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Study of a region on yeast chromosome XIII that complements *pet* G199 mutants (*COX7*) and carries a new non-essential gene

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Abstract

The mutants of *Saccharomyces cerevisiae* assigned to complementation group G199 are deficient in mitochondrial respiration and lack a functional cytochrome oxidase complex. Recombinant plasmids capable of restoring respiration were cloned by transformation of mutants of this group with a yeast genomic library. Sequencing indicated that a 2.1-kb subclone encompasses the very end (last 11 amino acids) of the *PET111* gene, the *COX7* gene and a new gene (*YMR255W*) of unknown function that potentially codes for a polypeptide of 188 amino acids (about 21.5 kDa) without significant homology to any known protein. We have shown that the respiratory defect corresponding to group G199 is complemented by plasmids carrying only the *COX7* gene. The gene *YMR255W* was inactivated by one-step gene replacement and the disrupted strain was viable and unaffected in its ability to grow in a variety of different test media such as minimal or complete media using eight distinct carbon sources at three pH values and temperatures. Inactivation of this gene also did not affect mating or sporulation.

Key words
- *Saccharomyces cerevisiae*
- Gene cloning
- Cytochrome oxidase
- Subunit VII
- Gene disruption

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Introduction

In the yeast *Saccharomyces cerevisiae*, cytochrome oxidase is composed of eleven different polypeptides (1) and the enzyme contains two spectrally distinct heme *a* groups and two copper atoms. The three large subunits (*COX1*, *COX2*, and *COX3*) which are generally believed to represent the major catalytic centers of cytochrome oxidase are encoded by mitochondrial DNA and possess significant similarity in their primary sequence to the three subunits of prokaryotic cytochrome oxidases (2). All additional subunits are encoded by the nuclear genome. These subunits are thought to function in the regulation of catalysis or in the assembly of the holoenzyme (3). The main function of cytochrome oxidase, the terminal enzyme in the respiratory chain, is energy conservation. This hetero-oligomeric lipoprotein complex of the mitochondrial inner membrane is a molecular pump that uses redox chemistry to drive protons from the mitochondrial matrix across the membrane (4). Besides the eleven enzyme subunits, about 60 more nuclear genes are known to be necessary for the activity of cytochrome oxidase (5,6), an unexpected and exciting problem for study.

In order to define the role of one more nuclear-encoded subunit, we have characterized a *pet* mutant assigned previously to complementation group G199. *Pet* mutants are yeast strains which, due to a recessive

mutation in a nuclear gene affecting mitochondrial function, have lost the ability to grow on non-fermentable carbon sources, but grow normally on fermentable substrates (5).

The G199 gene was cloned by complementation of the inability to grow on glycerol/ethanol in mutant E880/U1, after transformation with a yeast genomic plasmid library. After subcloning, a 2.1-kb *Hin*dIII fragment still had the ability to restore respiration in the mutants. The sequence of this DNA segment contained the end of the *PET111* gene (7), the *COX7* gene (8,9) and a new ORF. A search at the *Saccharomyces* Genome Database (SGD), Stanford, CA, confirmed our results and showed that this region is located between coordinates 777570 and 779664 on yeast chromosome XIII. The function of this new ORF (*YMR255W*) was explored by phenotypic analysis after gene disruption.

Material and Methods

Yeast strains and media

The genotypes and sources of yeast strains used in this study are described in Table 1. The nuclear *pet* mutants were isolated by mutagenesis of the respiratory competent haploid *S. cerevisiae* strain D273-10B/A1 with ethyl methanesulfonate as described previously (5).

Yeast growth media were YPD (2% glucose, 2% peptone, 1% yeast extract), YEPG (1% yeast extract, 2% peptone, 2% glycerol, 2% ethanol), WO (2% glucose, 0.67% yeast nitrogen base without amino acids (Difco), amino acids and other supplements were added as required at 20 µg/ml), and YPGal (2% galactose, 2% peptone, 1% yeast extract). All solid media contained 2% agar. Plasmid-containing *Escherichia coli* RR1 (Δ*(gpt-proA)62 leuB6 thi-1 lacY1 hsdS*$_B$*20 rpsL20 (Str*^{r}*) ara-14 galK2 xyl-5 mtl-1 supE44 mcrB*$_B$) were grown in L-broth (0.5% yeast extract, 1.0% tryptone, 0.5% NaCl and 0.1% glucose) in the presence of 50 µg/ml ampicillin.

Transformation of *S. cerevisiae*

Mutant E880/U1 originated from a haploid spore derived from the sporulation of a diploid resulting from the mating of E880 and W303-1A. It was grown in 100 ml YPGal to an approximate density of 10^6 cells/ml and the entire culture was transformed by the method of Schiestl and Gietz (10) with a yeast genomic library. The library (provided by Dr. Marion Carlson, Department of Human Genetics, Columbia University) con-

Table 1 - *Saccharomyces cerevisiae* strains used.

ORF means gene *YMR255W*. [1]A. Tzagoloff, Columbia University; [2]R. Rothstein, Columbia University; [3]C.L. Dieckmann, University of Arizona.

Strain	Genotype	Source
D273-10B/A1	*MATα met6* ρ^+	Tzagoloff[1]
W303	*MATα/a ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1* ρ^+	Rothstein[2]
W303-1A	*MATa ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1* ρ^+	Rothstein[2]
W303-1B	*MATα ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1* ρ^+	Rothstein[2]
CB11	*MATa ade1* ρ^o	Tzagoloff[1]
KL14	*MATα his1 trp2* ρ^o	Tzagoloff[1]
LMY6	*MATα lys2* ρ^o	Dieckmann[3]
LMY7	*MATa lys2* ρ^o	Dieckmann[3]
E880	*MATα met6 pet-G199*	this study
E880/U1	*MATα met6 ura3-1 pet-G199*	this study
W303ΔORF-A1	*MATa ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 orfΔURA3* ρ^+	this study
W303ΔORF-A2	*MATa leu2-3,112 orfΔURA3* ρ^+	this study
W303ΔORF-B1	*MATα ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 orfΔURA3* ρ^+	this study
W303ΔORF-B2	*MATα his3-1,15 orfΔURA3* ρ^+	this study

sists of partial *Sau*3AI fragments (average length 12 kb) ligated to the *Bam*HI site of YEp24 (11). Uracil-independent clones were selected on minimal glucose medium and were subsequently checked for respiratory competence by the ability to grow on glycerol-rich medium (YEPG). Plasmid DNA was isolated from independent uracil$^+$ and glycerol$^+$ transformants and amplified in *Escherichia coli* RR1. One plasmid (pG199/T1) that was checked as positive after transformation of the original recipient was used to subclone and characterize the nuclear DNA insert.

Subcloning and DNA sequencing

Restriction fragments from the plasmid pG199/T1 were subcloned into YEp352/351 (12) and pKS+ (Bluescript® II KS (+) phagemid, Stratagene) and the DNA was sequenced by the method of Maxam and Gilbert (13). All restriction sites used for 5'-end labeling were crossed from neighboring sites and the sequence was confirmed from the complementary strands. The ends of the insert were sequenced by the method of Sanger et al. (14) using primers (New England Biolabs) adjacent to the *Bam*HI cloning site in the YEp24 vector.

Construction of a deletion strain at ORF *YMR255W*

A 1.1-kb *Taq*I fragment (from pG199/T5) containing the entire ORF was treated with Pfu DNA polymerase (Stratagene) to generate blunt ends beginning 336 bp before the ORF's ATG and extending 211 bp after the termination codon. The fragment was ligated to the pKS+ vector previously digested with *Apa*I, *Bst*XI and the Pfu DNA polymerase (for blunting), generating plasmid pKS/T8. To construct a disrupted allele of the *YMR255W* gene, plasmid pKS/T8 was digested with *Bst*EII and *Eco*RV, a procedure that removes 137 bp internal to the ORF. The deleted plasmid was ligated to a 1.1-kb *Hin*dIII fragment containing the yeast *URA3* gene. The disrupted *orf*::*URA3* allele was recovered from this construction as a linear 2.0-kb *Pvu*II fragment and was used to transform both the haploid W303-1A strain and the isogenic diploid strain W303 by the procedure of Rothstein (15).

DNA manipulation procedures and miscellaneous methods

Standard methods were used for the transformation of *E. coli*, the preparation of recombinant plasmid DNA, digestion of DNA with restriction endonucleases, isolation and ligation of DNA fragments, and nick translation (16). The isolation of yeast nuclear DNA and the conditions for Southern analysis have been described elsewhere (17).

Functional analysis

Media used to test the growth of gene disruptants included complete glucose medium (YPD) or synthetic medium with glucose (WO) and also both media supplemented with the following carbon sources replacing glucose: 2% fructose, 2% galactose, 2% maltose, 2% raffinose, 2% potassium acetate, 2% lactate, and 2% glycerol at three different pH values (pH 3.5, pH 6.6 and pH 8.5). Plates with the test media were incubated at three different temperatures (18, 30 and 37°C). The proper haploid derivatives were mated to generate diploids homozygous for the disruption of *YMR255W*. The sporulation of these cells was induced and examined as described by Rockmill et al. (18).

Results

Phenotype of *COX7* mutants

E880 is the mutant from the *pet* mutant collection of Tzagoloff and Dieckmann (5) carrying the mutation allelic to the comple-

mentation group G199. The mutant is unable to grow on non-fermentable carbon sources and is complemented by mating to a rho^o tester strain, indicating that the respiratory deficiency is caused by a recessive mutation in a nuclear gene. The phenotype of this mutant suggests that the respiratory deficiency stems from a specific defect in cytochrome oxidase. Mitochondria of the mutants lack cytochromes a and a_3 but have the absorption bands corresponding to cytochromes b, c and c_1 (data not shown).

Cloning and sequencing of *COX7* and ORF *YMR255W*

To clone the gene carrying the mutation in E880, strain E880/U1 was transformed with a yeast genomic library. Five of the uracil-independent and respiratory-competent clones obtained from the transformation were found to have plasmids with related inserts of yeast nuclear DNA. The nuclear DNA insert of those plasmids was ascertained to be approximately 9 kb long. An *Xba*I fragment of 6.9 kb was obtained from the original recombinant plasmid YEp24 and ligated to the *Xba*I site in the polylinker of plasmid YEp352 originating recombinant plasmid pG199/T2 (Figure 1). Initially, a total of three subclones were generated from this *Xba*I fragment (T3, T4 and T5; Figure 1). T3 is a 2.9-kb *Eco*RV fragment, T4 is a 3.2-kb *Xba*I-*Hin*dIII fragment and T5 is a 2.1-kb *Hin*dIII fragment. The DNA fragments were cloned in YEp352 and their ability to complement the respiratory-deficient phenotype of G199 mutants was investigated. The 2.1-kb recombinant plasmid pG199/T5 was the smallest complementing clone and its sequence was determined. It was found that this region contained the C-terminal end of the *PET111* gene, the *COX7* gene and another open reading frame of sufficient length to qualify as a protein-coding gene. Three new subclones were generated from pG199/T5. Subclone pG199/T6 (*Eco*RV-*Hin*dIII, 1.3 kb; Figure 1), that contained the entire *COX7* gene and part of gene *YMR255W*, complemented the respiratory defect in the mutants. Subclone pG199/T7 (*Hin*dIII-*Eco*RV, 0.7 kb; Figure 1) contained part of gene *YMR255W* and did not complement mutants. Subclone pG199/T8 was constructed (1.1 kb, *Taq*I fragment from pG199/T5; Figure 1) to contain the entire *YMR255W* gene including its upstream (promoter) region. The sequence of this region is shown in Figure 2 with the centromere proximal region located at the beginning and the telomere proximal region at the end of the sequence. The ORF of *YMR255W* encodes a polypeptide of unknown function with 188 amino acids and a calculated molecular mass of 21,584 Da (Figure 2) without significant homology to any known protein.

In situ disruption of the *YMR255W* gene

Plasmid pKS/T8 was constructed to explore the function of this new gene by one-step gene disruption. A null allele was created by insertion of the yeast *URA3* gene replacing the *Bst*EII-*Eco*RV fragment (137 bp) internal to the coding sequence (Figure 3). The disrupted allele was isolated on a linear (*Pvu*II) fragment and used to transform the respiratory-competent haploid strain W303-1A and the isogenic diploid strain W303. Southern analysis showed that the uracil-independent clones obtained from the transformation had acquired the disrupted *ymr255w*::*URA3* allele. Nuclear DNA from the parental strains, as well as from each transformant, was digested with *Hin*dIII and probed with a 833-bp *Kpn*I-*Sac*I fragment of pKS/T8. The probe recognizes a 2.1-kb fragment in the genomic DNA of the parental strain. The genomic DNA of the knockout mutant strain has a new species cross-hybridizing at about 3.1 kb, the size expected after the manipulations used in the construction and after the insertion of the 1166-bp fragment bearing the *URA3* gene (Figure 3).

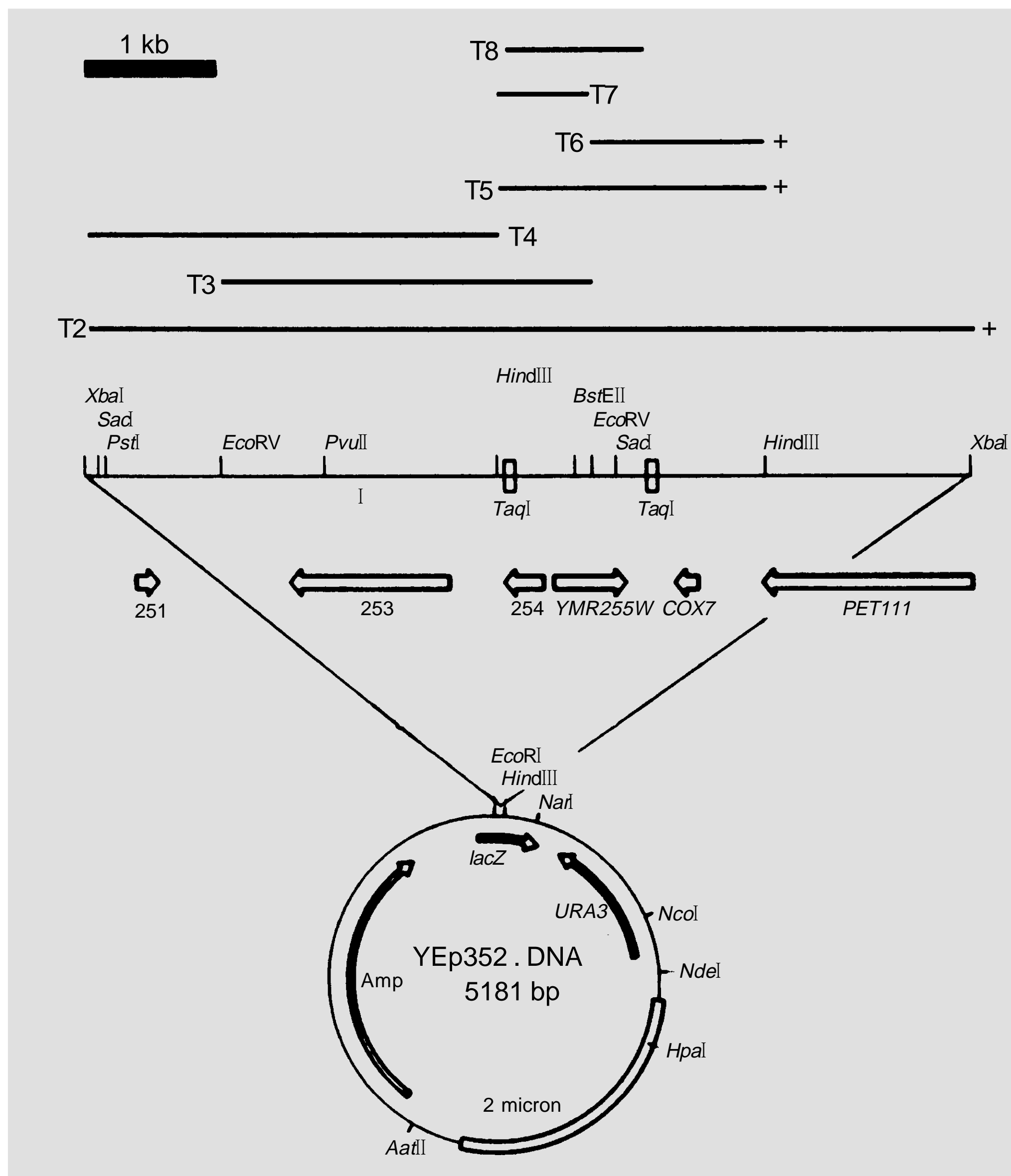

Figure 1 - Partial restriction maps of pG199/T2 and derivative plasmids. The larger insert (T2) is a 6.9-kb fragment of nuclear DNA ligated at the *Xba*I site of vector YEp352. The locations in YEp352 of the *URA3* and the ß-lactamase genes are shown. The bars in the upper part of the figure represent the size and position of the indicated subclones relative to the larger (T2) insert. The arrows below the restriction map represent the main ORFs present in this segment of chromosome XIII. The two *Taq*I sites that define subclone T8 are indicated. The plus signs denote the recombinant plasmids that complement the respiratory deficiency of group G199 mutants.

Phenotypic analysis of ORF *YMR255W* disruptants

The *YMR255W* gene was successfully disrupted in a haploid strain. Consequently the gene knockout is viable. Growth behavior of the disruptant in the different media and under the conditions listed in the Material and Methods section failed to disclose a behavior distinct from the wild-type strain. The disruptants also presented a normal frequency in the generation of spontaneous cytoplasmic petite mutants.

Discussion

The *Hin*dIII fragment characterized by sequencing is located between coordinates 777570 and 779664 on the right arm of chromosome XIII. The gene order (Figure 1) is centromere proximal, *YMR255W*, *YMR 256C* (*COX7*), and *YMR257C* (*PET111*). Our sequence analysis placed these genes together for the first time. This has been fully confirmed by the results of the systematic yeast genome sequencing project. The phenotype of the mutant E880 that originated

Figure 2 - Nucleotide sequence of *YMR255W* and flanking regions. The sense DNA strand from the *Taq*I fragment (pG199/T8) and the translated reading frame corresponding to YMR 255W are shown (GenBank accession number AF007064). Restriction enzyme sites used for cloning, disruption and probe preparation are identified.

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         TaqI
1        TCG AAA AAT CTA GCA CGT GGG AGG GTA AGC ATA AAC TAC CCA GAA ACG      48

49       CAT GTA TGG ACT GCA TTT GCT TCC TGA CCA AAA TAA GTA TTA TCG TCA      96

97       TAT AAA AAA CGT GCA TAG TAT ATG TAA CAT CAA TGA TGC TGG GCG TTG     144

145      TTT GCC ATT TTG TAT TTA CTA TGG CAG TGT ATT TTG TAA CGA GCA CGT     192

193      GAT TTA CAG GGC GCA GAA ATG TTG AAA ATT TAG AAA AAA GTA AGA TAA     240

241      GCA ATA TCA GTG GCA CCA TTG AGC TAG TCT CTA ACA GCG GGG TGA GAA     288

289      GCT ATT TTT GAT AGG AGA ATA CCT TCA ATA TCA TTT TTA CTA TTT ATC     336

1        Met Pro Leu Glu Ser Ile Trp Ala Asp Ala Pro Asp Glu Glu Pro Ile      16
337      ATG CCT TTA GAA TCT ATA TGG GCC GAT GCC CCT GAC GAA GAA CCA ATA     384

17       Lys Lys Gln Lys Pro Ser His Lys Arg Ser Asn Asn Asn Lys Lys Asn      32
385      AAG AAG CAG AAA CCA AGT CAC AAG CGG AGT AAT AAC AAT AAA AAG AAT     432

33       Asn Asn Ser Arg Trp Ser Asn Glu Ser Ser Ser Asn Asn Lys Lys Lys      48
433      AAT AAT AGC AGG TGG AGT AAT GAA TCA AGT TCA AAC AAT AAG AAA AAA     480
                                                      BstEII
49       Asp Ser Val Asn Lys Val Lys Asn Asn Lys Gly Asn His Glu Ser Lys      64
481      GAT TCT GTA AAC AAA GTG AAG AAT AAC AAA GGT AAC CAC GAA AGT AAA     528

65       Thr Lys Asn Lys Ile Lys Glu Thr Leu Pro Arg Glu Lys Lys Pro Pro      80
529      ACG AAA AAT AAA ATA AAG GAA ACT CTT CCC AGA GAA AAG AAG CCG CCT     576

81       His Ser Gln Gly Lys Ile Ser Pro Val Ser Glu Ser Leu Ala Ile Asn      96
577      CAT AGT CAG GGC AAA ATA TCG CCT GTA AGT GAA TCA TTG GCG ATA AAT     624
                                          EcoRV
97       Pro Phe Ser Gln Lys Ala Thr Glu Ile Ser Pro Pro Pro Val Ser Pro     112
625      CCT TTC TCC CAA AAA GCA ACA GAG ATA TCT CCT CCA CCA GTT TCA CCT     672

113      Ser Lys Met Lys Thr Thr Lys Thr Gln Ser Lys Gln Asp Thr Ala Ser     128
673      AGC AAG ATG AAA ACT ACC AAA ACA CAA TCC AAA CAG GAT ACC GCT TCT     720

129      Lys Met Lys Leu Leu Lys Lys Lys Ile Glu Glu Gln Arg Glu Ile Leu     144
721      AAG ATG AAG TTA TTA AAA AAG AAA ATT GAA GAG CAG AGG GAA ATA TTG     768

145      Gln Lys Thr His His Lys Asn Gln Gln Gln Gln Val Leu Met Asp Phe     160
769      CAA AAG ACT CAT CAC AAG AAT CAA CAG CAA CAA GTG TTG ATG GAT TTT     816
                                SacI
161      Leu Asn Asp Glu Gly Ser Ser Asn Trp Val Asp Asp Asp Glu Glu Glu     176
817      CTG AAC GAT GAA GGG AGC TCC AAC TGG GTT GAC GAT GAT GAA GAG GAG     864

177      Leu Ile Leu Gln Arg Leu Lys Thr Ser Leu Lys Ile ***                 189
865      CTT ATC CTT CAA CGT TTG AAG ACC TCT TTG AAA ATA TGA TCC CTC CCA     912

913      TCA CAT GGA CAC TTT TAA GCA CGT TTT GCT TGA AGA GAC ATT AGT TTA     960

961      CAC CAT TAT AAT TGT TTA ATA AAT ACT AGA CAT ACT TCA AGT GAG AAA    1008

1009     ATC AAT CAC ACC TTT TTC AAT GAT TAG CAA ATC TTT ATT GCC AGG CCT    1056

1057     AAC TGA AAC GAT TTA TTA CTG CGC AAG AAA ACA AAG ATG GAA AAG GCT    1104
                     TaqI
1105     ATG CTA GTT CGA                                                    1116
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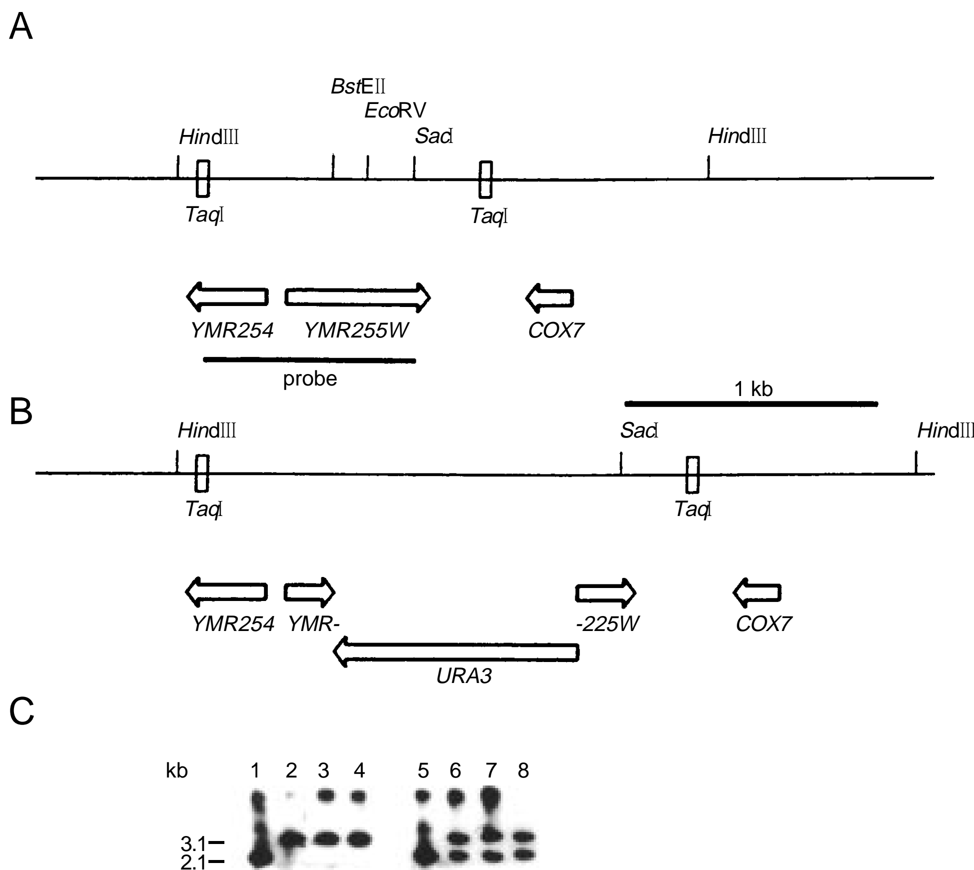

Figure 3 - Southern hybridization analysis of yeast genomic DNA from representative isolates of strain W303 after disruption of *YMR255W*. The location of the restriction sites is marked along the line that represents the DNA. The ORFs and respective orientations are shown below. *A*, Map of the *YMR255W* region indicating the *Bst*EII and *Eco*RV sites used to delete a 137-bp internal fragment of the gene before the insertion of a 1166-bp DNA fragment carrying the *URA3* gene. *B*, Map of the same chromosomal region after gene replacement with the construction explained above. *C*, Electrophoretogram of total yeast DNA prepared from the control strain W303-1A (lanes 1 and 5) or from individual transformants from experiments done with the W303-1A haploid strain (lanes 2, 3 and 4) or with the diploid W303 strain (lanes 6, 7 and 8). About 0.2 µg of DNA was digested with *Hin*dIII, fractionated by electrophoresis on 1% agarose gel and blotted onto Hybond N. The Southern blot was hybridized to the labeled *Taq*I-*Sac*I fragment (probe) and exposed to X-ray film. The size of the fragments detected is indicated on the left-hand margin.

this study was respiratory incompetence. The yeast strains belonging to this group were unable to grow on non-fermentable substrates and upon spectral and enzymatic examination revealed the absence of cytochrome *c* oxidase activity and a lack of the spectral bands assigned to this respiratory complex (heme a-a_3). The activities of the other membrane enzymes were present (data not shown). Here we demonstrate that the mutant phenotype could be completely rescued by transformation of the mutant with plasmid pG199/T6 carrying a 1.4-kb fragment that contained only the *COX7* gene and its promoter region. This gene has been sequenced previously (8,9) and the sequence of the fragments amplified by PCR using degenerate primers corresponded to the amino- and carboxyl-terminal regions of the sequenced subunit VII polypeptide. We now used a genetic method that independently led to the cloning of the same gene. Although cytochrome oxidase in yeast or mammals is a complex enzyme with 11 to 13 subunits, studies with the corresponding *Paracoccus denitrificans* enzyme showed that the mininal unit required for electron transfer-linked proton pumping consists of subunits I and II (4). Current research aims to understand the function of the other subunits. The recent elucidation of the three-dimensional structure of the mammalian enzyme (19) will certainly direct new attempts to define the role of the accessory polypeptides such as subunit VII (*COX7*). Another interesting problem is the detection and study of the many nuclear genes that affect cytochrome oxidase activity without being part of the purified active enzyme (6).

One of the genes adjacent to *COX7* is *PET111* (7). This is also a respiratory gene that functions as a translational factor for the mitochondrially encoded *COX2* mRNA re-

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                                                  A     A       B
  1   MPLESIWADAPDEEPIKKQKPSHKRSNNNKKNNNSRWSNESSSNNKKKDS

 51   VNKVKNNKGNHESKTKNKIKETLPREKKPPHSQGKISPVSESLAINPFSQ

                C
101   KATEISPPPVSPSKMKTTKTQSKQDTASKMKLLKKKIEEQREILQKTHHK

151   NQQQQVLMDFLNDEGSSNWVDDDEEELILQRLKTSLKI  188
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Figure 4 - Derived amino acid sequence corresponding to the coding region of gene *YMR 255W.* Sequence motifs identified by the Yeast Proteome Database as putative targets for N-glycosylation (A), protein kinase A (B) or Cdc28 kinase (C) are underlined. Heptad sequences that represent potential coiled-coil structures have been identified (24) and are displayed in bold characters.

quiring the 5'-untranslated portion of the messenger (20). The presence of two neighboring respiratory genes made us wonder if the next gene *YMR255W*, of unknown function, had a mitochondrial role. To that effect a specific null mutant was generated. The disruption of this gene behaved as many other inactivated yeast genes (21), i.e., its knockout does not result in an observable phenotype as examined by growth in rich and minimal media with different carbon sources and at different temperatures. The stability of the mitochondrial genome was found to be normal and was estimated by measuring the spontaneous generation of petite mutants. Also, the null mutant did not affect mating or sporulation in tests carried out with the appropriate haploids or the homozygous diploid yeast. The usual assumption in those cases is that the genome harbors at least one functional equivalent of the disrupted gene or that the experimental conditions tested in the laboratory do not include the physiological demanding conditions that would require this gene product.

Examination of the protein sequence encoded by *YMR255W* as analyzed by the Yeast Proteome Database shows that the 188-amino acid basic polypeptide (calculated pI of 9.95) is probably a gene with low abundance mRNA as suggested by the codon adaptation index of 0.138 (22) and low codon bias (0.039) (23). The sequence does not show any transmembrane domains but there are regions with the potential to generate coiled-coil domains (24) and potential phosphorylating sites for Cdc28 kinase and cAMP-dependent protein kinase (Yeast Proteome Database) as well as putative N-glycosylation sites (Figure 4). There is no extensive homology to any known protein. A similarity search within the yeast genome using the FASTA algorithm (25) through the SGD revealed 13 ORFs, 8 belonging to genes of unknown function and 5 to genes already named, all with 20 to 29% identity in overlaps that range from 120 to 188 amino acids. Another search using the National Center for Biotechnology Information (NCBI) BLAST program (26) against all non-redundant sequences deposited showed two or three regions (10 to 38 amino acids) of similarity in three additional yeast genes: *TAF61*, a subunit of transcription factor TFIID (27), *ROD1*, resistance to O-dinitrobenzene and ions such as zinc and calcium (28), and *MLP1*, a probable coiled-coil protein with myosin-like motifs and possible involvement in DNA repair (29). These proteins have similarity to *YMR255W* in its central region (residues 66 to 104 approximately) and also in the C-terminal region. Such similarities could eventually resolve the problem of finding a function for this gene. Further phenotypic testing (30), over-expression of the gene and a search for a synthetic lethal mutant (31) could provide important new clues.

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