c* oxidase assembly cause hypersensitivity to hydrogen peroxide (Pungartnik *et al.*, 1999; Williams *et al.*, 2005; Banting and Glerum, 2006), and a key component of the reactive prooxidant species is Cox1 (Khalimonchuk *et al.*, 2007). One feature of the assembly process that is likely to play a role in minimizing the level of such prooxidant species is the coupling of Cox1 synthesis to assembly of cytochrome *c* oxidase (Barrientos *et al.*, 2004).

Translation of *S. cerevisiae* mitochondrially coded mRNAs within the organelle is tightly controlled by nuclearly encoded mRNA-specific translational activators that, in most cases, recognize the 5'-untranslated regions (UTRs) of their target mRNAs (reviewed in Fox, 1996; Towpik, 2005). For example, Pet309 recognizes the leader of the *COX1* mRNA and specifically activates synthesis of the Cox1 protein (Manthey and McEwen, 1995). Furthermore, Pet309 also interacts with the activators of *COX2* and *COX3* mRNA translation to colocalize synthesis of the three core subunits of cytochrome *c* oxidase, promoting efficient assembly (Sanchirico *et al.*, 1998; Naithani *et al.*, 2003).

Mss51 is the second known *COX1* mRNA-specific translational activator (Decoster *et al.*, 1990; Siep *et al.*, 2000). It is of particular interest because dominant *MSS51* missense mutations can suppress the leaky cytochrome *c* oxidase assembly defect caused by a *shy1Δ* mutation in yeast (Barrientos *et al.*, 2002). Mutations affecting the human homologue of *SHY1*, *SURF1* (Mashkevich *et al.*, 1997), cause a similar cytochrome oxidase deficiency associated with

Leigh syndrome (Tiranti et al., 1998; Zhu et al., 1998). PSI-BLAST comparisons indicate that both the human and mouse genomes encode a possible orthologue of yeast Mss51 (NP_001019764 and XP_912342) (Saccharomyces Genome Database), although the function of this mammalian protein is unknown.

Yeast Mss51 has two genetically distinct activities that make it an excellent candidate for a regulatory protein coupling Cox1 synthesis to cytochrome c oxidase assembly (Perez-Martinez et al., 2003). First, Mss51 is required to translate an mRNA encoding a reporter gene, ARG8^m, inserted at the COX1 locus in place of the COX1 protein coding sequence, demonstrating that Mss51 has a target in either the 5'- or 3'-UTRs of the COX1 mRNA (or both). This activity may resemble that of other known translational activators. However, Mss51 (but not Pet309) is also required to express a chimeric mRNA bearing the untranslated regions of the COX2 mRNA flanking either the COX1 coding sequence or a COX1::ARG^m translational fusion gene, demonstrating that Mss51 has a second genetically defined target mapping in the COX1 coding sequence itself (Perez-Martinez et al., 2003). Furthermore, immune precipitation of epitope-tagged Mss51 efficiently coprecipitates newly synthesized, unassembled Cox1 (Perez-Martinez et al., 2003; Barrientos et al., 2004; Mick et al., 2007). This strongly suggests that the second target of Mss51 action is Cox1 itself. This protein-protein interaction seems to be necessary for Cox1 synthesis (Perez-Martinez et al., 2003) and is also likely to be required for early steps in the cytochrome c oxidase assembly pathway (Mick et al., 2007; Pierrel et al., 2007; Zambrano et al., 2007).

Evidence indicating a coupling of Cox1 synthesis to assembly emerged from a systematic study demonstrating that most yeast mutations which disrupt cytochrome c oxidase assembly reduce, but do not eliminate, in vivo pulse labeling of Cox1 (Barrientos et al., 2004), a phenomenon observed previously in a few mutants (Poutre and Fox, 1987; Calder and McEwen, 1991). These observations suggest the existence of an assembly–feedback control system similar to that discovered previously in the chloroplast of Chlamydomonas reinhardtii (Choquet et al., 1998). Overproduction of Mss51 in several of the yeast assembly mutants largely reversed their Cox1 synthesis reductions (Barrientos et al., 2004). Interestingly, one cytochrome c oxidase assembly mutation, cox14Δ, did not reduced Cox1 pulse labeling, indicating that Cox14 is required for the feedback. Coprecipitation experiments revealed that Cox14 interacts both with newly synthesized Cox1 and with Mss51. Based on these findings and the dual activities of Mss51 (Perez-Martinez et al., 2003), Barrientos et al. (2004) proposed a model in which sequestration of Mss51 in assembly intermediates containing Cox1 and Cox14 could limit Cox1 synthesis (Barrientos et al., 2004).

In this article, we present evidence that strongly supports a regulatory role for Mss51 in controlling Cox1 translation and demonstrates dynamic interactions among newly synthesized Cox1, Mss51, and Cox14 that couple Cox1 synthesis to early steps in cytochrome c oxidase assembly.

MATERIALS AND METHODS

Strains, Media, and Genetic Methods

The S. cerevisiae strains used in this study are listed in Table 1. Standard genetic methods and media recipes were as described previously (Rose et al., 1988; Fox et al., 1991). Complete fermentable media were YPD or YPRaf (containing 2% glucose or 2% raffinose). Nonfermentable medium was YPEG (3% glycerol and 3% ethanol) or 2% lactate. Minimal media contained 0.67% yeast nitrogen base, 2% glucose, and Complete Supplement Mixtures (CSMs) purchased from Bio 101 (Vista, CA). Sequences encoding 3xHA or 3xMyc

epitope tags were added to the 3' ends of nuclear gene coding sequences, without altering mRNA flanking sequences, by pop-in pop-out transformation as described previously (Schneider et al., 1995). Mitochondrial transformation, and integration of altered genes into rho° mtDNA, was as described previously (Perez-Martinez et al., 2003; Bonnefoy et al., 2007). Plasmids (see below) bearing cox1Δ::ARG8^m-1, cox1Δ::ARG8^m-2, and COX1::3xHA chimeric genes were transformed into the rho° strain NAB69 by high-velocity microprojectile bombardment. Mitochondrial transformants were identified by their ability to rescue arginine growth when mated with a rho⁺ cox1Δ::arg8^m-1 mutant (Bonnefoy and Fox, 2000) or by respiratory growth when mated with a rho⁻ strain carrying a cox1-D369N mutation. L45 (Meunier et al., 1993). Altered mitochondrial genes in the transformants were integrated by homologous recombination into rho⁺ mtDNA by isolating cytoductants issued from crosses of the transformants to either NB40-36a followed by selection for Arg+ growth, or to XPM10b followed by selection for respiratory growth, as appropriate.

Construction of Chimeric Mitochondrial Genes

Chimeric genes were generated by the fusion polymerase chain reaction technique (Ho et al., 1989) using Pfu polymerase (Stratagene, La Jolla, CA) or Taq polymerase (Invitrogen, Carlsbad, CA), as described previously (Perez-Martinez et al., 2003). The cox1Δ::ARG8^m-1 construct (pXPM76) consists of 395 base pairs of the COX1 5'-UTR sequence, followed by ARG8^m, and 119 base pairs encoding the COX2 3'-UTR replacing 525 base pairs corresponding to the COX1 3'-UTR. After the COX2 3'-UTR sequence, there are 466 base pairs of downstream COX1 flanking sequence to allow integration at the COX1 locus. The cox1Δ::ARG8^m-2 construct (pXPM80) consists of 568 base pairs of upstream COX1 flanking sequence, followed by 73 base pairs encoding the COX2 promoter and 5'-UTR, ARG8^m, and 990 base pairs of downstream COX1 flanking sequence encoding its 3'-UTR. The COX1::3xHA construct (pXPM63) consists of the intronless COX1 coding sequence (Labouesse, 1990) with 90 base pairs encoding three HA epitopes inserted upstream of the stop codon, flanked by 395 base pairs of upstream sequence and 990 base pairs of downstream sequence.

Analysis of Mitochondrial Proteins

Yeast cells were grown in 10 ml of complete raffinose medium until late log phase. Cells were disrupted by vortexing with glass beads, and crude mitochondria were obtained as described by Diekart et al. (2001), except that protease inhibitor mini-tablets (Roche Diagnostics, Indianapolis, IN) were added instead of phenylmethylsulfonyl fluoride. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970). For Western blots, proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Billerica, MA), or nitrocellulose where indicated, and probed with an anti-Arg8 antibody (Steele et al., 1996), anti-Cox1 (Mito-Sciences, Eugene, OR), anti-hemagglutinin (HA) (Roche Diagnostics), or anti-Myc (Roche Diagnostics). Immune complexes were detected with either goat anti-rabbit immunoglobulin (Ig)G or anti-mouse IgG conjugated to horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA) and the enhanced chemiluminescence (ECL) kit (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom), or when using anti-Cox3, the ECL Plus kit (GE Healthcare). Alternatively, for the experiment of Figure 2A and similar experiments, the secondary antibody was AlexaFluor488-conjugated goat anti-mouse IgG (Invitrogen). Fluorophore signals were detected using a Storm Phosphorimager 840 (GE Healthcare). The signals were quantitated by subtracting the sum of pixel intensities of a background area from the sum of pixel intensities of an equal sample area as described (Demlow and Fox, 2003). For immunoprecipitation, mitochondria were solubilized in 1% digitonin and incubated with anti-HA agarose (Roche Diagnostics) as described previously (Herrmann et al., 2001; Perez-Martinez et al., 2003). Total proteins were precipitated from solubilized extracts and immune supernatants using StrataClean resin (Stratagene, San Diego, CA) (Ziegler et al., 1997). Mitochondrial translation products were radiolabeled in 300–700 µg of highly purified mitochondria for 30 min at 25°C in the presence of [³⁵S]methionine, as described previously (Westermann et al., 2001; Perez-Martinez et al., 2003).

RESULTS

Mss51 Acts on the COX1 mRNA 5'-UTR and Is Present at Levels near Rate Limiting for Expression

Mss51 is required posttranscriptionally for expression of a chimeric mitochondrial mRNA composed of the COX1 5'- and 3'-UTRs flanking the mitochondrial reporter gene ARG8^m, demonstrating that one of its activities is mediated through untranslated COX1 mRNA sequences (Perez-Martinez et al., 2003). Other well studied mitochondrial translational activators target exclusively mRNA 5'-UTRs (Towpik, 2005). However, the unusual nature of Mss51's dual activities raised the possibility that it might target the

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Table 1. Yeast strains[a] used in this study

Strain	Genotype	Reference
CAB267	MATα, ade2, ura3, cor1Δ::URA3, MSS51::3xHA [ρ⁺]	This study
CAB268	MATα, ade2, ura3, cor1Δ::URA3 [ρ⁻]	This study
CAB299	MATα, ura3, leu2-3,112, lys2, his4-519, MSS51::3xHA, COX14::3xMYC	This study
CAB300	MATα, ura3, leu2-3,112, lys2, his4-519, MSS51::3xHA, COX14::3xMYC [ρ⁻, ΔΣΜ, ΔΣΝ]	This study
CAB302	MATα, ura3, leu2-3,112, lys2, his4-519, MSS51::3xHA, COX14::3xMYC, pet309::URA3	This study
CAB306	MATα, ura3, leu2-3,112, lys2, his4-519, MSS51::3xHA, COX14::3xMYC, cbs2::URA3 [ρ⁻, ΔΣΜ, ΔΣΝ]	This study
CAB312	MATα, ura3 ΔpMX3, leu2-3,112, his4-519, MSS51::3xMYC, [ρ⁻]	This study
CAB313	MATα, ura3-52, leu2-3,112, lys2, his4-519, MSS51::3xMYC, MSS51::3xMYC	This study
CAB315	MATα, ura3-52, leu2-3,112, lys2, his4-519, MSS51::3xMYC, mss51::LEU2 [ρ⁻, ΔΣΜ, ΔΣΝ]	This study
DAU1	MATα, ade2 ura3Δ [ρ⁺]	
NAB69	MATα, ade2-101, arg8::hisG, ura3-52, kar1-1 [ρ⁺]	
NB40-3α	MATα, lys2, leu2-3,112, arg8::hisG, ura3-52 [ρ⁺]	
SB7	MATα, ade2, ura3Δ, MSS51::3xHA [ρ⁺]	
TF258	MATα, ura3, leu2-3,112, lys2, his4-519, MSS51::3xHA, COX14::3xMYC [ρ⁺]	This study
TF250	MATα, ura3, leu2-3,112, lys2, his4-519, MSS51::3xHA, COX14::3xMYC [ρ⁻] cox1Δ	This study
TF256	MATα, ura3, leu2-3,112, lys2, his4-519, MSS51::3xHA, COX14::3xMYC [ρ⁻, cox2-N15I]	This study
XPM100	MATα, lys2, leu2-3,112, arg8::hisG, ura3-52 [ρ⁻, arg8::ARG8^m]	[Perez-Martinez et al., 2003]
XPM34	MATα, lys2, leu2-3,112, arg8::hisG, ura3-52 [ρ⁻, cox2-N15I]	This study
XPM63	MATα, lys2, leu2-3,112, arg8::hisG, ura3-52, mss51::LEU2 [ρ⁻, cox1Δ::ARG8^m]	[Perez-Martinez et al., 2003]
XPM271a	MATα, lys2, leu2-3,112, arg8::hisG, ura3-52 [ρ⁻, cox1Δ::ARG8^m-1]	This study
XPM275a	MATα, lys2, leu2-3,112, arg8::hisG, ura3-52, mss51::LEU2 [ρ⁻, cox1Δ::ARG8^m-1]	This study
XPM296	MAT=MATα, ade2/ADE2, lys2/LYS2, leu2-3,112/LEU2, arg8::hisG/arg8::hisG ura3-52/ura3Δ	This study
	[ρ⁻, cox1Δ::ARG8^m]	
XPM287	MAT=MATα, pet309::URA3/PET309, ade2/ADE2, lys2/LYS2, leu2-3,112/LEU2,	This study
	arg8::hisG/arg8::hisG, ura3-52/ura3Δ [ρ⁻, cox1Δ::ARG8^m]	
XPM288	MAT=MATα, mss51A::LEU2/MSS51, ade2/ADE2, lys2/LYS2, leu2-3,112/LEU2,	This study
	arg8::hisG/arg8::hisG, ura3-52/ura3Δ [ρ⁻, cox1Δ::ARG8^m]	
XPM290	MAT=MATα, mss51A::LEU2/MSS51, pet309::URA3/PET309, ade2/ADE2, lys2/LYS2,	This study
	leu2-3,112/LEU2, arg8::hisG, ura3-52/ura3Δ [ρ⁻, cox1Δ::ARG8^m]	
XPM30b	MATα, lys2, leu2-3,112, arg8::hisG, ura3-52 [ρ⁻, cox1Δ::ARG8^m-2]	This study
XPM312	MATα, lys2, leu2-3,112, arg8::hisG, ura3-52, mss51A::LEU2 [ρ⁻, cox1Δ::ARG8^m-2]	This study

[a] All strains are congenic or isogenic to D273-10b, except for NAB69. Mitochondrial genotypes are shown in brackets. ΔΣΜ and ΔΣΝ indicate intronless mtDNA (Labouesse, 1990).

[b] Chimeric cox2Δ::COX1 gene inserted nonjointly upstream of COX2 (Perez-Martinez et al., 2003).

3′-UTR, the mRNA region mediating many other cases of translational control (Kuersten and Goodwin, 2003). To determine whether the 5′- or 3′-UTR could be a sole target of Mss51 in vivo, we created strains in which COX1 5′- and 3′-UTRs individually replaced the COX1 5′- or 3′-UTRs flanking ARG8^m by inserting chimeric genes into mtDNA at the COX1 locus (see Materials and Methods). We deleted the 525 base pairs encoding the COX1 3′-UTR, replacing them with 118 base pairs encoding the COX23-UTR. In a separate strain, we deleted the 505 base pairs encoding the COX1 5′-UTR, replacing them with 73 base pairs encoding the COX2 promoter and COX2 mRNA 5′-UTR, which contains the target of the COX2-specific translational activator Pet111 (Dunstan et al., 1997; Green-Willms et al., 2001) (Figure 1A). In strains containing a wild-type nuclear genome both chimeric mitochondrial mRNAs supported Arg⁺ growth. In strains containing the mss51Δ mutation, the chimeric ARG8^m mRNA bearing the COX1 5′-UTR and COX2 3′-UTR failed to support Arg⁺ growth. However, the mss51Δ strain containing the chimeric mRNA bearing the COX2 5′-UTR and COX1 3′-UTR grew well in the absence of arginine. As expected, expression of this ARG8^m mRNA bearing the COX2 5′-UTR was also independent of the COX1-specific

translational activator Pet309 (Manthey and McEwen, 1995; Perez-Martinez et al., 2003) but was dependent upon the COX2-specific translational activator Pet111 (data not shown). Thus, Mss51 has a translational activation target in the COX1 mRNA 5′-UTR and may act there together with Pet309. This target could correspond to a region of the COX1 mRNA 5′-UTR that interacts with Mss51 in the yeast three-hybrid system (Zambrano et al., 2007).

For mRNA-specific translational activation through the COX1 5′-UTR to play a role in mediating Cox1 synthesis, Mss51 and/or Pet309 should be present at or near rate-limiting levels in mitochondria. To test whether this is so, we examined expression of ARG8^m, inserted in place of the COX1 codons, in diploid strains lacking one copy of MSS51, PET309, or both. Expression of the reporter in heterozygous diploids relative to homozygous wild type was assayed by growth on medium lacking arginine (Figure 1B). In both cases, reduced gene dosage decreased the rate of growth on medium lacking arginine, with pet309Δ/PET309 having a stronger effect than mss51Δ/MSS51. A diploid heterozygous for both nuclear mutations was more strongly affected than either single mutant. We conclude that the levels of Mss51
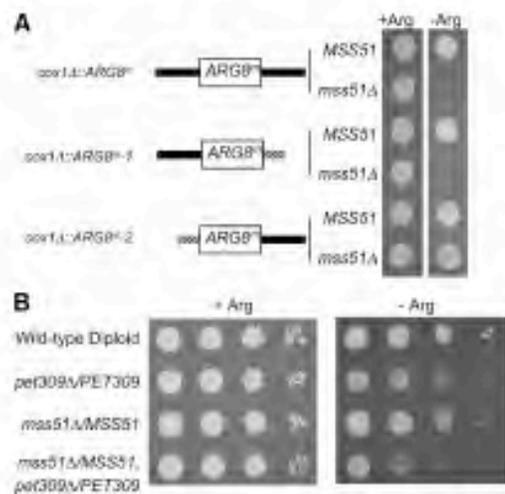
Figure 1. Mss51 acts on the COX1 mRNA 5'-UTR and is rate limiting, along with Pet309, for mRNA translation. (A) Cells grown in liquid YPD were spotted on CSM raffinose medium containing (+Arg) or lacking (−Arg) arginine and incubated for 4 d at 30°C. The cells contained coxIΔ::ARG8^m genes at the COX1 locus in mtDNA encoding chimeric mRNAs: black bars, COX1 untranslated regions; stippled bars, COX2 untranslated regions. coxIΔ::ARG8^m (top diagram) has ARG8^m flanked by native COX1 5'- and 3'-UTRs, with (XPM10b) or without (XPM63a) MSS51. coxIΔ::ARG8^m-1 (middle diagram) has ARG8^m flanked by the native COX1 5'-UTR, and the COX2 3'-UTR (525 base pairs of COX1 downstream sequence replaced by 118 base pairs of COX2 downstream sequence) with (XPM271a) or without (XPM275a) MSS51. coxIΔ::ARG8^m-2 (bottom diagram) has ARG8^m flanked by the COX2 5'-UTR (505 base pairs of COX1 upstream sequence replaced by 73 base pairs of COX2 upstream sequence), and the native COX1 3'-UTR with (XPM304b) or without (XPM312a) MSS51. (B) Diploid cells containing the coxIΔ::ARG8^m reporter in mtDNA were grown in liquid YPD. Tenfold serial dilutions of diploid cells were spotted on CSM glucose media containing (+Arg) or lacking (−Arg) arginine, and incubated for 4 d at 30°C. The indicated relevant nuclear genotypes correspond to the following strains (Table 1): wild type, XPM286; pet309Δ/PET309, XPM287; mss51Δ/MSS51, XPM288; and pet309Δ/PET309 mss51Δ/MSS51, XPM291.

and Pet309 are at or near rate limiting for translational activation through the COX1 5'-UTR.

A Significant Fraction of Total Mss51-3xMyc Interacts with Cox1-3xHA

Mick et al. (2007) have shown that affinity purification of Mss51 from unlabeled wild-type cells yields cytochrome c oxidase assembly intermediate complexes that contain unassembled Cox1. If intermediate complexes containing Cox1 bind a significant fraction of total Mss51, then the levels of assembly intermediates could affect the availability of rate-limiting Mss51 for translational activation through the COX1 mRNA 5'-untranslated leader (UTL). To ask whether such complexes contain a significant fraction of total cellular Mss51, we examined the efficiency with which Mss51 would coimmune precipitate with Cox1 from digitonin solubilized extracts of mitochondria. We were unable to immune precipitate Cox1 with the commercially available anti-Cox1 antibody (M5418; MitoSciences). We therefore modified the

COX1 gene in mtDNA by the addition of sequences encoding three HA-epitopes at the Cox1 C terminus (see *Materials and Methods*). Otherwise wild-type cells containing this mtDNA grew normally on nonfermentable carbon sources. To allow detection of Mss51 in fractions derived from this strain, we added sequences encoding three Myc-epitopes at the C terminus of Mss51 without altering sequences flanking MSS51 (Schneider et al., 1995). These epitopes did not affect respiratory growth of otherwise wild-type strains.

Mitochondria were isolated from log-phase cells containing both Cox1-3xHA and Mss51-3xMyc, as well as a control strain lacking the tag on Cox1, and were solubilized with digitonin. One-half of each extract was immune precipitated with anti-HA coupled to agarose beads. Total proteins were collected from the other half of each extract, and from the immune supernatants, by precipitation with StrataClean resin (Stratagene). The precipitated fractions were analyzed by semiquantitative Western blotting using anti-Myc primary antibody and fluorescein-labeled secondary antibody. Signals were detected digitally by phosphorimaging (see *Materials and Methods*). The average percent of total of Mss51-3xMyc recovered in the anti-HA immune precipitates from four trials was 41% (SD = 22%). One such experiment is shown in Figure 2A.

The average percentage of total Cox1-3xHA precipitated by anti-HA was 24% (SD = 10%). This relatively low efficiency of direct immune-precipitation suggests that the three HA-epitopes may be sterically blocked in a high fraction of fully assembled cytochrome c oxidase complexes. Thus, the immune precipitates may be enriched for assembly intermediates. These data are consistent with the hypothesis that a significant fraction of Mss51 may be sequestered with Cox1 in assembly intermediates and thus unavailable for translational activation through the COX1 mRNA 5'-UTL.

If cytochrome c oxidase assembly is blocked at a step downstream in the pathway, then the fraction of Mss51-3xMyc present in assembly intermediates could be higher than in wild type. We tested this by measuring coimmune precipitation from a strain lacking MSS2, a nuclear gene required for export of the Cox2 C-tail domain to the intermembrane space (Broadley et al., 2001). However, in this case the average percentage of Mss51-3xMyc precipitating with Cox1-3xHA was 50% in four trials (SD = 35%) (Figure 2A), not significantly higher than in wild type. In addition, the epitope tag apparently stabilized unassembled Cox1 in the mss2 mutant. Although these data do not provide supporting evidence for increased sequestration of Mss51 with Cox1 in the assembly defective mutant, it must be noted that the measurements are inherently imprecise, and the physiologically relevant differences between wild type and mutant could be small.

We also compared wild type to an assembly defective strain by asking what fraction of total unmodified Cox1 protein would coimmune precipitate with tagged Mss51-3xHA. Here, we disrupted cytochrome c oxidase assembly using the mitochondrial cox2-N15I mutation, which prevents processing of the pre-Cox2 leader peptide (Saracco, 2003). Solubilized extracts of mitochondria were subjected to immune precipitation with immobilized anti-HA, and the precipitates were analyzed by Western blotting (Figure 2B). Owing to the weak immune reaction of the available anti-Cox1 monoclonal antibody with Cox1, we had to use for the Westerns an enhanced chemiluminescence detection system that does not allow quantitative analysis of signal strength. The steady-state level of Cox1 in extracts of COX2 mitochondria was far higher than that in the cox2-N15I mutant extracts, as expected. We therefore overexposed the anti-Cox1
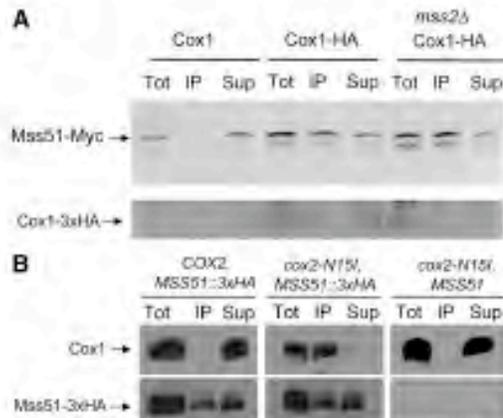
Figure 2. A large fraction of total Mss51-3xMyc coimmune precipitates with Cox1-3xHA from mitochondrial extracts. (A) Mitochondria were isolated from three strains that each contained Mss51-3xMyc encoded by a modified chromosomal *MSS51* gene. CAB312 (Cox1) was otherwise wild-type; CAB313 (Cox1-HA) has a modified intronless mitochondrial *COX1* gene encoding Cox1-3xHA; CAB315 (mss2Δ Cox1-HA) had both Cox1-3xHA and a nuclear mss2Δ mutation. Mitochondria were solubilized with digitonin. Half of each extract was analyzed as total protein (Tot). The other half was subjected to immunoprecipitation with anti-HA antibody, yielding a precipitate (IP) and supernatant (Sup). The three fractions from each strain were subjected to SDS gel electrophoresis and Western blotting with anti-Myc antibody. Immune complexes were visualized by phosphorimaging after reaction with AlexaFluor coupled anti-mouse-IgG (see *Materials and Methods*). (B) Mitochondria were isolated from two strains that contained Mss51-3xHA encoded by a modified chromosomal *MSS51* gene. TF258 (COX2) contained wild-type mtDNA; TF266 (cox2-N15i) had a missense substitution mutation that prevents processing of the pre-Cox2 precursor. In addition, the control strain XPM34 contained unmodified Mss51 and the cox2-N15i mutation. Mitochondria were extracted and subjected to immune precipitation as described in A. The Western blots were probed with anti-Cox1 antibody and subsequently with anti-HA antibody, then visualized by ECL detection (see *Materials and Methods*). The cox2-N15i blots probed with anti-Cox1 were overexposed relative to the others (see text).

blot from the mutant relative to wild type, to achieve comparable signal strength. We also probed the blots with anti-HA to detect Mss51-3xHA and exposed these wild-type and mutant blots equally. (It seems that the 3xHA epitope was partially destroyed by proteolysis during the immune precipitation, producing multiple bands and apparent incomplete recovery of total Mss51-3xHA in the precipitate plus supernatant fractions.) Importantly, the residual Cox1 present in the cox2-N15i mutant was highly enriched in the anti-HA immune precipitate (in an epitope-dependent manner), in contrast to wild-type where most of the Cox1, which is assembled into cytochrome oxidase, remained in the soluble extract after immune precipitation (Figure 2B). Thus, when assembly was disrupted the steady-state level of Cox1 was greatly reduced, but virtually all of the Cox1 present was associated with Mss51.

Coimmune Precipitation of Newly Synthesized Cox1 with Mss51-3xHA Depends upon Cox14

Cox14 is a short mitochondrial protein, encoded in the nucleus, that interacts with newly synthesized Cox1 and with
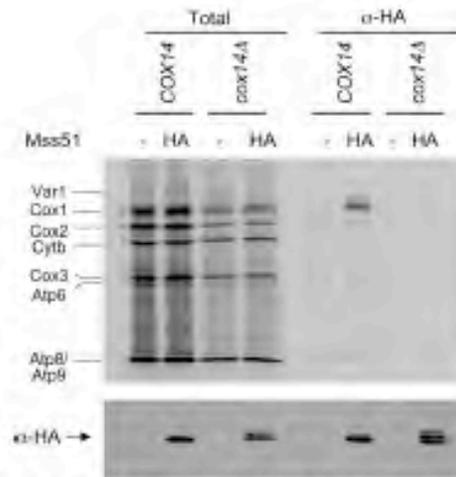
Figure 3. The interaction between Mss51 and newly synthesized Cox1 is greatly reduced in the absence of Cox14. Mitochondria were purified from four strains whose relevant genotype was either COX14 or cox14Δ and contained either Mss51 or Mss51-3xHA, as indicated. The mitochondria were allowed to synthesize mitochondrially coded proteins in the presence of [³⁵S]methionine (see *Materials and Methods*) and then solubilized with digitonin. 10% of the extract was subjected to SDS-PAGE (Total), whereas 90% was immunoprecipitated with anti-HA antibody and then subjected to SDS-PAGE (α-HA). The gel was blotted to a nitrocellulose membrane, which was autoradiographed (top). Labeled translation products are indicated as follows: cytochrome c oxidase subunit 1, Cox1; subunit 2, Cox2; subunit 3, Cox3; cytochrome b, Cytb; subunit 6 of ATPase, Atp6; subunit 8, Atp8; subunit 9, Atp9; and the ribosomal protein, Var1. After autoradiography, the membrane was probed with anti-HA antibody and visualized by ECL detection to confirm precipitation of Mss51-3xHA (bottom). The strains, from left to right, were DAU1, SB7, CAB268, and CAB267 (see Table 1).

Mss51 (Barrientos et al., 2004). Cox14 is required for assembly of active cytochrome c oxidase and accumulation of Cox1, but unlike most other assembly-defective mutants, cox14 mutants do not exhibit assembly feedback inhibition of COX1 mRNA translation as judged by pulse labeling (Barrientos et al., 2004).

Stable interaction of Mss51 with newly synthesized Cox1 could be dependent on Cox14. To test this idea, we asked whether Cox1, newly synthesized in mitochondria from a cox14Δ mutant, would coimmune precipitate with Mss51-3xHA. Mitochondria were isolated from COX14 and cox14Δ strains that contained either wild-type Mss51 or the functional Mss51-3xHA. After mitochondrial translation in the presence of [³⁵S]methionine and solubilization, anti-HA antibody was added to the mitochondrial lysates and immune precipitates were analyzed by SDS gel electrophoresis and autoradiography. Mss51-3xHA selectively coprecipitated Cox1 from the wild-type COX14 strain but failed to do so from the cox14Δ strain (Figure 3), contrary to results reported previously (Barrientos et al., 2004). Probing of a Western blot of these lysates with anti-HA antibody confirmed that Mss51-3xHA was present in the absence of Cox14. These results strongly suggest that Cox14 is required for Mss51 to form complexes with newly synthesized Cox1 that are stable under our solubilization conditions.
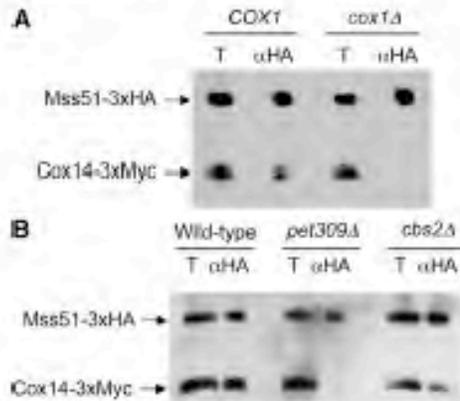
Figure 4. Cox1 synthesis is necessary for the interaction between Mss51 and Cox14. (A) Mitochondria were purified from cells containing Mss51-3xHA and Cox14-3xMyc, and either wild-type mtDNA (*COX1*) or a *cox1* deletion (*cox1Δ*) in mtDNA (strains TF258 and TF259). The mitochondria were solubilized with digitonin and the extracts were incubated with anti-HA antibody (αHA). The precipitates were analyzed by Western blot probed with anti-Myc and anti-HA antibodies. The total fractions (T) corresponds to 10% of the mitochondrial extract, the αHA precipitates were from the remaining 90%. (B) Mitochondria were purified from cells containing Mss51-3xHA, Cox14-3xMyc, and intronless mtDNA, that were otherwise wild type (Wild-type), *pet309::URA3* (*pet309*), or *cbs2::URA3* (*cbs2*) (strains CAB300, CAB302, and CAB306). Analysis was as described in A.

It is impossible to test reciprocally whether the interaction of Cox14 with newly synthesized Cox1 depends upon Mss51, because in the absence of Mss51, Cox1 cannot be synthesized.

Interaction between Mss51 and Cox14 Depends upon Cox1 Synthesis

Mss51 and Cox14 have been shown by coimmunoprecipitation to interact physically (Barrientos *et al.*, 2004). These nuclearly coded proteins could interact in a stable complex, or associate dynamically in response to synthesis of mitochondrially encoded Cox1. We first tested this by constructing strains containing Mss51-3xHA and Cox14-3xMyc, with either wild-type mtDNA, or mtDNA bearing a *cox1Δ* mutation that eliminates the coding sequence, as well as 787 base pairs of upstream, and 525 base pairs of downstream sequence. Mitochondria were isolated from both strains and solubilized with digitonin. The soluble extracts were immunoprecipitated with an anti-HA antibody, and the precipitates were analyzed by Western blot probed with both anti-HA and anti-Myc (Figure 4A). As expected, Mss51-3xHA coimmunoprecipitated Cox14-3xMyc from the extract of the *COX1* mitochondria. However, Cox14-3xMyc was not coprecipitated from the extract of the *cox1Δ* mitochondria.

To ask whether the Cox1 protein or the *COX1* mRNA was necessary for the interaction between Mss51-3xHA and Cox14-3xMyc, we tested for coimmunoprecipitation in a *pet309Δ* strain whose mtDNA does not contain introns. Strains of this genotype contain near wild-type levels of mature *COX1* mRNA but do not translate it (Manthey and McEwen, 1995). Consistent with the hypothesis that Cox1 synthesis is necessary for the interaction between Mss51 and

Cox14, the *pet309Δ* mutation prevented coimmune precipitation of Cox14-3xMyc by Mss51-3xHA (Figure 4B). As a control, we tested whether a *cbs2Δ* mutation, which produces a respiratory negative phenotype by specifically preventing translation of apo-cytochrome *b* from the *COB* mRNA (Rödel, 1986), would prevent the Mss51–Cox14 interaction. It did not, confirming the specific requirement for Cox1 synthesis of the Mss51–Cox14 interaction (Figure 4B).

Mss51 Can Interact with Newly Synthesized Cox1 and with Cox14 Independently of the COX1 mRNA 5′-UTR

Cox1 protein can be synthesized and assembled into functional cytochrome oxidase from a chimeric mRNA bearing the *COX1* coding sequence flanked by *COX2* 5′- and 3′-UTRs, transcribed from an ectopic *cox2Δ::COX1* locus in mtDNA. Translation of this chimeric mRNA is reduced relative to wild type, but nevertheless dependent upon *MSS51* function, demonstrating a second function for Mss51 protein, distinct from its interaction with the *COX1* mRNA 5′-UTR (Perez-Martinez *et al.*, 2003). If the interaction between Mss51 and newly synthesized Cox1 corresponds to this second function, then this physical interaction also should be independent of the *COX1* 5′-UTR on the mRNA. To test this, we radiolabeled newly synthesized Cox1 in mitochondria isolated from strains containing Mss51-3xHA and either the *COX1* gene in wild-type mtDNA, or the ectopic *cox2Δ::COX1* locus in mtDNA with *cox1Δ::ARG8ᵐ* at the *COX1* locus. After solubilization with digitonin and addition of anti-HA antibody, immune precipitates were isolated and analyzed by SDS gel electrophoresis and autoradiography of a gel-blot (Figure 5A). Newly synthesized radiolabeled Cox1 was selectively precipitated with Mss51-3xHA from extracts of mitochondria bearing either the wild-type *COX1* mRNA or the chimeric *cox2Δ::COX1* mRNA, demonstrating that the Mss51–Cox1 physical interaction does not require the *COX1* mRNA 5′-UTR in *cis* to the coding sequence. As a further control, we also probed the blot with anti-Arg8 antibody. As expected, no coimmune precipitation of Arg8 protein with Mss51-3xHA was detectable despite its translation from an mRNA bearing the *COX1* mRNA 5′-UTL (Figure 5B).

We also tested by coimmune precipitation whether the Cox1-translation-dependent interaction between Mss51 and Cox14 requires prior interaction of Mss51 with the *COX1* mRNA 5′-UTR. Cox14-3xMyc was coprecipitated with Mss51-3xHA from extracts of mitochondria containing the chimeric *cox2Δ::COX1* mRNA, albeit at lower efficiency than from mitochondria with the wild-type *COX1* mRNA (Figure 5C). Thus, the interaction between Mss51 and Cox14 can occur in the absence of the *COX1* mRNA 5′-UTR in *cis* to the coding sequence. The lower efficiency of Cox14 coprecipitation may reflect the lower level of Cox1 synthesis observed from the chimeric mRNA (Perez-Martinez *et al.*, 2003).

DISCUSSION

Mss51 has functional interactions with both the *COX1* mRNA and with newly synthesized unassembled Cox1 protein. Its involvement in both gene regulation and assembly of the protein whose synthesis it regulates suggests a role for Mss51 in homeostatic coupling of Cox1 synthesis to its assembly into cytochrome *c* oxidase (Perez-Martinez *et al.*, 2003). Our data strongly support this hypothesis.

By manipulation of the mtDNA sequences flanking the *COX1* coding sequences in *rho⁺* mtDNA, we genetically mapped the Mss51 target in the *COX1* mRNA to the 5′-UTR. This A+U-rich 450 nucleotide mRNA leader also contains

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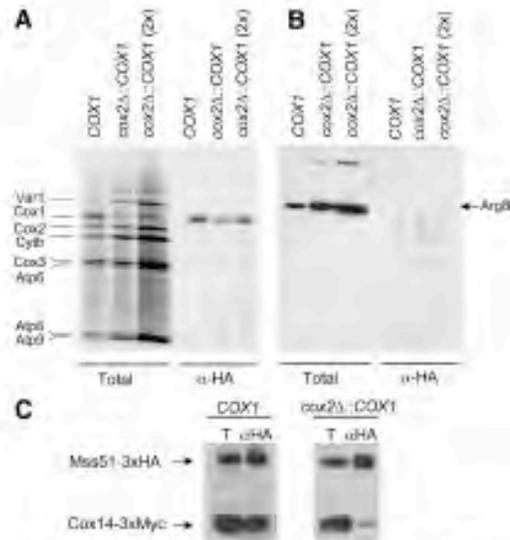
Figure 5. Mss51 interaction with newly synthesized Cox1 is not dependent on the COX1 mRNA UTRs. (A) Mitochondria were purified from two strains containing Mss51-3xHA, with either wild-type miDNA (SB7) or modified miDNA bearing the cox1Δ::ARG8ᵐ deletion and cox2Δ::COX1, an ectopic chimeric gene encoding an mRNA with the COX1 codons flanked by COX2 5′- and 3′-UTRs (XPM270a). The mitochondria were allowed to synthesize labeled proteins, and Total and α-HA precipitated proteins from wild-type (COX1) and the ectopic chimeric (cox2Δ::COX1) mRNAs were analyzed as described in the experiment in Figure 3. The third lane in each series had twice the amount of mitochondria (700 μg), as indicated. (B) After autoradiography, the membrane was probed with anti-Arg8 antibody and visualized by ECL detection. (C) Extracts of unlabeled mitochondria from strains containing Mss51-3xHA and Cox14-3xMyc, and either wild-type miDNA (TF258) or modified miDNA the encoding the ectopic chimeric mRNA with COX1 codons flanked by COX2 5′- and 3′-UTRs (CAB299) were analyzed by immune precipitation and Western blot as described in Figure 4.

the target of the COX1 mRNA-specific translational activator Pet309 (Manthey and McEwen, 1995). Although neither of these targets have been further localized genetically, the downstream 245 nucleotides of the COX1 mRNA 5′-UTR interact in a yeast three-hybrid assay with an N-terminal portion of Mss51 (but not full-length Mss51), suggesting that the biological target of Mss51 is relatively close to the protein coding sequence (Zambrano et al., 2007). Although the mechanism(s) by which these, and other, mitochondrial translational activators function is unknown, it is interesting to note the overall similarities to the control of cytoplasmic translation of mRNAs bearing internal ribosome entry sites (IRES) in their 5′-UTRs by IRES-transacting factors (Komar and Hatzoglou, 2005).

mRNA-specific translational activation is known to be rate limiting for expression of reporter genes inserted into the COX2 and COX3 mitochondrial loci in yeast (Steele et al., 1996; Cohen and Fox, 2001; Green-Willms et al., 2001), and we found that PET309 gene dosage is similarly limiting for expression of COX1. Interestingly, Mss51 is approximately fourfold more abundant than Pet309 in S288c-related cells grown on glucose (Ghaemmaghami et al., 2003) and approx-

imately eightfold more abundant in D273-10B-related cells grown on raffinose (unpublished data). Despite this relative abundance of Mss51, we nevertheless detected reduced expression of the ARG8ᵐ reporter inserted into the COX1 locus in diploid cells containing a single MSS51 nuclear gene, relative to homozygous wild-type diploids. Thus, Mss51 is present at levels that are near rate limiting for translational activation of the COX1 mRNA, consistent with the possibility that it has a role in regulating the level of Cox1 synthesis in mitochondria. Reduced gene dosage of both PET309 and MSS51 together had a stronger negative effect on reporter gene expression than reduced dosage of either alone. This suggests that when both Mss51 and Pet309 levels are lowered, relatively few mRNAs are simultaneously occupied by both necessary factors. However, this result does not distinguish whether the Mss51 and Pet309 have distinct functions in 5′-UTR-dependent translational activation, or work together to execute a single activity.

Barrientos et al. (2004) observed that that the synthesis of Cox1 within mitochondria, as measured by pulse labeling in vivo, was reduced in several mutant strains unable to assemble cytochrome c oxidase. However, this assembly-feedback regulation was not observed in assembly-defective cox14 mutants, nor in double mutants lacking both COX14 and other genes necessary for assembly. Thus, the 70-amino acid Cox14 protein is required both for assembly and for feedback regulation. Furthermore, Cox14 coprecipitated with both newly synthesized Cox1 and with Mss51. Based on these data, Barrientos et al. (2004) proposed that reduced synthesis of Cox1 in most assembly-defective mutants could be due to Cox14-dependent sequestration of Mss51 in assembly intermediates, although they reported that the association of Mss51 with newly synthesized Cox1 was not dependent upon Cox14.

Our findings, together with those of previous studies, support sequestration of Mss51 in early assembly intermediates as a mechanism for coupling Cox1 synthesis and assembly, in a sequence of events depicted in Figure 6. First, on the inner surface of the inner membrane (Siep et al., 2000; Tavares-Carreon et al., 2008) Mss51 and Pet309 activate translation of the COX1 mRNA (Manthey and McEwen, 1995; Perez-Martinez et al., 2003) through functional interactions with a site or sites in its 5′-UTR. Here, it is important to note that the levels of both Pet309 and Mss51 are at or near rate limiting for translational activation of chimeric COX1 mRNA encoding the reporter ARG8ᵐ. Thus, this is likely to be a point of regulation for Cox1 synthesis, as well as its localization (Naithani et al., 2003).

Second, as the completed or nearly completed Cox1 polypeptide emerges, it interacts with Mss51 (Figure 6, step 2). This step is inferred from the fact that Mss51 is required for Cox1 synthesis even when the COX2-mRNA specific translational activator Pet111 carries out the upstream activation function on a COX2 mRNA 5′-UTR fused to the COX1 coding sequence in a chimeric mRNA (Perez-Martinez et al., 2003). It presumably precedes involvement of Cox14, because Cox1 synthesis is robust in cox14Δ mutants (Barrientos et al., 2004). This inferred interaction must be weak because we did not detect coimmune precipitation of Cox1 with Mss51 from solubilized extracts in the absence of Cox14 (Figure 3). The role of Mss51 at this step is unknown. However, it may function by antagonizing a late Cox1 translation elongation arrest, a regulatory mechanism that has been documented in bacteria (Perez-Martinez et al., 2003; Woolhead et al., 2006).

Third, a complex containing at least Mss51 and Cox14 assembles dynamically with newly synthesized Cox1 (Fig-

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Further assembly
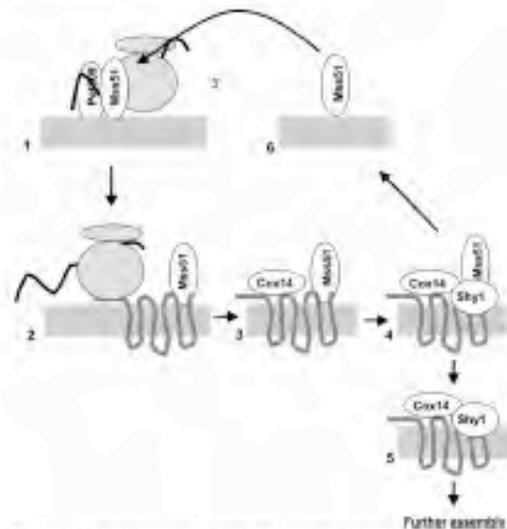
Figure 6. Dual activities of Mss51, and the stabilization of Mss51–Cox1 interaction by Cox14, couple Cox1 synthesis to assembly (see Discussion). 1) Mss51 and Pet309 activate COX1 mRNA through the 5'-UTR. 2) Mss51 interacts with newly synthesized Cox1 and allows completed translation through an unknown mechanism (Perez-Martinez et al., 2003). 3) Cox14 enters a complex that stabilizes the binding of Mss51 to newly synthesized Cox1. 4) Shy1 associates with the complex shown in 3 and facilitates dissociation of an early assembly intermediate (5) and Mss51 (6). Released Mss51 is available to activate another round of COX1 mRNA translation. Not shown: the protein Coa1 enters the pathway in step 3 or 4 (Pierrel et al., 2007), and Coa2 enters the assembly complex downstream of step 4 (Pierrel et al., 2008). Cox14 and Shy1 remain associated with assembled cytochrome oxidase supercomplexes (Mick et al., 2007).

ure 6, step 3). It is possible that Mss51 molecules interacting with the COX1 mRNA 5'-UTR normally transit in cis directly to a nascent Cox1 polypeptide translated from that mRNA molecule. This would generate a requirement for another Mss51 molecule to interact with the COX1 mRNA to activate the next round of translation. However, we found that Mss51 does interact physically with newly synthesized Cox1 translated from the chimeric COX1 mRNA bearing the COX2 mRNA 5'-UTR. Thus, Mss51 is capable of binding to nascent Cox1 (and to Cox14) in the absence of a COX1 mRNA 5'-UTR in cis to the coding sequence. The Cox1–Mss51–Cox14 complex may also contain the assembly factor Coa1 (Pierrel et al., 2007), not depicted in Figure 6.

The interaction between Mss51 and Cox14 must be bridged dynamically by newly synthesized Cox1, because Mss51 and Cox14 do not coimmune precipitate from extracts of mitochondria from either cox1Δ mutant, or a pet309 mutant, that specifically fail to synthesize Cox1. This finding is consistent with the observation of high molecular weight complexes containing Mss51 that are absent after treatment of wild-type cells with chloramphenicol or in a pet309 mutant (Pierrel et al., 2008). The assembly intermediate complex(es) containing Mss51, Cox14 and newly synthesized Cox1 contain roughly half of total Mss51, based on our coimmune precipitation with epitope-tagged Cox1. This finding is consistent with the observation that ~50% of Mss51 is associated with Cox14 (Barrientos et al., 2004).

Interestingly, these complexes seem to contain >0.1% of total cellular Cox1 based on data reported by Mick et al. (2007), indicating a significant pool of Cox1 is present in early assembly intermediates containing Mss51.

Fourth, Shy1 associates with Cox1-containing complexes, probably after Mss51, Cox14, and Coa1, although the evidence for this order is not conclusive (Figure 6, step 4) (Mick et al., 2007; Pierrel et al., 2007). Both Shy1 and Cox14 are associated physically with Mss51, with downstream assembly intermediates lacking Mss51, and with fully assembled respiratory supercomplexes containing active cytochrome c oxidase (complex IV) and the cytochrome bc_1 complex (complex III) (Mick et al., 2007). We found that a cox1 deletion mutation in mtDNA prevented coimmune precipitation of Shy1 with Mss51 (unpublished data), consistent with the dynamic assembly of Shy1-containing complexes nucleated by newly synthesized Cox1. Mutations inactivating Shy1 in yeast, and its orthologues in humans and bacteria, decrease but do not eliminate cytochrome c oxidase activity, apparently by decreasing the efficiency of heme a_3 insertion into Cox1 (Smith et al., 2005; Khalimonchuk et al., 2007; Bundschuh et al., 2008; Pierrel et al., 2008). Interestingly, respiratory growth of a yeast shy1Δ mutant is improved by dominant suppressor mutations in MSS51 and by overexpression of wild-type MSS51 (Barrientos et al., 2002).

Finally, Cox1-containing assembly intermediates that retain Cox14 and Shy1 but not Mss51 proceed toward further assembly by insertion of metal ions and heme a moieties into Cox1, and association of additional enzyme subunits (Mick et al., 2007; Pierrel et al., 2007; Khalimonchuk and Winge, 2008; Barrientos et al., 2009). At this step, Mss51 is released from the assembly pathway and available to activate additional rounds of COX1 mRNA translation (Figure 6, steps 5 and 6).

Sequestration of Mss51 in early complexes formed during the assembly process could cause decreased COX1 mRNA translation if the early complexes over accumulate due to downstream blocks in assembly, as proposed previously (Barrientos et al., 2004). Our data support this model by showing that Mss51 levels can limit COX1 mRNA translation and that a significant fraction of cellular Mss51 is associated with early assembly complexes containing Cox1. Furthermore, our data explain why mutations eliminating Cox14 do not prevent Cox1 synthesis, despite preventing cytochrome c oxidase assembly: Mss51 is not stably associated with newly synthesized Cox1 in the absence of Cox14 and therefore remains available to activate futile synthesis of Cox1. However, the Cox1 produced in the absence of Cox14 is highly unstable and present at lower steady-state levels than those observed in mutants blocked further downstream in the assembly process (Barrientos et al., 2004). This feedback system, observed in mutants, may reflect a mechanism that normally coordinates the level of newly synthesized Cox1 with the levels of assembly factors in wild-type cells and thereby helps to protect against oxidative damage (Khalimonchuk et al., 2007).

In contrast to expectation, we did not observe a significant increase in the fraction of total Mss51-3xMyc coprecipitated with Cox1-3xHA when cytochrome c oxidase assembly was blocked by a nuclear mss2Δ (Broadley et al., 2001) mutation. However, the available methodology for quantifying these solubilized coprecipitated complexes is not highly accurate, and the physiologically relevant differences may not be great. Furthermore, we cannot determine whether the presence of epitope tags exerts subtle effects on the behavior of these proteins. Thus, these data do not argue strongly against the model. When we compared wild type with an

assembly defective mitochondrial cox2 mutant (Saracco, 2003) for the fraction of total unmodified Cox1 coprecipitated with Mss51-3xHA, we observed a dramatic difference. In contrast to wild type, virtually all of the Cox1 present in the mutant was associated with Mss51, consistent with the idea that early assembly intermediates containing Mss51 and Cox1 accumulate when assembly is blocked.

Mutants lacking the assembly factor Coa1 seem to resemble cox14Δ mutants insofar as they exhibit normal pulse labeling of Cox1 (Mick et al., 2007; Pierrel et al., 2007, 2008). However, in contrast to the tight respiratory negative phenotype of cox14Δ mutants, coa1Δ mutants exhibit low levels of respiration (Pierrel et al., 2007) and weak growth on nonfermentable carbon sources (Mick et al., 2007). In these respects, and their suppressibility by over expressed MSS51, the coa1Δ and shy1Δ mutations are similar (Barrientos et al., 2002; Pierrel et al., 2007). Furthermore, coa1Δ does not disrupt the interaction between Mss51 and Cox14 (Pierrel et al., 2007). Thus, Coa1 is probably not required for the interaction between Mss51 and newly synthesized Cox1 but could reinforce it. Robust Cox1 synthesis in the coa1Δ mutant may depend on the low level of cytochrome c oxidase assembly that takes place in its absence.

Photosynthetic complexes are composed of protein subunits encoded by genes in both chloroplast and nuclear DNA, analogously to mitochondrial respiratory complexes. In Chlamydomonas reinhardtii, translation of chloroplast encoded mRNAs specifying certain key subunits of photosystem I, photosystem II, and the cytochrome $b_6 f$ complex has been shown to be coupled to the assembly of those complexes (Choquet and Wollman, 2002). In each case, regulation of translation by assembly was dependent upon the 5'-UTRs of the chloroplast encoded mRNAs (Choquet et al., 1998; Wostrikoff et al., 2004; Minai et al., 2006). In the case of cytochrome f synthesis, assembly feedback regulation depends upon C-terminal residues of cytochrome f itself, which is hypothesized to interact with an effector protein that directly regulates translation (Choquet et al., 2003). These findings suggest that mRNA-specific coupling of organellar translation to the assembly of energy transducing complexes may be widespread in eukaryotes, and raise interesting questions about how such regulation could be achieved in mammalian mitochondria whose mRNAs lack 5'-UTRs.

ACKNOWLEDGMENTS

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Protein Synthesis and Assembly in Mitochondrial Disorders

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Abstract: Human mitochondrial DNA (mtDNA) codes for 13 polypeptides which constitute the central core of the oxidative phosphorylation (OXPHOS) complexes. The machinery for mitochondrial protein synthesis has a dual origin: a full set of tRNAs, as well as the 12S and 16S rRNAs are encoded in the mitochondrial genome, while most factors necessary for translation are encoded by nuclear genes. The mitochondrial translation apparatus is highly specialized in expressing membrane proteins, and couples the synthesis of proteins to the insertion into the mitochondrial inner membrane. In recent years it has become clear that defects of mitochondrial translation and protein assembly cause several mitochondrial disorders.

Since direct studies on protein synthesis in human mitochondria are still a relatively difficult task, we owe our current knowledge of this field to the large amount of genetic and biochemical studies performed in the yeast *Saccharomyces cerevisiae*. These studies have allowed the identification of several genes involved in mitochondrial protein synthesis and assembly, and have provided insights into the conserved mechanisms of mitochondrial gene expression. In the present review we will discuss the most recent advances in the understanding of the mechanisms and factors that govern mammalian mitochondrial translation/protein insertion, as well as known pathologies associated with them.

Keywords: Assembly, mitochondria, mitochondrial disease, mitochondrial DNA (mtDNA), mitoribosomes, oxidative phosphorylation (OXPHOS), translation, yeast.

1. INTRODUCTION

Oxidative phosphorylation, the metabolic pathway that couples respiration to ATP production, takes place within mitochondria. These organelles arose from an endosymbiotic process that involved α-proteobacteria [1]. As a result of this process, mitochondria have their own genome, which is highly reduced. Human mitochondrial DNA (mtDNA) is a circular, double stranded DNA molecule of 16.6 kb that codes for 13 proteins, which are subunits of the OXPHOS complexes [2]: *ND1* to *ND6* and *ND4L* code for subunits 1 to 6 and 4L of the NADH dehydrogenase complex (Complex I); *CYTB* codes for cytochrome *b*, a component of the *bc₁* complex (Complex III); *COX1*, *COX2* and *COX3* code subunits 1, 2 and 3 of cytochrome *c* oxidase (complex IV); *ATP6* and *ATP8* code subunits a and A6L from the F₁F₀-ATP synthase (Complex V). In addition, human mtDNA encodes 22 tRNAs, as well as the 12S and 16S rRNAs, which are components of the mitochondrial ribosomes.

The polypeptides encoded by mitochondrial genes are highly hydrophobic, exhibiting two to 15 transmembrane stretches. These proteins are part of the catalytic core of the OXPHOS complexes, and the majority is highly conserved from bacteria to animals. The mitochondrial translation machinery is highly specialized for expression of membrane proteins. Therefore mitochondrial ribosomes are associated to the inner membrane [3, 4]. In *S. cerevisiae*, this association is mediated by proteins like Oxa1p [5, 6], Mba1p [7] and Mdm38p [8]. In addition, yeast translational activators

are associated with the mitochondrial inner membrane, and are thought to tether initiation of translation to the region where newly made polypeptides are to be inserted (reviewed in [9, 10]). Assembly of OXPHOS complexes is associated with protein synthesis. For example, Cox11p is a protein involved in delivering of Cu_B to subunit 1 of the cytochrome *c* oxidase, and is associated with mitochondrial ribosomes [11]. All these observations strongly suggest that translation of mitochondrial mRNAs is coupled with insertion of newly made proteins into the mitochondrial inner membrane, as well as with the incorporation of prosthetic groups and assembly of the OXPHOS complexes [12].

In addition, mitochondrial translation seems to be coupled with transcription. In yeast these processes are physically and genetically coupled through interaction of the mitochondrial RNA polymerase with Nam1p, a protein involved in translation [13, 14]. In humans mitochondrial translation and transcription could be coupled by the interaction of the RNA polymerase with the ribosomal protein MrpL12 [15].

The mitochondrial translation system is more closely related to the bacterial system than to the eukaryotic apparatus found in the cytosol. However, important differences from the prokaryotic system are present: *i)* Mitochondrial genetic code is different from the standard one and reveals variability between species [16]. In human mitochondria, AUG and AUA (as well as AUU exclusively in the case of *ND2*) serve as methionine codons [17]. TGA, which is a stop codon in the universal genetic code, is read as tryptophan in human mitochondria, whereas AGA and AGG, conventionally encoding arginine, are used as stop codons in this system, in addition to UAA and UAG. *ii)* The polypeptides encoded by the mammalian mitochondrial genome are

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synthesized from nine monocistronic and two dicistronic transcripts with overlapping open reading frames [2, 18]. Some of the transcripts lack a complete stop codon, and at these locations, the post-transcriptional addition of poly(A) tails produces a functional termination codon [19]. *iii)* In mammalian mitochondria the same tRNA^Met is used for both initiation and elongation. The mitochondrial tRNA^Met bears the unusual modified base 5-formyl cytidine (f5C) in the first position of the anticodon [20]. This base might be responsible for the ability of this tRNA to decode the AUG and AUA codons [21]. *iv)* Mammalian mitochondrial ribosomes have a relatively low RNA content and a higher protein content than the prokaryotic ribosomes. About half of these proteins are homologs of bacterial proteins, while the rest are unique to mitochondria (reviewed in [22]). *v)* Of particular interest is the observation that the mammalian mitochondrial mRNAs have an almost complete lack of untranslated regions. Thus, a Shine/Dalgarno interaction between the mRNA and the 12S rRNA is not used during mammalian mitochondrial translation. The yeast mitochondrial mRNAs also lack a typical Shine/Dalgarno element. However, in this organism translational activators that are mRNA-specific, could be involved in the localization of the small ribosomal subunit near the translational start codon [9].

Although our understanding of the mechanisms of mitochondrial protein synthesis and assembly is far from complete, in this review we discuss the current understanding of mammalian mitochondrial protein synthesis, as well as the pathologies associated with defects in this process. These pathologies are caused by mutations in nuclear or mitochondrial genes that affect translation (reviewed in [23]), as well as assembly of newly made proteins into the OXPHOS complexes. All reported mutations causing mitochondrial disease in humans can be found in the Mitomap (http://www.mitomap.org/) and OMIM (Online Mendelians Inheritance in Man; http://www.ncbi.nlm. nih.gov/sites/entrez?db=OMIM) databases.

In this review we will also address some aspects of the mitochondrial translation in the yeast *S. cerevisiae*. This microorganism is widely used for the study of mitochondrial functions, as its experimental manipulation is relatively easy as compared to mammalian systems. This budding yeast is a facultative aerobic organism where the presence of a functional OXPHOS system is not essential for the survival of the cell, hence it can tolerate the absence of any of the mitochondrial components that are related to the biogenesis of the OXPHOS complexes. In addition, *S. cerevisiae* has been particularly suitable to explore the mechanisms for coupling translation to assembly of newly synthesized proteins in the inner membrane.

2. TRANSLATION MACHINERY

2.1. Mitochondral Ribosomes

Mitochondrial ribosomes (mitoribosomes) are very complex structures whose assembly requires the coordinated expression of mitochondrial and nuclear genes. The mitochondrial rRNAs are encoded in the mitochondrial genome of all the organisms reported to date, while the genes for mitochondrial ribosome proteins (MRPs) are predominantly encoded by the nuclear genome [24]. In mammalian mitochondria every MRP is encoded in the nuclear genome, while in yeast the only MRP encoded by the mitochondrial genome is Var1p, a component of the small subunit of the mitoribosome [25, 26]. In contrast, the mitochondrial genomes of many protozoa and plants encode numerous MRPs [27].

As a reminiscence of their ancestral prokaryotic origins, it was expected that organellar ribosomes would be very similar to the eubacterial ones. However, even though the main features of mitoribosomes are comparable with those of bacterial or eukaryotic cytoplasmic ribosomes, they present striking differences (reviewed in [22]). Mammalian mitoribosomes have a low RNA content, and have a smaller sedimentation coefficient as compared to those of eubacteria and to the cytoplasmic ribosomes (70S for eubacterial –*E. coli*-, 80S for cytoplasmic and 55S for human mitochondrial ribosomes). Nevertheless, they are larger in mass and dimensions than 70S bacterial ribosomes [28]. The mitoribosome has a protein:RNA ratio of 69% protein and 31% RNA, whereas the bacterial ribosome has a protein:RNA ratio of 33% protein and 67% RNA [28].

The rRNAs found in the mitoribosomes are also variable in length and sequence. Within the small ribosomal subunit, the rRNA molecule present in yeast mitochondria is 15S, while in human mitochondria this molecule is only 12S. The size of the latter shows a reduction of about 40% when compared to the equivalent 16S rRNA found in bacterial small ribosomal subunits. Within the large ribosomal subunit, the yeast mitochondria contain a 21S rRNA molecule whereas in human mitochondria this is a 16S molecule, about half the size of the bacterial 23S [22, 24, 29]. An interesting feature shared by the animal and fungal mitochondrial ribosomes is that they lack 5S rRNA, which is an essential component of the large subunit of other ribosomes, i.e. *E. coli* ribosomes and plant mitoribosomes [24, 30]. However, the 5S rRNA is encoded in the nuclear genome and imported into the mitochondrial matrix. The functional relevance of this process remains to be elucidated [31, 32].

Mitoribosomes have gone through major adaptations during mitochondrial evolution. One of the most striking adjustments, was the increase in protein content by elongation of its proteins and the acquisition of new ribosomal proteins. However, during evolution several MRPs from bacterial origin have been lost as well [27]. The total number of identified MRPs range from around 81 in most metazoan species, to 80 in yeast, 63 in plants, and 39 in the apicomplexan *Plasmodium falciparum* [27]. Through proteomic analysis, around 80 MRPs have been identified in mammalian mitochondria (reviewed in [22]). Currently, information regarding the functions of the MRPs that are not present in bacterial ribosomes is rather limited. Since mitochondrial encoded proteins are inserted into the inner membrane in a co-translational manner, at least some of the metazoa-specific supernumerary MRPs might be important for this process [27].

Since the core components of the OXPHOS complexes are encoded in the mitochondrial genome and need to be synthesized by mitochondrial ribosomes, in principle any

mutation altering the assembly and/or function of the mitochondrial ribosomes either at the MRPs or the rRNAs, could result in a human disorder. However, there are just a few cases reported where a link between mutations in components of the mitochondrial ribosome and a human disorder have been established with certainty: point mutations at nt 1494 and 1555 in the 12S rRNA gene are some of the mutations associated with maternally inherited non-syndromic sensoneuronal deafness and aminoglycoside-induced deafness [33-35]. These mutations decrease the accuracy of translation and make the ribosomal decoding site hypersusceptible to aminoglycoside antibiotics [36]. An extensive analysis in japanese diabetic patients revealed two point mutations, C1310T and A1438G, in the 12S rRNA [37], whereas the mutation T1119C was found in young obese adults but not in diabetic patients [38]. An unusual 12S rRNA mutation was reported (T1095C) in a pedigree with maternally inherited sensorineural deafness and Parkinson's disease, which suggested that the last one could be another manifestation of mutations affecting the mitochondrial translation machinery [39].

Regarding the MRPs, a nonsense mutation in the gene coding for MRPS16 was associated with fatal, neonatal encephalopathy. This patient had a marked reduction of the 12S rRNA transcript level, and translation was severely affected [40]. In addition, many MRPs have been proposed as candidates for human mitochondrial disorders, as they map to loci associated with mitochondrial pathologies. These pathologies include deafness, Usher syndrome 1E, retinitis pigmentosa, Leigh syndrome, Spinocerebellar ataxia, Russel-Silver syndrome, Struve-Wiedemann syndrome, among others [41-43]. In Table 1 we summarize the human mitochondrial disorders that are possibly related to MRPs. It also compares these MRPs with the *S. cerevisiae* MRPs, and the phenotype observed of the disruptions made in yeast.

There are fewer cases that have been associated with the assembly of the mitoribosome. This depends among other factors on a protein called paraplegin. Mutations in the gene *SPG7*, which encodes paraplegin, are associated with hereditary spastic paraplegia (HSP) [44]. Paraplegin is a highly conserved AAA-protease present in the mitochondrial inner membrane from yeasts and mammals (reviewed in [45]). One of the fundamental roles of paraplegin is the cleavage of the N-terminal targeting sequence of MrpL32p after its import into mitochondria. MrpL32p is an essential component of the mitoribosome, and its processing is a prerequisite for assembly into the mitoribosome [46].

To add another degree of complexity to the assembly of the mitoribosome in human mitochondria, some MRPs have isoforms, an example of this is MRPS18, which has three isoforms [47]. The implication of this finding is that human mitochondrial ribosomes are heterogeneous, depending on which isoform they contain, and raises the possibility of functional specialization for the different mitochondrial ribosomes. This could imply that mutations in specific MRP isoforms may affect specific tissues, giving rise to a wide spectrum of disorders [48].

2.2. Mitochondrial tRNAs

Human mtDNA encodes 22 tRNAs. For each amino acid there is only one tRNA, except for leucine and serine, which require two tRNAs, dedicated to different codon groups: $Leu^{(UUR)}$ and $Leu^{(CUN)}$, and $Ser^{(UCN)}$ and $Ser^{(AGY)}$ respectively. Mitochondrial tRNAs can decode two codons. This is achieved by "wobble" base pairing, where in the third position of an anticodon, a purine can be replaced with another purine, or pyrimidine with another pyrimidine [49]. In mitochondria, the most studied example of wobble binding is the tRNA $Leu^{(UUR)}$ mentioned above. The anti-codon can read both UUA and UUG codons. This occurs via a modification of the wobble base by taurine [50]. The same kind of modification by taurine also occurs in the tRNA Lys. These modifications have a crucial role in stabilizing the codon-anticodon interaction in the ribosome [51].

Human mitochondrial tRNAs have minimized structures compared to other species or to cytoplasmic tRNAs: they have shortened stems and loops or even lack entire domains [52]. They have a decreased GC content and an increased amount of non-Watson-Crick base pairing in the stems, leading to a thermodynamic instability. This has been proposed to increase the susceptibility of mitochondrial tRNAs to suffer pathogenic mutations (reviewed in [53]).

tRNA mutations are the most common group of mtDNA mutations. They can cause an impairment of the mitochondrial protein synthesis and therefore can be associated with several syndromes [54]. A typical feature for mitochondrial tRNA mutations is that the same mutation can cause very different disease phenotypes in different families, and different mutations can cause similar disease phenotypes in different individuals.

The most studied tRNA mutations are located in the tRNA $Leu^{(UUR)}$ which is known to be a hot spot for mitochondrial mutations. Mutations within the tRNA $Leu^{(UUR)}$ are associated with MELAS (myopathy, encephalopathy, lactic acidosis and stroke-like episodes). For instance, in Finland the MELAS mutation has arisen at least 9 different times [55]. 80% of the identified patients bearing a defect in tRNA $Leu^{(UUR)}$ present an A3243G mutation, and about 10% a T3271C mutation. Both mutations prevent the taurine modification in the wobble base of the tRNA, leading to a defect in reading UUG codons, but not UUA codons [51]. Complex I is particularly affected by this mutation because ND6 has many UUR codons [56], and 42% of leucine codons are UUG codons. Mutations in the ND6 gene itself give a MELAS type disease, which even further supports the hypothesis [57]. In addition, the A3243G mutation has been shown to cause impaired transcription termination [58], impaired pre-tRNA processing [59, 60], decreased stability and aminoacylation [59, 61], and abnormal tRNA conformation [62]. The same mutation can cause a wide variety of symptoms, the mildest being diabetes mellitus (with or without hearing loss), developed by 85% of the patients before the age of 70 [63]. Interestingly, the point

Table 1.   **Comparison of MRPs from Human and Yeast that are Candidates for Mitochondrial Disorders**

Human MRP	Yeast MRP [24, 27, 176]	Deletion phenotype in yeast	Candidate mitochondrial disorder	Reference
Small subunit				
MRPS2	Mrp4p	YPG-[b]	DFNB33*	[43]
			Leigh syndrome	[42]
MRPS10	Rms10p	lethal	Spinocerebellar ataxia	[42]
MRPS11	Mrps18p	lethal	DFNA30*	[43]
MRPS12	Ynr036cp	YPG-	DFNA4	[41, 43]
MRPS14	Mrp2p	YPG-	DFNA7	[41]
MRPS15	Mrps28p	YPG-	DFNA2	[43]
			Stuve-Wiedemann syndrome	[42]
MRPS17	Mrps17p	YPG-	Russell-Silver syndrome	[42]
MRSP18A MRPS18B	Rsm18p	YPG-	Spinocerebellar ataxia	[42]
MRPS21	Mrp21p	YPG-	DFNA7	[43]
MRPS23	Rsm25p	YPG-	Retinitis pigmentosa 17	[41]
			Russell-silver syndrome	[42]
MRPS26	-	-	Hallervorden-Spatz syndrome	[41]
MRPS29 (DAP3)	Rsm23p	YPG-	DFNA7	[43]
			Apoptotic protein	[47]
MRPS30	-	-	Leigh syndrome	[42]
MRPS33	Rsm27p	YPG-	DFNB13	[43]
Large Subunit				
MRPL2	Rml2p	YPG-	Spinocerebellar ataxia	[42]
MRPL3	Mrpl9p	YPG-	DFNA18	[43]
			Moebius syndrome 2	[41]
MRPL4	YmL6p	lethal	DFNB15	[41, 43]
MRPL9	-	-	DFNA7	[43]
			Retinitis pigmentosa 18	[41]
MRPL12	Mrp1p	lethal	DFNA20	[43]
MRPL14	MrpL38p	YPG-	Spinocerebellar ataxia	[42]
MRPL19	Img1p	YPG-	DFNA43	[43]
MRPL24	-	-	DFNA7	[43]
MRPL38	MrpL35p	YPG-	DFNA20	[43]
MRPL39	-	-	Usher syndrome, type IE	[41, 42]
MRPL41	MrpL27p	YPG-	DFNB33	[43]
			Leigh syndrome	[42]
MRPL42	-	-	DFNA25	[43]
MRPL46	MrpL17p	YPG-	DFNA30	[43]
MRPL48	-	-	Leigh syndrome	[42]

Human MRP	Yeast MRP [24, 27, 176]	Deletion phenotype in yeast	Candidate mitochondrial disorder	Reference
MRPL49	Img2p	YPG-	Leigh syndrome	[42]
MRPL53	-	-	MMD*	[42]
MRPL54	MrpL37p	YPG-	Leigh syndrome	[42]
			DFNB15	[43]

*DFNA: autosomal dominant non-syndromic hearing loss; DFNB: autosomal recessive non-syndromic hearing loss; MMD: Multiple mitochondrial dysfunctions syndrome. * YPG-: respiratory deficient cells.

mutation A12300G on tRNA Leu[CUN] has been shown to suppress the pathological phenotype caused by the A3243G mutation [59]. Mitochondrial translation, complex assembly and cell respiration are normal in cells containing 99% of pathological MELAS mutation and moderate amount of the suppressor mutation. This kind of suppression has never been found *in vivo* in patients nor healthy relatives of the patients. Interestingly, the tRNA Leu[CUN] has taurine modifications in some of its tRNA, but not in all, enabling to read UUR codons, but not UUY codons for phenylalanine [64].

The other well-studied tRNA mutation is the A8344G mutation in the tRNA Lys causing myoclonic epilepsy associated with ragged-red fibers (MERRF) [65]. This mutation decreases the aminoacylation of the tRNA and premature termination of translation takes place at, or near of, each lysine codon [66]. In addition, this mutation prevents the taurine modification in tRNA Lys [51]. It has been suggested that the decreased efficiency of the wobble base modification caused by the lack of taurine modification, can be one explanation for the differences in the degree of the disorder observed in different patients. It is also known that this mutation is tolerated at high levels: healthy relatives can present mutant loads up to 70% [67].

tRNA molecules must be modified in order to be functional. Therefore, mutations present in the components required to complete any of the modifications required by the tRNAs, can also be found associated to human disorders. One example of this, are mutations found in the gene encoding the pseudouridylate synthase 1 (*PUS1*). This enzyme is responsible for the pseudouridylation of both cytoplasmic and mitochondrial tRNAs. The identified mutations have been linked to cause mitochondrial myo-pathy, lactic acidosis and sideroblastic anemia (MLASA), a progressive disorder affecting muscle and erythroid cells [68].

One common symptom caused by mtDNA mutations is deafness. Some tRNA mutations, like tRNA Ser[UCN], Leu[UUR], Lys and Glu cause syndromic or non-syndromic hearing losses. (reviewed in [69]).

Mitochondrial Aminoacyl-tRNA Synthetases

Mitochondrial aminoacyl –tRNA synthetases are encoded by nuclear genes and are imported into mitochondria. Defects in these genes have been recently shown to cause mitochondrial disease by affecting mitochondrial translation. Mitochondrial leucyl tRNA synthetase (*LARS2*) has been

shown to be a type 2 diabetes susceptibility gene in the Netherlands and Denmark [70].

An intronic mutation in the arginine tRNA synthetase gene (*RARS2*) causes severe infantile encephalopathy associated with pontocerebellar hypoplasia and multiple mitochondrial respiratory-chain defects. The mutation produces a short *RARS2* transcript and reduction in both, the amount of tRNA Arg and its aminoacylation status [71].

Recently, mutations in the mitochondrial aspartyl-tRNA synthetase (*DARS2*) were found to cause leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation (LBSL). Enzyme activities of mutant proteins were decreased, but no mitochondrial phenotype was seen in patient's fibroblast and lymphoblast cell lines [72]. The precise role that this mutation plays during the development of the observed malfunctions remains to be elucidated.

2.3. Translation Initiation, Elongation and Termination

Translation Initiation

In eubacteria, translation initiation depends on three factors: IF1, IF2 and IF3 (reviewed in [73]). In mammalian mitochondria, homologs of IF2 ($IF2_{mt}$) and IF3 ($IF3_{mt}$) can be clearly identified [74-76]. In contrast, no IF1 homolog has been detected in mitochondria, however, $IF2_{mt}$ can perform the functions that both essential factors IF2 and IF1 play in *E. coli*. A conserved insertion of 37 amino acids in $IF2_{mt}$ substitutes the function of IF1 [77]. This insertion is only found in mitochondrial IF2s, and could be in close proximity to the small ribosomal subunit to directly influence its binding to the ribosome [78]. In eubacteria IF1 is the smallest of the three initiation factors, with a molecular mass of 8.2 kDa. IF1 binds to the A-site of the small ribosomal subunit and is thought to direct the initiator tRNA to the P site in the ribosome by blocking the A site. In addition IF1 enhances the dissociation and association of the ribosome: in the presence of IF1, the interaction between IF2 and the small ribosomal subunit is enhanced, and the release of IF2 is indirectly promoted when IF1 is ejected (reviewed in [73]).

$IF2_{mt}$ is a GTP/GDP binding protein that promotes the binding of tRNA fMet to the small ribosomal subunit. This binding is dependent on the presence of mRNA, closely resembling the prokaryotic IF2 [75]. Yeast $IF2_{mt}$ is encoded by the *IFM1* gene [79]. It has the same domain structure and biochemical properties as the mammalian mitochondria $IF2_{mt}$, but with enhanced ability to bind unformylated

initiator tRNA Met [80, 81]. Genetic evidence suggests that Rsm28p, a dispensable component of the yeast mitochondrial ribosomal small subunit, could have partially overlapping roles with $IF2_{mt}$ [82]. The *rsm28Δ* mutation, together with mutations in the *IFM1* and *FMT1* (which encodes the methionyl-tRNA-formyltransferase) genes caused a synthetic respiratory defective phenotype. This suggests that in yeast, Rsm28p participates in the assembly of the initiation complex [82]. To date, no ortholog of Rsm28p has been identified in mammalian mitochondria.

The bacterial IF3 has several roles in translation initiation (reviewed in [73]): *i)* It prevents the association of the ribosomal subunits by binding to the small subunit; *ii)* stimulates the rapid formation of codon-anticodon interaction at the ribosomal P-site; *iii)* proofreads the selection of the initiator tRNA fMet and the AUG codon in the P-site by promoting dissociation of initiation complexes with incorrectly bound tRNAs; and *vi)* promotes the shift in position of the mRNA from the standby site to the decoding position in the P-site of the small ribosomal subunit.

$IF3_{mt}$ has a central portion bearing only 20.8% identity to the *E. coli* IF3 [76]. $IF3_{mt}$ does not appear to be highly conserved among animals. For example, BLAST searches failed to identify the homolog of $IF3_{mt}$ in *C. elegans* [76]. $IF3_{mt}$ promotes the dissociation of the ribosome, as it does in bacteria. It stimulates tRNA fMet binding to the mRNAs on the ribosome [76]. Structural modeling of $IF3_{mt}$ suggests that the central region of this protein is similar to the bacterial IF3 [76, 83]. However, the mammalian $IF3_{mt}$ has extensions of around 30 amino acids at the N and C termini surrounding a central region, which is homologous to the bacterial IF3. These extensions on $IF3_{mt}$ are not essential for promoting initiation complex formation on mitochondrial ribosomes. However, the C-terminal extension appears to be important to reduce the affinity of $IF3_{mt}$ for the large subunit, preventing improper joining of the ribosomal subunits during initiation complex formation [83, 84]. BLAST searches allowed the tentative identification of the *Schizosaccharomyces pombe* $IF3_{mt}$, which shows 25% and 20.9% sequence identity to the bacterial and human $IF3_{mt}$, respectively. In contrast to the human factor, *S. pombe* $IF3_{mt}$ has no predicted N and C- terminal extensions. To date, the *S. cerevisiae* homolog has not been firmly established [76].

Selection of Initiation Site and Translational Activators in Yeast Mitochondria

A feature of the human mitochondrial translation system is that the mRNAs in this organelle have an almost complete lack of 5' untranslated regions (5'-UTR). The start codon is generally located within three nucleotides of the 5' end [2]. Thus, mammalian mitochondrial ribosomes cannot recognize the start codon using the Shine/Dalgarno interaction between the mRNA and the 12S rRNA as observed in bacteria. Further, this system does not use a cap-binding and scanning mechanism such as observed in the eukaryotic cytoplasm. To date, it is not understood how mitochondrial ribosomes select the translation initiation site in the mRNAs. Jones et al. (2008) [85] found that the first 35 nucleotides of the mammalian mitochondrial mRNAs are highly unstructured. The start codons could be accessible within single stranded motifs, making them potentially accessible for ribosome

binding. These data are consistent with a model in which the specialized mitochondrial ribosome preferentially allows passage of unstructured 5' sequences into the mRNA entrance site to participate in translation initiation.

In the yeast *S. cerevisiae*, translation initiation is controlled by a family of proteins known as translational activators (reviewed in [9, 10]). Each member of this family is specific for a mitochondrial mRNA. These proteins act on the 5'-UTR of the target mRNA to promote translation initiation. Genetic and biochemical evidences indicate that translational activators interact with the ribosome, and at least for some activators, this interaction is mediated by their carboxy terminal portion (for examples see [86, 87]). This suggests that, through the interaction with the small ribosomal subunit and with the mRNA's 5'-UTR, translational activators could participate in the recognition of the start codon by the ribosome. In addition, translational activators are associated with the mitochondrial inner membrane and with themselves, suggesting that these proteins tether mRNAs to the mitochondrial inner membrane, where newly made polypeptides will be integrated [14, 88-90]. Table 2 shows the yeast translational activators known and their target mRNAs. *ATP8* and *VAR1* are the only genes for which no translational activators have been found.

Table 2.   Translational Activators Found in *S. cerevisiae* Mitochondria

Translational activator*	Target mRNA
Pet309p	*COX1*
Pet111p	*COX2*
Pet54p	*COX3*
Pet122p	*COX3*
Pet494p	*COX3*
Cbs1p	*CYTB*
Cbs2p	*CYTB*
Atp22p	*ATP6*
Aep1p (Nca1p)	*ATP9*
Aep2p (Atp13p)	*ATP9*

*Taken from [9, 10], except for Atp22p, which is described in [90].

Translational activators have highly diverged, so clear homologs can only be found among fungi [91]. However, this does not necessarily imply that there is no equivalent system to the translational activators in mammals. To date, only Pet309p has been found to have an ortholog in humans: the protein LRPPRC. The role of this protein during the synthesis of Cox1p will be discussed later. It has been suggested that in contrast to what has been found in yeast, the mammalian translational activators could interact with the coding regions of the mitochondrial mRNAs [23].

One characteristic of the yeast translational activators is that the non-respiratory phenotype produced by mutations on the mRNA's 5'-UTR can be experimentally bypassed by the exchange of this region with the 5'-UTR from a different

mRNA [9]. There is a group of translational activators whose effect is not restricted to the 5'-UTR region, making the bypass mentioned above impossible. This is the case for Cbp6p, which is a protein required for *CYTB* mRNA translation [92]. A better understood example is Mss51p, a protein necessary for *COX1* mRNA translation. The action of Mss51p maps to the *COX1* 5'-UTR, as well as the coding region [93]. In addition Mss51p physically interacts with the newly made Cox1p, suggesting that this protein is involved in the coordination of Cox1p synthesis and insertion into the mitochondrial inner membrane [93, 94]. Together with Mss51p, the assembly regulation of Cox1p synthesis could be mediated by factors like Cox14p [94] and Coa1p [95, 96].

PPR Proteins and Translation

PPR (pentatricopeptide repeat) proteins are a recently discovered family of proteins, which has essential roles in mitochondrial and chloroplast biogenesis (reviewed in [97, 98]). In general, members of this family are involved in RNA metabolism [97]. PPR motifs are 35 amino acids repeats usually present as a tandem within a protein. These repeats are predicted to form a superhelix that encloses a central groove with an RNA-binding surface [99]. Predicted models of PPR proteins indicate that the bottom of the central groove is positively charged, which could facilitate the binding of RNA molecules [99, 100].

Some PPR-containing proteins are part of the mitochondrial translation machinery. PPR proteins are associated with mitoribosomes, although their role is not clear at present [101-104]. Some PPR-containing proteins act as translational activators [105-107]. This is the case for the yeast protein Pet309p and its human ortholog, LRPPRC, with 7 and 11 predicted repeats, respectively [100, 107]. The presence of these motifs in Pet309p is necessary for the production of the protein Cox1p, but it is dispensable for the transcription or stability of the *COX1* mRNA, indicating that the 7 PPR motifs present in Pet309p are required for *COX1* mRNA translation [100]. LRPPRC is an RNA binding protein that it is localized in mitochondria and nucleus [108]. The role of LRPPRC in both nuclear and mitochondrial mRNA metabolism suggests that this protein could coordinate gene expression between the two organelles.

Translation Initiation and Disease

Currently, two genes have been associated to defects in translation initiation. One candidate gene is *LRPPRC*, which is implicated in the mitochondrial disease Leigh Syndrome of the French-Canadian type [107]. The C1119T transition of *LRPPRC* causes an A354V missense mutation, resulting in significantly lower levels of the LRPPRC protein in mitochondria. It is not clear whether import of the mutant protein is affected, or if the protein is degraded after import. The mutation A354V is associated with reduced levels of *COX1* and *COX3* mRNA transcripts, [109], and could affect the *COX1* mRNA translation initiation, as the yeast counterpart does [107].

Another candidate factor is IF3$_{mt}$, which was found to interact with the mitochondrial serine-threonine kinase PINK1. Mutations in the later protein cause the early-onset autosomal recessive PARK6 variant of Parkinson's disease [110]. The *MTIF3* gene, which codes for IF3$_{mt}$, was shown

to present an allelic association with Parkinson's disease. Since an altered function of IF3$_{mt}$ may affect the availability of mitochondrial encoded proteins, it was proposed as a possible link between mitochondrial protein synthesis, oxidative stress and an increased vulnerability for Parkinson's disease [111].

Translation Elongation

Mammalian mitochondrial translation elongation requires three factors: EF-Tu$_{mt}$ [112-114], EF-Ts$_{mt}$ [115] and EF-G$_{mt}$ [116]. Prior to ribosome binding, EF-Tu$_{mt}$ forms a complex with the incoming aminoacyl-tRNA in a GTP dependent manner. After codon-anticodon interaction in the A-site and hydrolysis of GTP, EF-Tu$_{mt}$ is released from the ribosome [117]. EF-Ts$_{mt}$ works as a guanine exchange factor during the functional recycling of EF-Tu$_{mt}$ [118]. EF-Ts$_{mt}$ forms a stable complex with EF-Tu$_{mt}$ to promote release of GDP from EF-Tu$_{mt}$. The complex formed by EF-Tu$_{mt}$ and EF-Ts$_{mt}$ dissociates upon binding of a new molecule of GTP and aminoacyl-tRNA to EF-Tu$_{mt}$ [117]. The crystal structure of the bovine EF-Tu-Ts$_{mt}$ complex suggests that the interaction between EF-Tu$_{mt}$ and EF-Ts$_{mt}$ results in disruption of the Mg^{2+} binding site, which lowers the affinity of EF-Tu$_{mt}$ for guanine nucleotides [119]. EF-Tu$_{mt}$ has been identified in *S. cerevisiae* [120, 121]. While EF-Ts$_{mt}$ is absent in this organism, in the yeast *S. pombe*, both EF-Tu$_{mt}$ and EF-Ts$_{mt}$ are present [122], as it is the case for mammalian elongation factors. However, *S. cerevisiae* EF-Tu$_{mt}$ has been found to be functionally equivalent to the *S. pombe* EF-Tu/EF-Ts$_{mt}$ couple [122]. It was also suggested that the GTPase activity of *S. cerevisiae* EF-Tu$_{mt}$ is independent of an exchange factor and therefore it would not require EF-Ts$_{mt}$ [122].

EF-Tu$_{mt}$ binds to the mitochondrial inner membrane independently of the presence of mitochondrial ribosomes. In addition to the role in translation elongation, bovine EF-Tu$_{mt}$ was found to carry chaperone properties [123]: *In vitro*, it protects proteins from thermal aggregation and promotes refolding of denatured proteins. It might be involved in mediating degradation of misfolded, newly synthesized mitochondrial proteins [123]. The chaperone activity has also been documented for bacteria, chloroplasts and cytosolic EF-Tu (for examples see [124-126]).

Mitochondrial EF-G$_{mt}$ was first characterized in bovine [116]. There are two forms of this factor, EF-G1$_{mt}$ and EF-G2$_{mt}$, which are coded by two different genes, from yeast to mammals [79, 127]. Both genes have strong homology to bacterial EF-G. The prokaryotic EF-G catalyzes the translocation step, during which the A- and P-site tRNAs move to the P and E sites of the elongating ribosome, and mRNA is advanced by one codon (Nierhaus 1996; Caldas *et al.* 2000; Rodnina *et al.* 2000). Bacterial EF-G has also been suggested to display chaperone activities, with a possible function in protein folding and protection from stress [128]; however this role has not been established yet for mitochondrial EF-G$_{mt}$.

Mutations in the human mitochondrial elongation factors manifest in a variety of symptoms that have been reported in a few case-reports. A homozygous C997T mutation in *TSFM*, the gene coding for the mitochondrial translation elongation factor EF-Ts$_{mt}$, is associated with encephalo-

myopathy and hypertrophic cardiomyopathy [129]. This mutation predicts a R333W substitution that is likely to disrupt the EF-Tu-EF-Ts$_{mt}$ complex. A patient with severe infantile macrocystic leukodystrophy with micropolygyria had the mutation R339Q in EF-Tu$_{mt}$ [130]. Mutations in EF-G1$_{mt}$ are the cause of *i)* progressive hepatoencephalopathy due to the substitution N174S affecting a highly conserved residue of the GTP–binding domain [131], *ii)* fatal hepatopathy, due to heterozygous mutations leading to a protein with the mutation S321P, as well as a product with a premature stop at amino acid 607 [132], and *iii)* early-onset Leigh syndrome, with allelic mutations that produce a stop codon at position 47 and a M496R substitution [130].

Translation Termination

The human mitochondrial genetic code employs four termination codons [16]: AGA and AGG (these two codons conventionally would encode arginine), UAA and UAG. Once the translation complex has reached the termination codon, the completed protein must be dissociated from the final tRNA, ribosome, and its mRNA. The proteins responsible for fulfilling these functions are the release factors (RFs). Recently, the mitochondrial release factor mtRF1a was found to decode the stop codons UAA and UAG [133]. *In silico* analyses identified an additional candidate for a release factor protein, mtRF1 [134]; however, *in vitro* experiments did not show a release activity [133]. The lack of activity could be due to the use of heterologous bacterial ribosomes rather than 55S mitoribosomes. The putative mtRF1 could decode the stop codons AGG and AGA *in vivo* [133].

Two termination factors have been identified in *S. cerevisiae* mitochondria: mRF1p and Rrf1p. mRF1p is responsible for stop codon recognition and peptide release [135, 136], whereas Rrf1p is the mitoribosome recycling factor [137]. Rrf1p promotes the dissociation of the tRNA, mRNA and the ribosome, so the components are available for another round of translation.

3. HOW MITOCHONDRIAL TRANSLATION IS COUPLED TO INSERTION OF NEWLY MADE PROTEINS?

Although mitochondria evolved from bacteria, there are several aspects that have changed since the organelle originated. In the case of the proteins delivered to the inner membrane, almost the entire bacterial mechanism of recognition and targeting of membrane proteins seems to be replaced in mammalian and yeast mitochondria. Since most, if not all, mitochondrial encoded proteins are highly hydrophobic, there is no need for a system that distinguishes between soluble and membrane proteins. Accordingly, all the components of the SRP and Sec translocon are absent from mammalian and yeast mitochondria [138, 139], favoring the hypothesis that the translation mechanism in mitochondria is specialized to deal exclusively with membrane proteins. The translation machinery is normally associated with the mitochondrial inner membrane, allowing synthesis of proteins and their insertion to occur in a simultaneous and coordinated manner [3, 4, 139, 140]. Most of our current knowledge in the field of membrane insertion of mitochondrial encoded proteins comes from studies made

in the yeast *S. cerevisiae*, therefore, we will describe the findings made in this microorganism and describe what has been observed in the case of human mitochondria.

Oxa1p: A Ribosome Anchor that Works as an Insertase

One of the first factors involved in inner membrane protein insertion that was characterized was the insertase Oxa1p [141, 142]. This protein belongs to the conserved family of proteins called Alb3/Oxa1/YidC, which includes homologs present in chloroplasts, mitochondria and bacteria respectively [143, 144]. It is the only known component of the inner membrane insertion machinery that has been conserved during evolution in mitochondria from all organisms studied to date. Oxa1p was originally identified as a component required for the biogenesis of the respiratory chain complexes, in particular, for the cytochrome *c* oxidase (thus it was named Oxa1p for *oxidase assembly*) [141, 142]. This protein forms a homo-oligomeric complex that facilitates the integration of mitochondrial encoded proteins into the inner membrane, as well as of some nuclear encoded proteins that are encoded in the nuclear genome, imported into the mitochondrial matrix and finally reach the inner membrane in an export-like step [145-148]. Oxa1p spans the mitochondrial inner membrane five times, exposing its N-terminus to the intermembrane space and its long C-terminus to the matrix [149]. It can be divided structurally into two functional domains: *i)* the membrane core that constitutes the insertase section of the protein; and *ii)* the C-terminus predicted to form a coiled coil structure that possess the ability to bind mitochondrial ribosomes [5, 6, 150].

The role of Oxa1p during the insertion of mitochondrial encoded proteins can be clearly monitored for the subunits of the cytochrome *c* oxidase that are encoded by the mitochondrial genome (Cox1p, Cox2p and Cox3p, as well as for the cytochrome *b* (the core subunit of the *bc₁* complex). The interaction between Oxa1p and the newly synthesized Cox1p, Cox2p and Cox3p is very transient and occurs essentially during the insertion process [147]. Complex I is completely absent in the yeast *S. cerevisiae* where most of the studies addressing the function of Oxa1p have been performed. However, in the filamentous fungus *Neurospora crassa* the depletion of Oxa1p produced a decrease on the steady state levels of Complex I, suggesting that it also plays a role during the biogenesis of this complex [148].

The C-terminal domain of Oxa1p is predicted to form an α-helical domain that has the ability to bind to mitochondrial ribosomes. The deletion of this ribosome binding domain (RBD) prevents the interaction of the ribosomes with the insertase region of Oxa1p leading to the release of the translation products from the mitoribosomes, into the matrix and their subsequent accumulation within that subcompartment of the organelle [5, 6]. Although the precise site of interaction is currently unknown, Oxa1p can be cross-linked to Mrp20p, a component of the large ribosomal subunit. Mrp20p is the homolog subunit to the bacterial L23, which is located next to the peptide exit tunnel of the ribosome [5]. The interaction between Oxa1p and translating ribosomes, constitutes one of the crucial steps during the co-translational insertion of mitochondrial translation products by facilitating the interaction of the newly synthesized proteins with the insertion machinery.

The role of Oxa1p during the biogenesis of the OXPHOS complexes is not limited to the insertion of some of their core subunits, but it is also involved in their further assembly. In the case of the F_1F_0-ATP synthase, Oxa1p is not strictly required for the insertion of the three subunits that are mitochondrial encoded (Atp6p, Atp8p and Atp9p) [151]. Rather, Oxa1p directly interacts with newly synthesized Atp9p after its insertion and mediates the assembly of its oligomeric form into the functional F_1F_0-ATP synthase. This role during the assembly is not dependent on the C-terminus, and occurs after the insertion of the proteins [152].

Since the function of Oxa1p in yeast has a significant impact on the assembly of functional OXPHOS complexes Coenen *et al* reasoned that patients where the activity of more than one OXPHOS complex is compromised could carry mutations in Oxa1L. In order to address this, they analyzed fibroblasts isolated from patients with combined deficiencies in complex I and IV [153]. In this study no mutations in Oxa1L were associated with the observed phenotypes. Yet, since this study was reduced to the analysis of few patients, it is not possible to discard the possibility that cases where the gene Oxa1L contains pathogenic mutations would be found in the future. Alternatively, since mutations in the human Oxa1L would presumably produce severe alterations in several OXPHOS complexes simultaneously, such severe alterations could result in lethal effects in humans preventing embryonic development.

There is little experimental evidence about the function of Oxa1L in mammalian cells. In a recent report, a knock down of the Oxa1L gene in HEK293 cells affected the biogenesis of the NADH:Ubiquinone oxidoreductase (Complex I) of which the steady state levels were decreased. In this study, it was also observed that both the steady state levels and the activity of the F_1F_0-ATP synthase were strongly affected. Intriguingly, in contrast with what is observed in yeast cells, the absence of Oxa1L did not compromise the assembly and/or activity of the cytochrome *c* oxidase [154].

Mba1p: A Membrane Associated Ribosome Receptor

Mba1p is a membrane associated protein located in the mitochondrial inner membrane facing the matrix. This protein belongs to a high molecular weight complex whose components have not yet been identified [155, 156]. In a similar way than Oxa1p, Mba1p interacts transiently with mitochondrial translation products, in particular with the subunits of the cytochrome *c* oxidase Cox1p, Cox2p and Cox3p, at early steps of their synthesis [155]. Mba1p binds to the large subunit of the mitochondrial ribosomes independently of the protein synthesis process or of the presence of Oxa1p [7]. Mba1p overlaps in function and substrate specificity with Oxa1p. However, they seem to belong to independent export machineries, since the combined absence of Mba1p and the C-terminal RBD of Oxa1p impairs almost completely the insertion of the three subunits of the cytochrome *c* oxidase as well as the cytochrome *b* [7]. Mba1p and Oxa1p seem to coordinate the co-translational membrane insertion specially by positioning of the ribosome and its exit tunnel to the proximity of the place where the actual insertion process occurs [7].

Although Mba1p clearly plays an important role during the insertion of inner membrane proteins in mitochondria of yeast, it is a protein that seems to be conserved only in fungi. However, database searches identified the ribosomal protein L45 (Mrpl45) as a related protein [7]. Mrpl45 was found as a protein present in purifications of mitochondrial ribosomes in animals, and it was observed to be missing in fungi [157]. And although it has been proposed that these two proteins are functional homologs, experimental evidence supporting this idea is still missing [7].

Mdm38p and Ylh47p

In yeast, two additional homolog proteins have been identified which form stable complexes with mitochondrial ribosomes: Mdm38p and Ylh47p [8]. An *mdm38Δ* strain shows a disrupted mitochondrial network, an apparent swelling of the mitochondria [158], and carries defects in K^+ homeostasis [159]. Recently, it was shown that mitochondria from an *mdm38Δ* strain exhibit a severe reduction in the amounts of cytochrome *b* and Atp6p [8]. This was also reflected in a strong growth defect observed in non-fermentable carbon sources. In the same study, Mdm38p was shown to interact with mitochondrial nascent chains. Consequently it was proposed to function as a component of the mitochondrial export machinery in an Oxa1p-independent pathway [8]. In addition, Mdm38p has been proposed to work as an essential component of the K^+/H^+ exchange system, and the changes observed in the mitochondrial encoded proteins were attributed to be a secondary effect [159, 160]. The role that Ylh47p plays is even more conspicuous than the one of Mdm38p: in the yeast deletion strain, the protein insertion is almost not affected and there is no growth defect observed on non-fermentable carbon sources [8].

The human homolog of Mdm38p and Ylh47p is called LETM1 [159, 161]. It has been identified as one of the possible candidates responsible for the Wolf-Hirschhorn syndrome (WHS, OMIM 194190), a complex malformation syndrome caused by the deletion of parts of the distal short arm of chromosome 4 [162, 163]. The core characteristics of WHS are growth retardation, microcephaly and mental retardation, epilepsy and cranio-facial dysgenesis. In human mitochondria, *LETM1* is located in the inner membrane, exposed to the matrix, and oligomerized in high molecular weight complexes of unknown composition [158]. Down-regulation of *LETM1* led to fragmentation of the mitochondrial network but was not associated with changes in the levels of respiratory chain complexes. Furthermore, the observed fragmentation was recovered by the ionophore nigericin, which catalyzes the electroneutral exchange of K^+ against H^+. The analysis of fibroblasts obtained from WHS patients did not resemble the results observed in the cell line where *LETM1* was depleted, therefore the possible role of mutations within this gene during the development of WHS remains to be clarified [158].

Assembly Factors for the OXPHOS Complexes

Mitochondrial proteomes include much more proteins than those that are encoded in the mitochondrial genome and are translated within the matrix. In *S. cerevisiae* there are between 700 and 800 proteins [164] and in mammalian

Table 3.   Assembly Factors of the Mitochondrial OXPHOS Complexes Known to be Linked to Human Disorders

Protein name in yeast	OXPHOS complex	Known function in yeast or human	Human homolog	Linked disorders	References
-	I	Assembly chaperone	CIA30	Cardioencephalomyopathy	[201, 202]
-	I	Assembly chaperone	B17.2	Progressive encephalopathy	[200, 201]
Bcs1p	III	ATP dependent chaperone for a pre-Complex III until the assembly of Rieske-FeS protein occurs	BCS1L	Tubulopathy, hepatic involvement and encephalopathy; GRACILE syndrome; Visceral and neurological involvement with lactic acidosis; Björnstad syndrome	[17], 194-199]
Cox10p	IV	Heme A biosynthesis	COX10	Ataxia, tubulopathy and Leukodystrophy; Leigh syndrome; Anemia, sensorineural hearing loss, fatal infantile hypertrophic cardiomyopathy	[190-193]
Cox15p	IV	Heme A biosynthesis	COX15	Leigh syndrome; Fatal infantile hypertrophic cardiomyopathy	[187-189]
Sco1p	IV	Copper metallation of the CuA site in Cox2	SCO1	Hepatic failure and encephalopathy	[182-186]
Sco2p	IV	Copper metabolism and insertion	SCO2	Hypertrophic cardiomyopathy and encephalopathy	[181]
Shy1p	IV	Assembly factor	SURF1	Leigh syndrome	[179, 180]
Atp12p	V	Formation of dimmers of subunits alpha and beta	ATPAF2	Resembling COFS syndrome	[177, 178]

mitochondria this number increases to 1,500 polypeptides [165]. The vast majority of the mitochondrial proteins are encoded in the nuclear genome, synthesized in the cytosol, and transported post-translationally to the organelle. As a consequence, functional mitochondria are the result of a tightly regulated expression, targeting and assembly of both nuclear and mitochondrial encoded proteins. The mechanisms used by the cytosolic proteins to reach their correct destinations within mitochondria have been studied intensively for a few decades (for reviews see [166-168]). After the sorting has occurred, the proteins should fold and incorporate into their functional complexes. This process occurs by the help of assembly factors, a general group of chaperones that do not belong to the functional complex but are necessary throughout their construction. Each one of the OXPHOS complexes has a very precise course of assembly (for reviews see [169-172]). This implies that even when each of the subunits of one complex are synthesized and inserted correctly, further steps on the assembly line of the complex could occur improperly and result also in some disorder of mitochondrial origin. Table 3 summarizes the assembly factors that have been identified in patients, the disorder linked to them and their identified molecular function.

4. CONCLUDING REMARKS

The correct development of any aerobic organism strongly depends on the mitochondrial OXPHOS. To achieve this, the machineries involved in protein synthesis within the organelle, protein insertion into the inner membrane, and

functional assembly of each of the OXPHOS complexes have to be tightly coordinated. Until now, we owe most of our understanding on these mechanisms on studies made in the model organism *S. cerevisiae*. However the understanding of the mechanisms of human mitochondrial translation and assembly remains far from complete. Further studies in human cell lines, the identification of more patients, and the availability of more efficient protocols for genome sequencing, will increase our knowledge about the mutations that are associated with disorders of mitochondrial origin, in particular as a result of problems with protein synthesis, membrane insertion, and assembly of the OXPHOS complexes.

For example, it will be exciting to explore the mechanisms by which the mitoribosomes locate the translation initiation site within mitochondrial mRNAs, as well as how the mitoribosomes are recycled [173]. Moreover, in the yeast *S. cerevisiae*, mitochondria with reduced or imbalanced translation capacity exhibit increased ROS production, with an impact on chronological lifespan. This establishes a link between ageing and the control of mitochondrial translation [174]. It will be interesting to see whether perturbations in the mitochondrial gene expression, particularly at the level of translation are associated with differences in aging and lifespan in higher organisms [175].

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ABBREVIATIONS

5'-UTR	=	5' Untranslated regions
DFNA	=	Autosomal dominant non-syndromic hearing loss
DFNB	=	Autosomal recessive non-syndromic hearing loss
HSP	=	Hereditary spastic paraplegia
LBSL	=	Leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation
MMD	=	Multiple mitochondrial dysfunctions syndrome
MELAS	=	Myopathy, encephalopathy, lactic acidosis and stroke-like episodes
MERRF	=	Myoclonic epilepsy associated with ragged-red fibers
MLASA	=	Mitochondrial myopathy, lactic acidosis and sideroblastic anemia
MRPs	=	Mitochondrial ribosomal proteins
mtDNA	=	Mitochondrial DNA
OXPHOS	=	Oxidative phosphorylation
PPR	=	Pentatricopeptide repeat
RBD	=	Ribosome binding domain

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The Pentatricopeptide Repeats Present in Pet309 Are Necessary for Translation but Not for Stability of the Mitochondrial *COX1* mRNA in Yeast[*]

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Pet309 is a protein essential for respiratory growth. It is involved in translation of the yeast mitochondrial *COX1* gene, which encodes subunit 1 of the cytochrome *c* oxidase. Pet309 is also involved in stabilization of the *COX1* mRNA. Mutations in a similar human protein, Lrp130, are associated with Leigh syndrome, where cytochrome *c* oxidase activity is affected. The sequence of Pet309 reveals the presence of at least seven pentatricopeptide repeats (PPRs) located in tandem in the central portion of the protein. Proteins containing PPR motifs are present in mitochondria and chloroplasts and are in general involved in RNA metabolism. Despite the increasing number of proteins from this family found to play essential roles in mitochondria and chloroplasts, little is understood about the mechanism of action of the PPR domains present in these proteins. In a series of *in vivo* analyses we constructed a *pet309* mutant lacking the PPR motifs. Although the stability of the *COX1* mRNA was not affected, synthesis of Cox1 was abolished. The deletion of one PPR motif at a time showed that all the PPR motifs are required for *COX1* mRNA translation and respiratory growth. Mutations of basic residues in PPR3 caused reduced respiratory growth. According to a molecular model, these residues are facing a central cavity that could be involved in mRNA-binding activity, forming a possible path for this molecule on Pet309. Our results show that the RNA metabolism function of Pet309 is found in at least two separate domains of the protein.

Biogenesis of the mitochondrial cytochrome *c* oxidase (COX)[2] complex depends on a large set of proteins. In the yeast *Saccharomyces cerevisiae* more than 20 nuclear genes have been found to be necessary for assembly and maintenance of the functional COX (1–3). The enzyme in mammals and yeast is composed of 13 and 12 subunits, respectively. The core of the enzyme is formed by subunits Cox1, Cox2, and Cox3, which are encoded in the mitochondrial DNA. Expression of the mitochondrial-encoded subunits is highly regulated by proteins involved in transcription, transcript stability and processing, translation, and assembly into the mitochondrial inner membrane (3–5).

In humans, deficiency in COX assembly is associated with mitochondrial disorders. The majority of these are caused by autosomal recessive mutations that affect COX assembly factors (6, 7). An example of such a factor is the mRNA-binding protein Lrp130 (8, 9). Mutations in the *LRP130* gene have been associated with the neurodegenerative disorder Leigh syndrome of the French Canadian type (10). These patients lack fully functional COX activity, associated with defects in the *COX1* and *COX3* transcripts (9).

It has been proposed that *PET309* is the yeast homologue of *LRP130* (10), with 37% of similarity over 300 amino acids. Both genes seem to participate in mRNA processing and may have similar functions in mitochondria. Pet309 is a translational activator necessary for Cox1 synthesis. It specifically acts on the 5'-UTR of the *COX1* mRNA to activate translation. In addition, it is required to stabilize the pre-*COX1* transcript (11). It has been observed that translational activators specific for the *COX1*, *COX2*, and *COX3* mRNAs interact with each other and with the mitochondrial inner membrane (12–15), suggesting that the activators promote that translation initiation takes place close to the insertion and assembly sites of the three COX subunits in the mitochondrial inner membrane (15, 16).

Both Lrp130 and Pet309 contain several pentatricopeptide repeats (PPRs). These repeats belong to a protein family that is very large in plants, with at least 442 members in *Arabidopsis thaliana*. However, there are fewer examples of these proteins in fungi, animals, and protists (17, 18). Pet309 is the only yeast translational activator that has been found to contain PPR motifs. In general, PPR proteins are usually found to localize in mitochondria and chloroplasts. It is known from the small set of PPR proteins studied to date that they participate mostly in different steps of sequence-specific RNA metabolism. They are implicated in precursor transcript stability and processing (which includes splicing and editing) (19–23), as well as in translation (11, 19, 24, 25). However, in a few examples specific RNA-binding activity or their natural RNA targets has been demonstrated (21, 26). These proteins play essential roles in plant embryogenesis, cytoplasmic male sterility restoration,

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TABLE 1

S. cerevisiae strains used in this study

Strain name	Nuclear (mitochondrial) genotype	Reference
XPM2s2	*Matα ura3-52 leu2-3,112 lys2 arg8::hisG pet309Δ::LEU2 (ρ+, ΔΣai)*	This study
XPM2s1	*Matα ura3-52 leu2-3,112 lys2 arg8::hisG pet309Δ::LEU2 (ρ+, cox1Δ::ARG8m)*	This study
XPM201	*Matα ura3-52 leu2-3,112 lys2 arg8::hisG (ρ+, ΔΣai)*	This study
XPM10b	*Matα ura3-52 leu2-3,112 lys2 arg8::hisG (ρ+, cox1Δ::ARG8m)*	52
SB5	*Matα ura3Δ ade2 PET309::3xHA (ρ+)*	S. A. Broadley

and chloroplast to nucleus retrograde signaling (for examples see Refs. 17, 27, 28).

PPRs are degenerated 35-amino acid motifs proposed to consist of two antiparallel α helices. There is no structural information about PPR proteins, but models based on the closely related TPR (tetratricopeptide repeat) proteins suggest that the tandem repeats of these domains form a solenoid-like structure with a hydrophilic cavity where the phosphate skeletons of RNA might interact (29).

Despite the growing number of PPR proteins discovered and characterized to date, very little is understood about the specific role of the PPR motifs present in these proteins. Yeast Pet309 provides a useful model of a PPR protein to elucidate the mechanism of action of the PPR motifs. Pet309 is predicted to contain at least seven PPR motifs located in the central portion of the protein. To test the function of the repeats present in Pet309, a set of deletions of the PPR motifs was constructed and analyzed. A model of the Pet309 PPR region was generated, and site-directed mutagenesis was carried out on residues that are predicted to be necessary for mRNA binding. It was shown that all the seven PPR repeats present in Pet309 are necessary for *COX1* mRNA translation, and that mutation of basic residues that could be facing the inner cavity of the PPR structure decrease Pet309 activity. Surprisingly, the *COX1* mRNA levels were not affected by the PPR deletions, showing that the mRNA stability function of Pet309 is independent of the PPR domains.

EXPERIMENTAL PROCEDURES

Strains, Media, and Genetic Methods—The *S. cerevisiae* strains used in this study are listed in Table 1. All strains are derived from strain D273-10B. Genetic manipulation and standard media recipes were as previously described (30). Yeast were cultured in complete fermentable media (1% yeast extract, 2% Bacto-peptone) or synthetic complete media (0.67% yeast nitrogen base, supplemented with the appropriate amino acids), containing 2% glucose, 2% raffinose or 3% ethanol-3% glycerol. The *pet309Δ::LEU2* deletion construct was obtained by PCR. Strains XPM201 and XPM10b were transformed with the PCR product (31), and correct integration of the *pet309Δ::LEU2* construct was confirmed by PCR.

Plasmid Constructs—Total DNA from the strain SB5 was used to amplify the PET309::HA sequence, including 310 and 205 bp of the PET309 5′- and 3′-UTR, respectively. This product was ligated into XbaI-XhoI sites of pBluescript to generate plasmid pXP96. In addition, the product was subcloned into XbaI-XhoI sites of yeast expression vectors pRS416 (32) and YEp352 (33) to generate pXP97 and pXP104, respectively. All pet309 mutant sequences were generated by fusion PCR (34), using Accuzyme DNA polymerase (Bioline) and pXP96 as the DNA template. The PCR products obtained from the PPR

region of *pet309* were ligated into PstI-EcoRI pXP96. After sequencing the constructs the complete *pet309* genes were subcloned into XbaI-XhoI pXP97 or pXP104 to generate yeast expression plasmids.

Analysis of Mitochondrial Proteins—Mitochondria were isolated from late logarithmic phase cells grown on synthetic complete media without uracil, containing 2% raffinose. Crude mitochondria were isolated and purified by centrifugation on 5–25% Nycodenz gradients (35).

Mitochondria separation into membrane and soluble fractions, and alkaline carbonate extractions of membranes were as described (36–38). Mitoplasting and proteinase K treatment were carried out as previously described (38). Total cellular extracts were isolated from cells grown to mid-log phase on synthetic complete media without uracil, containing 2% raffinose (39).

Proteins were separated by SDS-PAGE on a 12.5% gel (40). Western blots were probed with anti-HA-horseradish peroxidase (Roche Biochemicals), anti-Cox1 (Molecular probes), anti-citrate synthase, anti-Arg8p, anti-Yme1p (the three provided by T. D. Fox), anti-cytochrome *c1* (provided by D. González-Halphen) or anti-glucose-6-phosphate dehydrogenase (Sigma) antibodies. Secondary goat anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (Sigma or Bio-Rad) was detected with the ECL or ECL+ kits (GE Healthcare).

Synthesis of Mitochondrial Proteins—Translation in isolated mitochondria in the presence of [^{35}S]methionine was performed as described (41). After translation, mitochondria were washed with 0.6 M sorbitol, 20 mM HEPES, pH 7.4, and the radiolabeled proteins were separated on a SDS-PAGE gel, blotted into protran nitrocellulose membrane, and analyzed with a Typhoon 8600 PhosphorImager (Amersham Biosciences).

Northern Blot Analyses—Total RNA was prepared using the TRIzol reagent (Invitrogen) from yeast cultures grown to late log phase on raffinose-synthetic complete media lacking uracil. RNA was blotted to Hybond XL membrane (GE Healthcare). Blots were probed sequentially with the radioactively labeled *COX1* exon 4, *COX2* and with the 15S rRNA gene (42) to standardize the loading. Blots were analyzed with a Typhoon 8600 PhosphorImager and quantitated with ImageQuaNT 5.1 software.

Modeling for the PPR Region in Pet309—A search using the TPRpred server (43) against the whole Pet309 sequence revealed the presence of 11 putative PPR motifs located between residues 312 and 759. The six motifs with the lowest *p* value (~1e-07-1e-06, which indicates 1×10^{-7} to 1×10^{-6}) correspond to one segment of the protein comprising residues 347–560. Using this fragment of the sequence, the HHpred server (44) revealed an alignment with the six tandem TPRs of

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the crystal structure of PilF (45) (PDB code 2ho1) from *Pseudomonas aeruginosa*, with 10% identity extending over 213 residues. Only five residue insertions of one residue each occur in the alignment, all at the level of the junctions between the repeats. The three-dimensional model of the PPR repeats was constructed using SWISSMODEL (46) alignment interface mode. Fig. 6 was created with PyMOL, and the electrostatic potentials were calculated with the program APBS (47).

RESULTS

The PPR Region of Pet309 Is Necessary for Respiratory Growth—A comparison of the Pet309 sequence against the TPRpred server reveals the presence of 11 PPR motifs in the central portion of the protein. To understand the role of the PPR domain we created a deletion from residues 347 to 632 (*pet309Δppr*), which corresponds to the 7 most strongly predicted PPR motifs (having the lowest *p* values) (Fig. 1*A*). To facilitate detection of Pet309, the protein was tagged at its C terminus with three tandem copies of an HA epitope. The presence of the triple-epitope in wild-type Pet309 did not interfere with the respiratory growth of cells as judged by the ability of *PET309-HA* to fully complement the *pet*⁻ phenotype of a *pet309Δ::LEU2* mutant. Both the wild-type *PET309-HA* and the *pet309Δppr-HA* genes were cloned in the vectors pRS416 and YEp352 to allow expression in yeast in low copy or multiple copy plasmids, respectively. The plasmids were transformed into a yeast *pet309Δ::LEU2* mutant, and the respiratory growth of the resulting strains was examined (Fig. 1*B*). The wild-type *PET309-HA* supported normal growth on non-fermentable carbon sources, whereas the *pet309Δppr-HA* strain could not grow on a non-fermentable carbon source. A similar phenotype was observed in cells expressing the single copy or multiple copy expression plasmids, suggesting that overexpression of the mutant protein did not compensate for the absence of the PPR domain of Pet309.

To investigate the basis of the non-respiratory phenotype of the *pet309Δppr-HA* strain we first looked to see if the mutant protein was localized in mitochondria. Mitochondrial and post-mitochondrial supernatant fractions were obtained from strains transformed with the low copy and high copy plasmids bearing the wild-type *PET309-HA* or the mutant *pet309Δppr-HA* genes (Fig. 2*A*). The protein Pet309 was specifically recognized by the anti-HA antibody as a 118-kDa band for the wild-type *PET309-HA* or 85-kDa band for the *pet309Δppr-HA* strain. These polypeptides were not detectable in mitochondria bearing the untagged *PET309* gene or the empty plasmids
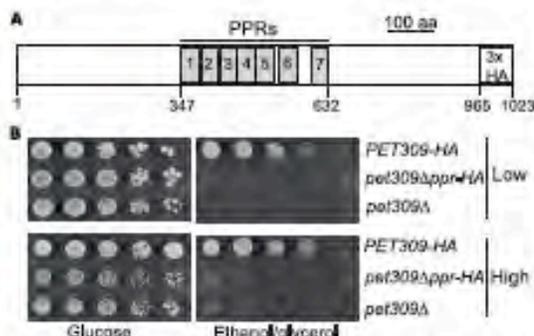
FIGURE 1. **The PPR motifs present in Pet309 are necessary for respiratory growth.** *A*, diagram showing the seven predicted PPR motifs (*gray boxes*) to be present in the central region of Pet309. Residue numbers are as indicated. *B*, the strain XPM232 was transformed with low copy number plasmids (*Low*) or high copy plasmids (*High*) bearing the wild-type *PET309-HA* gene, the *pet309Δppr-HA* gene, or empty vector (*pet309Δ*). 10-fold serial dilutions of the transformants were spotted on synthetic complete medium lacking uracil with either glucose or ethanol/glycerol, and incubated for 4 days at 30 °C.
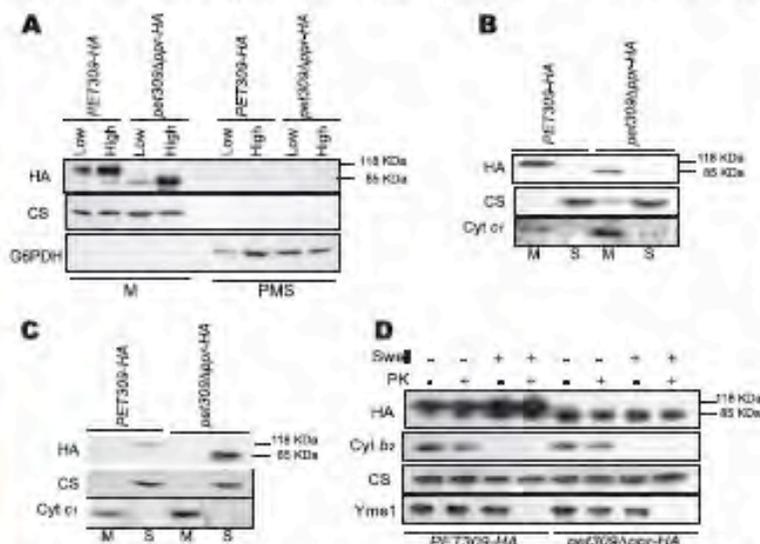
FIGURE 2. **Localization of the Pet309Δppr-HA protein in mitochondria.** Mitochondria were prepared from strains expressing the wild-type *PET309-HA* or the *pet309Δppr-HA* constructs, in a low copy (*Low*) or high copy plasmid (*High*). *A*, 10 μg of protein from mitochondria (*M*) and post-mitochondrial supernatant (*PMS*) were analyzed by SDS-PAGE, and the Western blot was probed with the antibodies anti-HA to detect Pet309, anti-citrate synthase (*CS*) as marker for a mitochondrial protein and glucose-6-phosphate dehydrogenase as marker for a cytosolic protein. *B*, 100 μg of mitochondria from strains bearing low copy plasmids were sonicated and separated into a membrane pellet (*M*) and a soluble supernatant (*S*) by centrifugation. The Western blot was decorated with the antibodies anti-HA, anti-CS as a soluble protein marker and anti-cytochrome *c*, (*Cyt c,*) as an integral membrane protein marker. *C*, 100 μg of mitochondria from strains expressing the *pet309-HA* alleles on low copy plasmids were subjected to alkaline Na₂CO₃ extraction. Integral membrane pellets (*M*) were separated from solubilized proteins (*S*) by centrifugation. After SDS-PAGE, the Western blot was probed with the indicated antibodies. *D*, 100 μg of mitochondria from strains bearing low copy plasmids were converted to mitoplasts by osmotic shock in the absence or presence of proteinase K (*PK*) (100 μg/ml). Samples were resolved by SDS-PAGE, immunoblotted, and probed with anti-HA, anti-cytochrome *b*, (*Cyt b,*) as an intermembrane space marker, anti-CS as a matrix marker and anti-Yme1, which is an inner membrane protein with a large domain facing the intermembrane space.

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(data not shown). Greater accumulation of the Pet309 polypeptides was observed under multiple copy expression. These polypeptides were not detectable in the post-mitochondrial supernatant fractions, indicating that the Pet309Δppr-HA protein co-purified with mitochondria.

Next, we investigated whether the Pet309Δppr-HA protein was membrane-bound or soluble. Mitochondria from strains bearing the *PET309-HA* or the *pet309Δppr-HA*, low copy plasmids were sonicated and centrifuged. Both the wild-type Pet309-HA and the mutant Pet309Δppr-HA proteins were present in the membrane pellet (Fig. 2*B*) and absent from the soluble supernatant. Alkaline Na₂CO₃ extraction of the mitochondrial membranes solubilized the wild-type Pet309-HA and the Pet309Δppr-HA proteins (Fig. 2*C*), indicating that both behave as peripheral membrane proteins.

To examine the submitochondrial location of the Pet309 proteins, purified mitochondria were converted to mitoplasts by osmotic shock treatment and were subjected to protease digestion. Both Pet309-HA and Pet309Δppr-HA proteins were protected from proteinase K treatment in mitochondria and in mitoplasts (Fig. 2*D*). This result indicates that both proteins are facing the matrix side of the inner membrane. These results are different from what was observed by Manthey (13), who reported that Pet309-c-Myc was an integral inner membrane protein. However, in that work, a high copy plasmid was used to overexpress the Pet309-c-Myc protein. Overexpression of translational activators has been associated with problems in mitochondrial gene expression (48, 49) and could affect their interaction with the mitochondrial inner membrane. For this reason we analyzed the Pet309-HA proteins expressed from low copy plasmids.

Taken together, these results indicate that the respiratory defect of *pet309Δppr-HA* mutants is not due to a mitochondrial mislocalization of the protein. The association of the mutant protein to the mitochondrial inner membrane and its submitochondrial localization were not altered by the absence of the PPR domains. These observations, together with the capacity of the mutated protein to stabilize the *COX1* mRNA (see below) strongly suggest that the Pet309Δppr-HA protein is not misfolded.

The PPR Motifs in Pet309 Are Required for Translation of the COX1 mRNA—Pet309 had been previously demonstrated to be necessary for the translation and stability of the *COX1* mRNA (11). We investigated the effect of the PPR domain deletion on expression of the *COX1* gene. Western blot analysis of mitochondrial protein extracts showed no accumulation of the Cox1 protein in the *pet309Δppr-HA* mutant (Fig. 3*A*). The mutant did not accumulate Cox1 even in *pet309Δppr-HA* high copy expression. Interestingly, overexpression of the wild-type Pet309-HA led to a substantial decrease in the Cox1 accumulation (3.5-fold). This observation is in agreement with the idea that overexpression of translational activators can lead to defects on the biogenesis of their target genes (49). Overexpression of Pet309 could lead to formation of inactive Pet309 aggregates that could affect accumulation of Cox1 (48).

To investigate the effect of the *pet309Δppr-HA* mutation on *COX1* translation, we first analyzed [³⁵S]methionine-labeled proteins from mitochondria carrying the *pet309Δppr-HA*
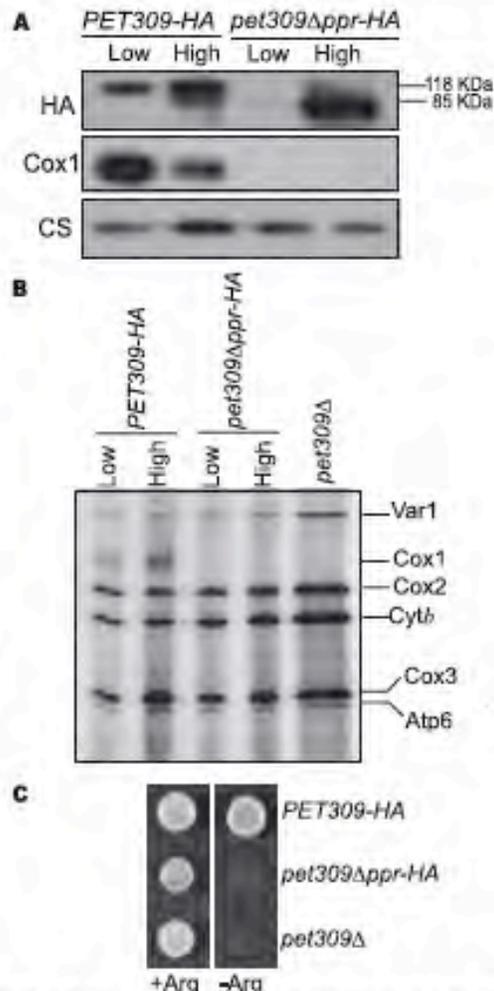
FIGURE 3. **The PPR domains present in Pet309 are required for COX1 mRNA translation.** *A*, mitochondria from strains expressing the wild-type PET309-HA, the pet309Δppr-HA, or empty vector (pet309Δ), in low copy (Low) or high copy plasmids (High) were separated by SDS-PAGE. Western blot was probed with antibodies anti-HA to detect Pet309, anti-Cox1, and anti-citrate synthase (CS) as loading control. *B*, translation in mitochondria (10 mg/ml) in the presence of [³⁵S]methionine was performed at 30 °C for 20 min. Translation products were analyzed as described under "Experimental Procedures." Cytochrome c oxidase subunit 1, Cox1; subunit 2, Cox2; subunit 3, Cox3; cytochrome b, Cytb; subunit 6 of ATPase, Atp6; ribosomal protein, Var1. *C*, growth phenotypes of strains carrying the cox1Δ::ARG8ᵐ mitochondrial gene. Cells from strain XPM231 bearing the high copy plasmids were grown on liquid minimal media lacking uracil and spotted on glucose minimal media lacking uracil (+Arg) or arginine (−Arg), and incubated for 3 days at 30 °C.

mutation in low copy or high copy expression plasmids (Fig. 3*B*). Labeling of Cox1 was reduced to undetectable levels by the *pet309Δppr-HA* mutation even in overexpression conditions. As expected, labeling of Cox1 in strains with the wild-type *PET309-HA* was normal, whereas a null mutation (*pet309Δ*) completely prevented Cox1 labeling. These results suggest that

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the PPR domain of Pet309 is necessary for the *COX1* mRNA translation. To corroborate this, we created a *pet309Δ::LEU2* strain in which the mitochondrial reporter gene *ARG8^m* replaced the *COX1* coding sequence (*cox1Δ::ARG8^m*). The *ARG8^m* product is a matrix-soluble protein involved in arginine biosynthesis (50). This reporter has been widely used to analyze translation of mitochondrial genes (49, 51–53). Translation of *cox1Δ::ARG8^m* has been demonstrated to be dependent upon Pet309 (52). In cells carrying the wild-type *PET309-HA*, the *cox1Δ::ARG8^m* gene supported growth in Arg– medium (Fig. 3C). In contrast, cells bearing the high copy or low copy (data not shown) *pet309Δppr-HA* gene required arginine to grow, confirming that the PPR domain present in Pet309 is necessary for the *COX1* mRNA translation.

The PPR Motifs in Pet309 Are Not Required for Stabilization of the COX1 mRNA—Pet309 is also involved in the *COX1* mRNA stability, as null mutants show a reduced accumulation of the mature *COX1* mRNA (11). This effect is particularly strong when the *COX1* gene has introns, but it is also observed with the intronless *COX1* gene (11). We analyzed whether deletion of the PPR repeats present in Pet309 could affect the *COX1* mRNA accumulation.

Levels of the *COX1* mRNA in cells bearing the intronless *COX1* gene were analyzed by Northern blot and normalized to the mitochondrial 15S rRNA (Fig. 4). In wild-type cells expressing the high copy *PET309-HA* gene, the *COX1* mRNA signal was increased 2-fold as compared with the low copy *PET309-HA* cells. A similar pattern was obtained for the *pet309Δppr-HA* cells. This effect was specific for *COX1*, because the *COX2* mRNA levels were not affected in any sample. It has been suggested that high levels of translational activators could stabilize their target mRNAs (49). This result indicates that Pet309 lacking the PPR repeats still has the capacity to stabilize the *COX1* mRNA. In contrast, the null *pet309* mutant showed a reduced accumulation of the *COX1* mRNA as compared with the *PET309-HA* or the *pet309Δppr-HA* cells.

We conclude that the PPR domains present in Pet309 are necessary for translation of the *COX1* mRNA. However, the absence of these repeats does not affect the *COX1* mRNA stability. Moreover, high expression of the pet309Δppr-HA protein caused accumulation of the *COX1* mRNA, as observed for the wild-type Pet309-HA protein.

Each One of the Seven PPR Repeats of Pet309 Is Necessary for Cox1 Synthesis—We next asked whether deletion of single PPR repeats could affect translation of the *COX1* mRNA. A *pet309Δ::LEU2* strain was transformed with high copy plasmids carrying single deletions of each PPR (*pet309Δppr₁₋₇-HA*). Western blot analysis of total cell extracts revealed the presence of a 114-kDa band whose migration in our SDS-PAGE system was indistinguishable from the wild-type Pet309-HA protein (Fig. 5A).

None of the seven PPR mutants were able to grow on the non-fermentable carbon source ethanol/glycerol (Fig. 5B), suggesting that translation of the *COX1* mRNA was affected. To evaluate this hypothesis we tested the Arg growth of cells carrying the *cox1Δ::ARG8^m* gene in the mitochondrial DNA (Fig. 5C). None of the seven mutants supported Arg growth in a media lacking arginine, indicating that each one of the PPR
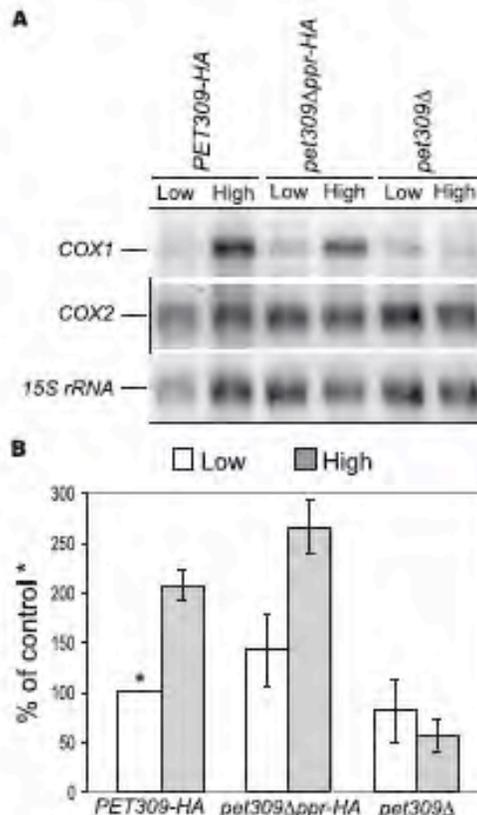
FIGURE 4. **The PPR region of Pet309 is not necessary to stabilize the COX1 mRNA.** *A*, 10 μg of total RNA from strains bearing the wild-type *PET309-HA*, the *pet309Δppr-HA* gene or empty vector (*pet309Δ*), in low copy (*Low*) or high copy plasmids (*High*) were analyzed by Northern blot hybridization and phosphorimaging. The blot was probed with the indicated probes. Cells carry the intronless *COX1* gene. *B*, quantification of the *COX1* signal normalized to the 15S rRNA signal was performed using the ImageQuaNT software. An asterisk indicates the 100% value of the control sample. Values are the mean of four independent experiments.

repeats present in Pet309 is necessary for *COX1* mRNA translation.

Similar Pet⁻ and Arg⁻ phenotypes were obtained with cells carrying the low copy plasmids (data not shown). Deletion of each PPR did not affect *COX1* mRNA levels, whereas overexpression of the mutant proteins led to increased accumulation of the *COX1* mRNA (data not shown). This strongly suggests that the translational activation and the mRNA stabilization activities of Pet309 are located on two different functional regions of the protein.

Mutagenesis of Basic Residues Inside the PPR Central Groove Affect the COX1 mRNA Translation—Based on similarities with the TPR motifs, PPR motifs are predicted to consist of two α helices (named A and B). Tandem PPR motifs are predicted to form a superhelix enclosing a groove, which is positively charged. This charge could be involved in nucleic acid binding. The side chains that face the inner groove are predicted to come

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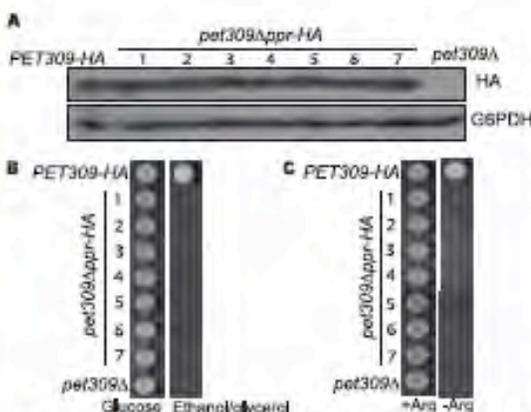
FIGURE 5. **Each PPR repeat present in Pet309 is necessary for translation of the COX1 mRNA.** Strains expressing the wild-type PET309-HA, the pet309Δppr, constructs bearing single deletions of each PPR, or empty plasmid (pet309Δ) were grown on synthetic complete liquid media lacking uracil. Strains bear the high copy plasmids. Each deleted repeat in Pet309 is indicated by numbers. A, total cell extracts were obtained. A sample of 50 μg of proteins was analyzed by SDS-PAGE and immunoblotting. Western blot was probed with anti-HA antibody to detect Pet309-HA and with anti-glucose-6-phosphate dehydrogenase antibody as loading control. B, cells were grown on liquid synthetic complete medium lacking uracil and spotted in the same medium with either glucose or ethanol/glycerol, and incubated for 3 days at 30 °C. C, cells carrying the cox1Δ::ARG8m mitochondrial gene were spotted on synthetic complete medium lacking uracil (+Arg) or lacking arginine (−Arg), and incubated for 4 days at 30 °C.
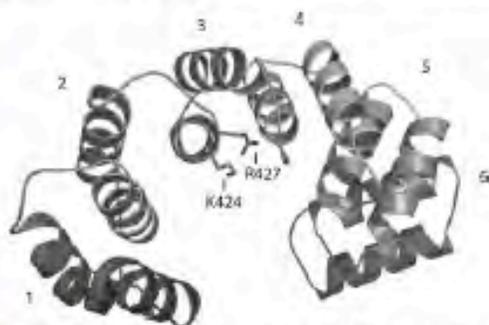
FIGURE 6. **Model of six PPR motifs present in Pet309.** The structure prediction was generated based on the crystal structure of the TPR protein PilF (45). The mutated residues are indicated by arrows, and each PPR is indicated by numbers.

from helix A (29). Based on the crystal structure of the TPR protein PilF (45), a model for six PPR repeats present in Pet309 was generated (Fig. 6). The model suggested that several side chains of basic residues are facing the central groove. This contributes to the calculated groove's highly positive electrostatic potential (data not shown) and is in agreement with the proposed structural model of PPR proteins (29). If the prediction is correct, lowering the positive charges within the central groove of Pet309 should affect the function of the protein.

To test this hypothesis, selected arginines or lysines from PPR3 were mutagenized to alanines (Fig. 6). The mutations were: K424A, R427A, and K424A/R427A. All these residues are presumably present in helix A of the PPR3 and are facing the

inner groove of the PPR region. The pet309-HA mutants were cloned in single copy or high copy expression vectors and transformed into a pet309Δ::LEU2 strain. When expressed on low copy plasmids, the mutants K424A and R427A failed to grow robustly on non-fermentable carbon sources (Fig. 7A); however, under high copy expression the mutants showed normal respiratory growth compared with the wild-type PET309-HA strain. Thus, overexpression of the mutant proteins could compensate for the respiratory defect observed under low expression of the Pet309 mutants. As expected, the double mutant K424A/R427A showed a stronger respiratory defect, compared with the single mutants. This defect could not be bypassed by overproduction of the mutant Pet309 protein.

The respiratory growth defect observed for the mutants was related to a defect in the COX1 mRNA translation. Arg growth of the mutants was analyzed in strains bearing the cox1Δ::ARG8m gene (Fig. 7B). The Arg phenotype of these strains followed the same pattern: the single mutants supported weak growth in media lacking arginine when expressed in single copy plasmids, whereas in high copy plasmids they showed wild-type Arg+ growth. The double mutant showed a weaker Arg growth than the single mutants, and its overexpression didn't compensate for the Arg growth defect.

These results indicate that basic residues that presumably are facing the PPR inner groove of Pet309 are important for translation of the COX1 mRNA. These residues could directly be involved in the specific interaction of Pet309 with the COX1 mRNA.

DISCUSSION

It is well established that a set of proteins from the PPR family are involved in mRNA translation in chloroplasts (25, 54, 55) and mitochondria (11, 24). Pet309 from yeast was the first PPR protein described to be essential for translation and stability of the COX1 mRNA. In this work, we have demonstrated that the PPR domains present in Pet309 are necessary for translation of the COX1 mRNA. Genetic evidence demonstrates that Pet309 associates with the 5'-untranslated region of the COX1 mRNA to activate translation (11). Although biochemical evidence for this is still lacking, the PPR domains of Pet309 might be involved in this interaction. PPR proteins are predicted to bind RNA sequences (29), and this prediction has been confirmed in several cases (8, 19, 25, 56, 57). The mutant Pet309 lacking seven PPR domains lost the capacity for translation but not the capacity for stabilization of the COX1 mRNA. This protein might have other domains that are important for either direct mRNA binding or RNA interaction through other factors, because Pet309 has been found to be part of a large protein complex in mitochondria (58).

As observed for the wild-type Pet309, the mutant protein was found to be associated with the mitochondrial inner membrane as a peripheral protein, and facing the matrix side. This indicates that deletion of the seven PPRs did not abolish the proper import of Pet309Δppr into mitochondria. As observed for the wild-type Pet309, overexpression of Pet309Δppr led to an increased accumulation of the COX1 mRNA. Together these observations indicate that the mutant Pet309 protein conserves

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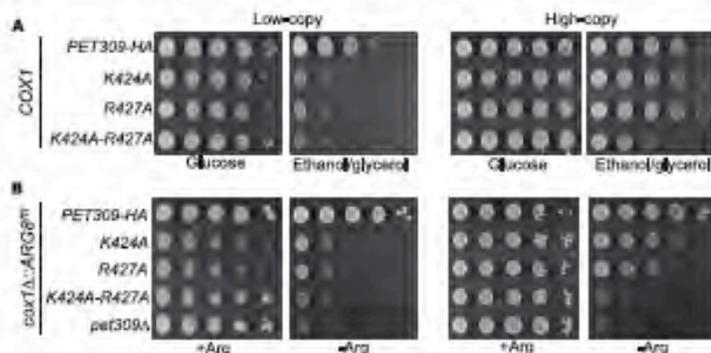
FIGURE 7. **Basic residues facing the PPR groove are important for the function of Pet309.** *A*, 10-fold serial dilutions of strains bearing the wild-type *PET309-HA*, the point mutations on Pet309 or empty vector (pet309Δ), in low copy or high copy plasmids were spotted on synthetic complete medium lacking uracil with either glucose or ethanol/glycerol, and incubated for 4 days at 30 °C. *B*, the same plasmids were transformed in a strain bearing the *cox1Δ::ARG8^m* gene. 10-fold serial dilutions were spotted on synthetic complete medium lacking uracil (+Arg) or arginine (−Arg) and incubated for 4 days at 30 °C.

some of its native properties and suggest that Pet309 can behave as a modular protein.

A series of deletions of one PPR motif at a time were generated. We found that *in vivo* each one of the seven PPR domains present in the central portion of Pet309 was necessary for *COX1* mRNA translation. This is not the case for other PPR proteins. The *Arabidopsis* HCF152 protein is composed of 12 PPR motifs. Only two PPR domains were found to be required for RNA binding but had low affinity. The affinity increased in the presence of more PPR motifs, and the highest affinity was obtained with the full-length protein (19). The human protein LRP130 was found to preferentially bind polypyrimidines, and this RNA-binding activity required only 2 of the 11 predicted PPR motifs (8). It should be noted, however, that these experiments were made in *in vitro* conditions and in the absence of the physiological RNA substrate. It is not known whether HCF152 or LRP130 require the complete set of PPR motifs to be active *in vivo*.

Deletion of any of the PPR motifs in Pet309 abolished respiratory growth even when the mutant proteins were overproduced. This suggests that in the absence of any of the PPR domains no residual activity is present that could be compensated for by overexpression of the mutant Pet309 proteins. PPR proteins belong to a large family of helical repeat proteins that include the RNA-binding protein Pumilio (29, 59, 60). The Puf domain of Pumilio binds RNA in an extended single-stranded conformation (59, 61). A similar model for the PPR domains was suggested, where RNA would be bound as an extended strand inside the cavity formed by the PPR motifs in tandem (29). Deletion of one of the PPR domains in Pet309 might interfere with the affinity and strength of binding for the rest of PPRs. Each PPR motif could bind a specific sequence of nucleotides in the *COX1* mRNA. Deletion of one of these motifs would leave a portion of the extended RNA "naked." This could affect the structure of the PPR-RNA complexes surrounding the deletion, leading to failed PPR-RNA interactions.

A structural model for PPR 1–6 of Pet309 was generated. In this model the repeats formed a superhelix enclosing a groove.

The majority of residues projecting into this cavity are hydrophilic, with positively charged amino acids facing the bottom of each repeat. These residues could be involved in RNA binding. The Pet309 model is in agreement with the proposed model for PPR proteins (29). When two basic residues from PPR 3 that are facing the inner groove were mutagenized to alanines we found that efficiency of translation of *COX1* was considerably affected. This effect was more pronounced in the double mutant. It is possible that the loss of these positive charges at the bottom of the PPR 3 could interfere with the strength of binding of the RNA phosphate backbone. A similar effect was observed when basic amino acids facing the concave surface of the Puf domain of Pumilio where mutagenized (59). In this work, a single point mutation (R1127A) abolished RNA binding. In addition to basic residues within the inner cavity of the Puf domain, side chains from polar and hydrophobic residues are facing the groove. Some of the polar residues at conserved positions interact with specific RNA bases, and solvent-exposed hydrophobic and basic residues stack against the bases in RNA (61). Similarly, the model of the PPR domains of Pet309 suggests that some polar and aromatic amino acids are facing the inner groove and could also be involved in the RNA sequence-specific binding of Pet309.

A common feature of proteins from the PPR family studied so far is that they are sequence-specific. This applies to Pet309, which functionally interacts with the *COX1* 5′-UTR and does not affect other mitochondrial mRNAs (11). The target for Pet309 in the *COX1* 5′-UTR remains to be elucidated. The sequence and length among the mitochondrial mRNAs are not conserved. This has hampered the identification of shared sequence or structural features that translational activators could recognize.

In addition to the PPR motifs, Pet309 has N-terminal and C-terminal regions of 346 and 333 amino acids, respectively. We propose that the PPR motifs present in Pet309 are necessary for sequence-specific binding to the 5′-untranslated leader of *COX1*, whereas other portions of the protein could be involved in the regulation of *COX1* translation. This regulation could be achieved by interaction with the mitochondrial ribosomes, because some PPR proteins in kinetoplasts have been found to be associated with ribosomes (18, 62). Alternatively, the N- and/or C-terminal ends of Pet309 could interact with other factors involved in the translation regulation of *COX1*. This could be the case for Mss51p, which acts on the *COX1* 5′-UTR, but in contrast to Pet309, it also interacts with newly made Cox1 (52, 63).

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Apéndice 2. Cepas de *S. cerevisiae* que se usaron en este estudio.

Cepa	Genotipo nuclear (mitocondrial)	Referencia
a Oxi3-7	Mata, *ade2-101, lys2, (ρ+, oxi3 pt6)*	(Perez-Martinez et al., 2003)
CAB295	*Matα, lys2, arg8::hisG, ura3-52, leu2-3, 112, cox14Δ::URA3 (ρ+, COX1(1-512)::ARG8^m, cox2-62, ΔΣai)*	Este trabajo
CAB297	*Matα, lys2, arg8::hisG, ura3-52, leu2-3, 112, cox14Δ::URA3 (ρ+, COX1(1-512)::ARG8^m, ΔΣai)*	Este trabajo
L74	*Matα, ade2-101 (ρ+, op1)*	(Perez-Martinez et al., 2003)
LSR2	Matα, *lys2, arg8::hisG, ura3-52, leu2-3, 112, cox4Δ::LEU2 (ρ+, ΔΣai[a], COX1ΔC15)*	Este trabajo
LSR3	Matα, *lys2, arg8::hisG, ura3-52, leu2-3, 112, cox7Δ::KanMX4 (ρ+, ΔΣai)*	Este trabajo
LSR4	*Matα, lys2, arg8::hisG, ura3-52, leu2-3, 112, cox7Δ::KanMX4, (ρ+, COX1(1-512)::ARG8^m, ΔΣai)*	Este trabajo
LSR5	Matα, *lys2, arg8::hisG, ura3-52, leu2-3, 112, cox7Δ::KanMX4 (ρ+, ΔΣai, COX1ΔC15)*	Este trabajo
LSR6	Matα, *lys2, arg8::hisG, ura3-52, leu2-3, 112, pet191Δ::LEU2 (ρ+, ΔΣai)*	Este trabajo
LSR7	*Matα, lys2, arg8::hisG, ura3-52, leu2-3, 112, pet191Δ::LEU2, (ρ+, COX1(1-512)::ARG8^m, ΔΣai)*	Este trabajo
LSR8	Matα, *lys2, arg8::hisG, ura3-52, leu2-3, 112, pet191Δ::LEU2 (ρ+, ΔΣai, COX1ΔC15)*	Este trabajo
LSR9	*Matα, lys2, arg8::hisG, ura3-52, leu2-3, 112, cox15Δ::LEU2, (ρ+, COX1(1-512)::ARG8^m, ΔΣai)*	Este trabajo
LSR10	Matα, *lys2, arg8::hisG, ura3-52, leu2-3, 112, cox15Δ::LEU2 (ρ+, ΔΣai)*	Este trabajo
LSR11	Matα, *lys2, arg8::hisG, ura3-52, leu2-3, 112, cox15Δ::LEU2 (ρ+, ΔΣai, COX1ΔC15)*	Este trabajo
LSR12	*Matα, lys2, arg8::hisG, ura3-52, leu2-3, 112, cox4Δ::LEU2, (ρ+, COX1(1-512)::ARG8^m, ΔΣai)*	Este trabajo
LSR13	Matα, *lys2, arg8::hisG, ura3-52, leu2-3, 112, pet122Δ::KanMX4 (ρ+, ΔΣai)*	Este trabajo
LSR14	*Matα, lys2, arg8::hisG, ura3-52, leu2-3, 112, pet122Δ::KanMX4 (ρ+, COX1(1-512)::ARG8^m, ΔΣai)*	Este trabajo
LSR26	*Matα, lys2, ura3-52 o Δ, his4-519, leu2-3, 112, MSS51::3xHA, COX14::3xMYC, cox4Δ::LEU2 (ρ+, ΔΣai, COX1ΔC15)*	Este trabajo
LSR28	Matα, *lys2, arg8::hisG, ura3-52, leu2-3, 112, pet122Δ::kanMX4 (ρ+, ΔΣai, COX1ΔC15)*	Este trabajo
LSR29	*Matα, lys2, ura3-52 o Δ, his4-519, leu2-3, 112, MSS51::3xHA, COX14::3xMYC, cox4Δ::LEU2 (ρ+,ΔΣai)*	Este trabajo
MS68	*Matα, lys2, ura3-52 o Δ, his4-519, leu2-3, 112, MSS51::3xHA, COX14::3xMYC, mss2Δ::KanMX4 (ρ+,ΔΣai, COX1ΔC15)*	Este trabajo
NB40-36a	*Matα, lys2, arg8::hisG, ura3-52, leu2-3, 112 (ρ+)*	(Perez-Martinez et al., 2003)
NAB69 rho0	*Mata, ura-, ade2-101, arg8-delta::hisG, ura3-52, kar1-1 (ρ0)*	(Perez-Martinez et al., 2003)
TF258	*Matα, lys2, ura3-52 o Δ, his4-519, leu2-3, 112, MSS51::3xHA, COX14::3xMYC (ρ+,ΔΣai)*	(Perez-Martinez et al., 2009)
TF272	*Matα, lys2, ura3-52 o Δ, his4-519, leu2-3, 112, MSS51::3xHA, COX14::3xMYC (ρ+, ΔΣai, COX1ΔC15)*	(Perez-Martinez et al., 2009)
XPM10b	*Matα, arg8::hisG, leu2-3,112, lys2, ura3-52 (ρ+, cox1-delta::ARG8^m)*	(Perez-Martinez et al., 2003)

XPM11	*Matα, lys2, arg8::hisG, ura3-52, leu2-3, 112 (ρ+, COX1(1-512)::ARG8^m, $\Delta\Sigma ai$)*	Este trabajo
XPM51	*Matα, lys2, arg8::hisG, ura3-52, leu2-3, 112, cox4Δ::LEU2, (ρ+, $\Delta\Sigma ai$)*	Este trabajo
XPM52	*Matα, lys2, arg8::hisG, ura3-52, leu2-3, 112, cox4Δ::LEU2, (ρ+, COX1(1-512)::ARG8^m, $\Delta\Sigma ai$)*	Este trabajo
XPM171	Matα, *lys2, arg8::hisG, ura3-52, leu2-3, 112, (ρ+, cox1Δ::ARG8^m, cox2Δ::COX1^b, COX2)*	(Perez-Martinez et al., 2003)
XPM183	Matα, *lys2, arg8::hisG, ura3-52, leu2-3, 112, mss2Δ::URA3 (ρ+, cox1Δ::ARG8^m, cox2Δ::COX1^b, COX2)*	(Perez-Martinez et al., 2003)
XPM184	Matα, *lys2, arg8::hisG, ura3-52, leu2-3, 112, mss2Δ::URA3 (ρ+,$\Delta\Sigma ai$)*	Este trabajo
XPM201	Matα, *lys2, arg8::hisG, ura3-52, leu2-3, 112, (ρ+, $\Delta\Sigma ai$)*	Este trabajo
XPM202	Matα, *lys2, arg8::hisG, ura3-52, leu2-3, 112, (ρ+, $\Delta\Sigma ai$, cox2-62)*	Este trabajo
XPM205	Matα, *lys2, arg8::hisG, ura3-52, leu2-3, 112, (ρ+, $\Delta\Sigma ai$, COX1ΔC5)*	Este trabajo
XPM206	Matα, *lys2, arg8::hisG, ura3-52, leu2-3, 112, (ρ+, $\Delta\Sigma ai$, COX1ΔC5, cox2-62)*	Este trabajo
XPM207	Matα, *lys2, arg8::hisG, ura3-52, leu2-3, 112, (ρ+, $\Delta\Sigma ai$, COX1ΔC11)*	Este trabajo
XPM208	Matα, *lys2, arg8::hisG, ura3-52, leu2-3, 112, (ρ+, $\Delta\Sigma ai$, COX1ΔC11, cox2-62)*	Este trabajo
XPM209	Matα, *lys2, arg8::hisG, ura3-52, leu2-3, 112, (ρ+, $\Delta\Sigma ai$, COX1ΔC15)*	Este trabajo
XPM210	Matα, *lys2, arg8::hisG, ura3-52, leu2-3, 112, (ρ+, $\Delta\Sigma ai$, COX1ΔC15, cox2-62)*	Este trabajo
XPM295	Matα, *lys2, arg8::hisG, ura3-52, leu2-3, 112, MSS51-3xHA, (ρ+, $\Delta\Sigma ai$)*	Este trabajo
XPM298	Matα, *lys2, arg8::hisG, ura3-52, leu2-3, 112, MSS51-3xHA (ρ+, $\Delta\Sigma ai$, COX1ΔC15)*	Este trabajo

XPM315	*Matα, lys2, arg8::hisG, ura3-52, leu2-3, 112, pet54Δ::KanMX4, (ρ+, COX1(1-512)::ARG8^m, ΔΣai)*	Este trabajo
XPM316	Matα, *lys2, arg8::hisG, ura3-52, leu2-3, 112, pet54Δ::KanMX4* (ρ+, ΔΣai)	Este trabajo
XPM317	Matα, *lys2, arg8::hisG, ura3-52, leu2-3, 112, pet54Δ::KanMX4* (ρ+, ΔΣai, COX1ΔC15)	Este trabajo
XPM318	*Matα, lys2, arg8::hisG, ura3-52, leu2-3, 112, coa1Δ::KanMX4, (ρ+, COX1(1-512)::ARG8^m, ΔΣai)*	Este trabajo
XPM319	Matα, *lys2, arg8::hisG, ura3-52, leu2-3, 112, coa1Δ::KanMX4* (ρ+, ΔΣai)	Este trabajo
XPM320	Matα, *lys2, arg8::hisG, ura3-52, leu2-3, 112, coa1Δ::KanMX4* (ρ+, ΔΣai, COX1ΔC15)	Este trabajo
YC60	*Matα, lys2, arg8::hisG, ura3-52, leu2-3, 112, cox11Δ::KanMX4, (ρ+, COX1(1-512)::ARG8^m, ΔΣai)*	Este trabajo
YC61	*Matα, lys2, arg8::hisG, ura3-52, leu2-3, 112, cox6Δ::KanMX4, (ρ+, COX1(1-512)::ARG8^m, ΔΣai)*	Este trabajo
YC62	Matα, *lys2, arg8::hisG, ura3-52, leu2-3, 112, cox6Δ::KanMX4* (ρ+, ΔΣai)	Este trabajo
YC63	Matα, *lys2, arg8::hisG, ura3-52, leu2-3, 112, cox6Δ::KanMX4* (ρ+, ΔΣai, COX1ΔC15)	Este trabajo
YC64	Matα, *lys2, arg8::hisG, ura3-52, leu2-3, 112, cox11Δ::KanMX4* (ρ+, ΔΣai)	Este trabajo
YC65	Matα, *lys2, arg8::hisG, ura3-52, leu2-3, 112, cox11Δ::KanMX4* (ρ+, ΔΣai, COX1ΔC15)	Este trabajo
YC66	*Matα, lys2, arg8::hisG, ura3-52, leu2-3, 112, pet100Δ::KanMX4, (ρ+, COX1(1-512)::ARG8^m, ΔΣai)*	Este trabajo
YC67	Matα, *lys2, arg8::hisG, ura3-52, leu2-3, 112, pet100Δ::KanMX4* (ρ+, ΔΣai, COX1ΔC15)	Este trabajo
YC68	Matα, *lys2, arg8::hisG, ura3-52, leu2-3, 112, pet100Δ::KanMX4* (ρ+, ΔΣai)	Este trabajo
YC75	Matα, *lys2, arg8::hisG, ura3-52, leu2-3, 112, cox14Δ::LEU2* (ρ+, ΔΣai)	Este trabajo
YC76	Matα, *lys2, arg8::hisG, ura3-52, leu2-3, 112, cox14Δ::LEU2* (ρ+, ΔΣai, COX1ΔC15)	Este trabajo
YC78	Matα, *lys2, arg8::hisG, ura3-52, leu2-3, 112, pet54Δ::KanMX4, cox14Δ::LEU2* (ρ+, ΔΣai, COX1ΔC15)	Este trabajo
YC78	Matα, *lys2, arg8::hisG, ura3-52, leu2-3, 112, pet54Δ::KanMX4, cox14Δ::LEU2* (ρ+, ΔΣai, COX1ΔC15)	Este trabajo

Todas las cepas son congénicas o isogénicas a la cepa D273-10b (ATCC # 24657).
a: Los genotipos mitocondriales se muestran en paréntesis. *ΔΣai* se refiere al gen COX1 sin intrones.
b: Inserción ectópica del gen quimérico *COX1* rio arriba del gen *COX2*.

Apéndice 3. Oligonucleótidos usados en este trabajo.

Nombre	Secuencia	Producto de PCR
COA1F2	5´-ACCCAAGCTTCATAGTCTGATTGTCCAGAGTCTCC-3´	*coa1Δ::KanMX4*
COA1R2	5´-ACGCGGATCCAGCACAGAAAATACTGCGTTACACG-3´	*coa1Δ::KanMX4*
CO1-C52	5´-ACTTAAGAATTATTTAATCCATTAACTAATTG-3´	*COX1ΔCterminal*
CO15´400F	5´-CATATATATATATTTAATGATATTAATACTCTC-3´	Región *5´UTR* de *COX1*
CO1-loopF	5´-GTTAATGGATTAAATAATAAATTAAATACAGTTAAATCTTCATCTATCG-3´	COX1Δloop
CO1-loopR	5´-TTTATTATTTAATCCATTAACTAATTGATC-3´	COX1Δloop
CO1NdeF	5´-GTATGATCACATCATATGTATATTGTAGG-3´	Región codificante de *COX1*
CO1NdeR	5´-CCTACAATATACATATGATGTGATCATAC-3´	Región codificante de *COX1*
CO1-521R	5´-ACTTAAGATTGTACAGCTGGTGTATTAAATGAGTGTACAGCTGCTGCAGAAGTTAATAAG-3´	*COX1 (PP521AA)*
CO1-524R	5´-ACTTAAGATTGTACAGCTGGTGTATTAAATGAGTGTTCAGCTGGTGG-3´	*COX1 (V524E)*
CO1-525R	5´-ACTTAAGATTGTACAGCTGGTGTATTAAATGAAGCTACAGCTGGTG-3´	*COX1 (H525A)*
CO1-527R	5´-ACTTAAGATTGTACAGCTGGTGTATTAGCTGAGTGTACAGC-3´	*COX1 (F527A)*
CO1-530R	5´-ACTTAAGATTGTACAGCTGCTGTATTAAATGAG-3´	*COX1 (P530A)*
CO1R14-3´	5´-TTAAAACTTAAGTATTAAATGAGTGTACAGCTGGTGGAG-3´	*COX1ΔC5*
CO1R15-3´	5´-TAAAACTTAAGCTGGTGGAGAAGTTAATAAGAATTCG-3´	*COX1ΔC11*
CO1R16-3´	5´-TTAAATTTTAAAACTTAAGTTAATAAGAATTCGATAGATG-3´	*COX1ΔC15*
COX6F1	5´-GCCAATCAGGGCCCCGCGCGTTATTTC-3´	*cox6Δ::KanMX4*
COX6R1	5´-ATATTAAAGGTAATCTGTGACCAGCCC-3´	*cox6Δ::KanMX4*
COX7F1	5´-TCAACTTATACCCTACATTTCTATAGACGC-3´	*cox7Δ::KanMX4*
COX7R1	5´-GTTTTGCTTGAAGAGACATTAGTTTACACC-3´	*cox7Δ::KanMX4*
COX11F1	5´-CTGCCATTCCAAAACATTATTCTCCCC-3	*cox11Δ::KanMX4*
COX11R1	5´-GCCTCTGAAATTTTCGGATGTGAGCG-3´	*cox11Δ::KanMX4*
COX15F2	5´-ACGCACAAATTAGAAGATAGATTGG-3´	*cox15Δ::LEU2*
COX15R2	5´-TTGAAGAGACTAATGGCTTGAACAGGG-3´	*cox15Δ::LEU2*
MSS2F1	5´-TGTGTGGAATGCTAACGATGAACCC-3´	*mss2Δ::URA3*
MSS2R1	5´-CTGATAGTGAACTCCCAAATACTCCAG-3´	*mss22Δ::URA3*
PET54F1	5´-CACAGTTCTTGATGTTGACCTCCCTCC-3´	*pet54Δ::KanMX4*
PET54R1	5´-GTTCTATAAAGACAGATGTAGAATTGGC-3´	*pet54Δ::KanMX4*
PET122F2	5´-GACGGATGGGGCCGGGGC-3´	*pet122Δ::KanMX4*
PET122R2	5´-CGTACAAATCTGCTCTCGC-3´	*pet122Δ::KanMX4*
PET191F1	5´-GCACGTTTGTTTACTTTCTCAATTGTTTGCGG-3´	*pet191Δ::LEU2*
PET191R1	5´-GGAACTTGTTAAGTGCATTTTTATTGTTCTACCC-3´	*pet191Δ::LEU2*
SS-77	5´-ACGAAACGGAAAAGAACTAAGAGA-3´	*cox4Δ::LEU2*
SS78	5´-GAGAACCCGTACAACCGACATA-3´	*cox4Δ::LEU2*

Apéndice 4. Plásmidos usados en este trabajo.

Plásmido	Características
pXPM54	pGEM T-easy, Amp^R, Región 3´ de COX1 sin los últimos 15 nucleótidos.
pXPM55	pGEM T-easy, Amp^R, Región 3´ de COX1 sin los últimos 33 nucleótidos.
pXPM56	pGEM T-easy, Amp^R, Región 3´ de COX1 sin los últimos 45 nucleótidos.
pXPM57	pBlueScript +, Amp^R, *COX1* completo flanqueado por sus extremos UTR.
pXPM59	pBlueScript +, Amp^R, *COX1* sin los últimos 15 nucleótidos, flanqueado por sus extremos UTR (Cox1ΔC5).
pXPM60	pBlueScript +, Amp^R, *COX1* sin los últimos 33 nucleótidos, flanqueado por sus extremos UTR (Cox1ΔC11).
pXPM61	pBlueScript +, Amp^R, *COX1* sin los últimos 45 nucleótidos, flanqueado por sus extremos UTR (Cox1ΔC15).
pXPM64	YEp352, URA3, Amp^R, 2μ, plásmido que sobreexpresa a Mss51.
pXPM104	YEp352, URA3, Amp^R, 2μ, plásmido que sobreexpresa a Pet309-3xHA.
pXP132	pGEM T-easy, Amp^R, Región 3´ de COX1 con el cambio de PP521,522AA.
pXP133	pGEM T-easy, Amp^R, Región 3´ de COX1 con el cambio de V524E.
pXP134	pGEM T-easy, Amp^R, Región 3´ de COX1 con el cambio de H525A.
pXP135	pGEM T-easy, Amp^R, Región 3´ de COX1 con el cambio de F527A.
pXP136	pGEM T-easy, Amp^R, Región 3´ de COX1 con el cambio de P530A.
pXP139	pBlueScript +, Amp^R, *COX1*, flanqueado por sus extremos UTR (Cox1PP521,522AA).
pXP140	pBlueScript +, Amp^R, *COX1*, flanqueado por sus extremos UTR (Cox1V524E).
pXP141	pBlueScript +, Amp^R, *COX1*, flanqueado por sus extremos UTR (Cox1H525A).
pXP142	pBlueScript +, Amp^R, *COX1*, flanqueado por sus extremos UTR (Cox1F527A).
pXP43	pBlueScript +, Amp^R, *COX1*, flanqueado por sus extremos UTR (Cox1P530A).
YEp352	pUC18, URA3, Amp^R, 2μ, plásmido de alto número de copia.

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