Highly Diverged Homologs of *Saccharomyces cerevisiae* Mitochondrial mRNA-Specific Translational Activators Have Orthologous Functions in Other Budding Yeasts

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ABSTRACT

Translation of mitochondrially coded mRNAs in *Saccharomyces cerevisiae* depends on membrane-bound mRNA-specific activator proteins, whose targets lie in the mRNA 5′-untranslated leaders (5′-UTLs). In at least some cases, the activators function to localize translation of hydrophobic proteins on the inner membrane and are rate limiting for gene expression. We searched unsuccessfully in divergent budding yeasts for orthologs of the *COX2*- and *COX3*-specific translational activator genes, *PET111*, *PET54*, *PET122*, and *PET494*, by direct complementation. However, by screening for complementation of mutations in genes adjacent to the *PET* genes in *S. cerevisiae*, we obtained chromosomal segments containing highly diverged homologs of *PET111* and *PET122* from *Saccharomyces kluyveri* and of *PET111* from *Kluyveromyces lactis*. All three of these genes failed to function in *S. cerevisiae*. We also found that the 5′-UTLs of the *COX2* and *COX3* mRNAs of *S. kluyveri* and *K. lactis* have little similarity to each other or to those of *S. cerevisiae*. To determine whether the *PET111* and *PET122* homologs carry out orthologous functions, we deleted them from the *S. kluyveri* genome and deleted *PET111* from the *K. lactis* genome. The *pet111* mutations in both species prevented *COX2* translation, and the *S. kluyveri pet122* mutation prevented *COX3* translation. Thus, while the sequences of these translational activator proteins and their 5′-UTL targets are highly diverged, their mRNA-specific functions are orthologous.

TRANSLATION of mitochondrially coded mRNAs in *Saccharomyces cerevisiae* is a surprisingly complex process. Most, if not all, of the seven major mitochondrially coded mRNAs (Dieckmann and Staples 1994) are translated under the direction of mRNA-specific translational activator proteins specified by nuclear genes (Fox 1996). The translational activator proteins interact functionally with targets in the mRNA 5′-untranslated leaders (5′-UTLs) and, in at least one case, also with the mitochondrial ribosomal small subunit (Fox 1996).

The activator proteins specific for translation of four of the mRNAs encoding integral membrane proteins, *COX1*, *COX2*, *COX3*, and *COB*, are themselves bound to the mitochondrial inner membrane (Michaelis *et al.* 1991; McMullin and Fox 1993; Manthey *et al.* 1998; N. S. Green-Willms and T. D. Fox, unpublished data), suggesting that they could localize translation to assembly sites of respiratory complexes (Costanzo and Fox 1990; Fox 1996). Indeed, the 5′-untranslated regions of the *COX2* and *COX3* mRNAs contain information necessary for proper targeting of the proteins they encode (Sanchirico *et al.* 1998). *COX2* and *COX3* mRNA-specific translational activation is also a rate-limiting step in gene expression (Steele *et al.* 1996; N. S. Green-Willms and T. D. Fox, unpublished data). Thus, the *S. cerevisiae* translational activator proteins appear to have dual roles in regulating mitochondrial gene expression and in localizing translation.

Why translation of the mitochondrially coded mRNAs specifying cytochrome *c* oxidase subunits Cox1p, Cox2p, and Cox3p should be dependent on distinct activators remains an open question. One possible rationalization is that mRNA specificity allows for relative topological distinctions between the sites where these mitochondrially coded proteins are synthesized, which could promote efficient assembly of cytochrome *c* oxidase complexes in the inner membrane. However, this effect would be relatively subtle since Cox1p, Cox2p, and Cox3p can be assembled into cytochrome *c* oxidase after translation of experimentally derived chimeric mRNAs bearing 5′-UTLs derived from certain other mitochondrial genes, under the direction of their respective activators (Müller *et al.* 1984; Costanzo and Fox 1986, 1988; Poutre and Fox 1987; Rödel and Fox 1987; Mulero and Fox 1993b; Manthey and McEwen 1995).

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These experiments shed no light on possible quantitative differences in efficiency of cytochrome *c* oxidase assembly, owing to the genetic instability of the mitochondrially heteroplasmic strains employed. However, they demonstrate that the one-to-one correspondence between translational activators and the mitochondrial genes they govern can be experimentally altered without destroying function. Thus, if there were no adaptive value in maintaining these correspondences, then they could diverge during evolution.

In this study we have sought to use phylogenetic comparisons to ask whether the correspondences between translational activators and mitochondrial mRNAs have been conserved and to shed light on the function of the activator proteins themselves. We have focused on the *COX2*-specific activator Pet111p (Strick and Fox 1987) and the subunits of the *COX3*-specific activator complex, Pet54p, Pet122p, and Pet494p (Brown *et al.* 1994), none of which had known homologs at the start of this study. We began a generally unsuccessful search for orthologous genes in the divergent budding yeasts *S. servazzii*, *S. kluyveri*, *Kluyveromyces lactis*, and the dimorphic yeast *Candida albicans*, using cross-hybridization and cross-complementation. These approaches worked only for a sister species of *S. cerevisiae*, *S. bayanus*. However, we were able to isolate *S. kluyveri* genes homologous to *PET111* and *PET122*, as well as a *K. lactis* homolog of *PET111*, by screening for complementation of highly conserved genes that are adjacent to *PET111* and *PET122* in *S. cerevisiae*. We found that Pet111p and Pet122p are among the most rapidly diverging proteins known in these species. They are nevertheless orthologous, since the mutations we constructed in the *S. kluyveri* and *K. lactis* homologs generated phenotypes in those species that are similar to those of the corresponding *S. cerevisiae* mutants. Thus, while the sequences of these translational activator proteins are highly diverged, their one-to-one correspondences with mitochondrial mRNAs have been conserved. These findings suggest that the specificity of translational activation plays an important role in fungal mitochondrial biogenesis.

MATERIALS AND METHODS

Yeast strains, media, and genetic methods: Strains used in this study are listed in Table 1. All *S. cerevisiae* strains were isogenic or congenic to the wild-type strain D273-10B (ATCC #25657), except strains JM43-GD7, NGB108, PTH43, and PTH352. Media and genetic methods were as described (Sherman *et al.* 1986). Respiratory growth was assessed on YPEG medium (3% ethanol, 3% glycerol, 1% yeast extract, 2% bactopeptone, 2% agar). Saccharomyces strains were transformed either by treatment with lithium acetate and polyethylene glycol (Ito *et al.* 1983) or by using the Yeast EZ Transformation Kit (Zymo Research, Orange, CA). *K. lactis* was transformed as described (Chen and Clark-Walker 1996).

Plasmid manipulations, nucleotide sequencing, and computer analysis: Plasmids were constructed and transformed into *Escherichia coli* DH5αF′IQ using standard techniques (Sambrook *et al.* 1989). Oligonucleotide synthesis and nucleotide sequencing were performed by DNA Services, Cornell University. Nucleotide sequence data were analyzed using Lasergene Biocomputing Software (DNAStar, Madison, WI) and Sequencher (Gene Codes, Ann Arbor, MI). The Basic Local Alignment Search Tool (BLAST; Altschul *et al.* 1990) program was accessed through the National Center for Biotechnology Information or the Saccharomyces Genome Database to search for nucleotide and protein sequence similarities. RNA structures were predicted using the mfold version 3.0 program accessed at http://mfold2.wustl.edu/~mfold/rna/form1-2.3.cgi (Zuker 1994). The *C. albicans* genomic sequence determined by the Stanford DNA Sequencing and Technology Center was searched at http://www-sequence.stanford.edu/group/candida. The *S. pombe* genomic sequence was searched at http://www.sanger.ac.uk/Projects/S_pombe/blast_server.shtml. The *C. elegans* genomic sequence was searched at http://www.sanger.ac.uk/Projects/C_elegans/blast_server.shtml.

Genomic libraries: Genomic DNA from *S. bayanus* (NRRL #Y-12624), *S. kluyveri* (NRRL #Y-12651), or *S. servazzii* (NRRL #Y-12661) was prepared using either the QIAGEN Genomic-tip 500 kit (QIAGEN Inc., Valencia, CA) or the procedure of Philippsen *et al.* (1991). DNA was partially digested with *Sau*3AI and the partial digestion products were separated by size in a 10–40% sucrose gradient as described by Rose and Broach (1991). Fragments of 15–20 kb were pooled and ligated to *Bam*HI-cleaved YEp352 (Hill *et al.* 1986) and the libraries were amplified by standard methods (Rose and Broach 1991). The *S. bayanus* library was composed of ~15,000 independent *E. coli* transformants, 45% with genomic DNA inserts; the *S. servazzii* library, of ~10,000 independent transformants, 70% with inserts; and the *S. kluyveri* library, of ~40,000 independent transformants, 74% with inserts. A *K. lactis* (strain CBS2359) genomic library of 4- to 10-kb *Bam*HI fragments cloned into YepLac195 (Mulder *et al.* 1994) was obtained from L. Grivell.

Isolation of complementing plasmids from libraries: In all cases, Ura^+ clones were selected on minimal glucose medium after transformation of host strains (Table 1) with library or defined plasmid DNAs. Respiring clones were then identified by replica plating to YPEG medium. *PET111* homologs were obtained either by complementation of *pet111* in strain NB39-5D or by complementation of *cox7* in strain JM43-GD7. We obtained only a 3′ fragment of *S. servazzii PET111*: the *cox7*-complementing clone carried DNA coding only the C-terminal 238 residues of the protein, and we failed to isolate a larger clone with the entire gene. *PET122* homologs were obtained either by complementation of *pet122* mutation in strain PTH43 or by complementation of *oxa1* in strain MCC318. *PET494* homologs were sought by complementation of *pet494* in strain NGB108. *S. bayanus PET54* was isolated as a 3.0-kb *Bam*HI fragment of *S. bayanus* genomic DNA that hybridized at high stringency to an *S. cerevisiae PET54* probe. Its ability to complement was demonstrated by transformation of strain PTH352.

Analysis of the *S. kluyveri LYS9* region: *PET494* and *LYS9* are ~2.4 cM (6.6 kb) apart on *S. cerevisiae* chromosome XIV (Müller and Fox 1984). We isolated the *S. kluyveri LYS9* region in an attempt to clone a *PET494* ortholog. Six plasmids were obtained from the *S. kluyveri* library that complemented *S. cerevisiae lys9* strain TF112 (Table 1). Sequencing the ends of each insert allowed orientation of the six fragments based on overlapping end sequences. Sequencing of the *LYS9*-containing region on one clone revealed that *S. kluyveri LYS9* is adjacent to a gene similar to *S. cerevisiae MSO1* (GenBank accession no. AF170311), the same arrangement as in *S. cerevisiae*. However, flanking *MSO1* on the other side is a homolog (GenBank accession nos. AF170309 and AF170310) of *S. cere-*

TABLE 1

Yeast strains used in this study

Strain name[a]	Genotype[b]	Source or reference
DL1	*MAT***a** *lys2* [*rho*$^+$]	Mulero and Fox (1993a)
GW226	*MAT***a** *pet122-7 ura3-52 ade2 leu2-3,-112* [*rho*$^+$]	G. Wiesenberger
JM43-GD7	*MAT*α *cox7-*Δ*1 leu2-3,-112 ura3-52 trp1-289 his4-590* [*rho*$^+$]	Calder and McEwen (1991)
MCC318	*MAT***a** *oxa1::LEU2 ade2 ura3-*Δ [*rho*$^+$]	This study
NB39-5D	*MAT*α *pet111-9 lys2 ura3-52* [*rho*$^+$]	This study
NGB108	*MAT*α *pet494::LEU2 ura3-52 leu2-3,-112 ade2-101* [*rho*$^+$]	Brown (1994)
PTH43	*MAT***a** *pet122-7 ade2-101 ura3-52* [*rho*$^+$]	P. Haffter
PTH352	*MAT***a** *pet54-5 ade2-101 ura3-52 leu2-*Δ*ClaI-EcoRV* [*rho*$^+$]	P. Haffter
PTY11	*MAT*α *ura3-52 trp1-*Δ*1* [*rho*$^+$]	P. E. Thorsness
TF112	*MAT***a** *pet494-2 lys9 ura3-52* [*rho*$^+$]	This study
TWM10-41	*MAT***a** *pet122-6 ade2 ura3-*Δ [*rho*$^+$]	T. W. McMullin
K. lactis 2105-1D	α *ade1 ade2 leu*$^-$	Gunge and Sakaguchi (1981)
K. lactis CK56-7A	α *ade1 lysA1 ura3 atp2.1*	Chen and Clark-Walker (1996)
K. lactis CW64-1C	α *pet111*Δ*::kan*^{r} *ade1 lysA1 ura3*	This study
S. bayanus NRRL #Y-12624	Wild type	C. P. Kurtzman
S. kluyveri NRRL #Y-12651	Wild type	C. P. Kurtzman
S. kluyveri GRY1175	*MAT*α *ura3-sk1*	J. N. Strathern
S. kluyveri MCC328	*MAT*α *pet122*Δ*:URA3 ura3-sk1*	This study
S. kluyveri NB180	*MAT*α *pet111*Δ*::URA3 ura3-sk1*	This study
S. servazzii NRRL #Y-12661	Wild type	C. P. Kurtzman

[a] Strains are *S. cerevisiae* unless otherwise noted.
[b] Mitochondrial genotypes are in brackets; genes not in brackets are nuclear.

visiae YKL215c, located on *S. cerevisiae* chromosome XI, instead of an *S. kluyveri PET494* homolog. Flanking *S. kluyveri LYS9* on the other side is a homolog (GenBank accession no. AF170312) of *S. cerevisiae* YCR095c, located on *S. cerevisiae* chromosome III. We did not obtain a contiguous sequence of the entire region.

Cloning the mitochondrial *COX2* and *COX3* genes from other yeasts: Probes corresponding to the *S. cerevisiae COX2* and *COX3* coding sequences were produced by PCR, ^{32}P-labeled using standard techniques, and used to probe Southern blots of restricted genomic DNA from *S. kluyveri* (strain GRY1175) or *K. lactis* (*COX3* only; strain 2105-1D). Hybridizations were done at low stringency (55° in aqueous hybridization solution containing 6× SSC, 0.9 m NaCl, and 0.09 m sodium citrate). DNA fragments within the size range of cross-hybridizing bands were isolated and cloned to create minilibraries that were screened by colony hybridization to the *COX2* and *COX3* probes. *S. kluyveri COX2* was cloned in part on a 1.1-kb *Dra*I fragment (Table 2) that lacked the 5′ end and 5′-UTL coding region. Sequence of the remaining coding sequence and upstream region was obtained by J. Piskur by direct sequencing of purified *S. kluyveri* mitochondrial DNA. *S. kluyveri COX3* was cloned as overlapping 1.0-kb *Ssp*I and 4.0-kb *Dra*I fragments (Table 2). *K. lactis COX3* was cloned on a 1.5-kb *Msp*I fragment. All genomic clones were confirmed to be devoid of rearrangements, either by Southern blot hybridization to genomic DNA or by production of fragments of the expected size using PCR on genomic DNA.

Construction of null alleles in *S. kluyveri PET111* and *PET122* and in *K. lactis PET111*: *S. kluyveri PET111* was subcloned on a 5.8-kb *Eco*RI-*Xho*I fragment in pBluescriptKS(−) (Stratagene, La Jolla, CA) to create plasmid pNB143. pNB143 was cleaved with *Hin*dIII to remove a 2.1-kb fragment of the *PET111* gene containing the C-terminal 689 codons. A fragment with *Hin*dIII ends carrying the *URA3* gene, generated by PCR, was ligated into the *Hin*dIII-cleaved pNB143 backbone to generate pNB145. The pNB145 *Eco*RI-*Xho*I insert was gel-purified and used to transform the *ura3* mutant *S. kluyveri* strain GRY1175 (Table 1) to uracil prototrophy. PCR reactions were performed using genomic DNA of a transformant as a template, with primers corresponding to the *PET111* region and *URA3*, to verify that the gene replacement occurred as expected, leaving only the N-terminal 108 codons of *PET111*.

S. kluyveri PET122 was subcloned on a 2.4-kb *Bgl*II-*Cla*I fragment in pBluescriptM13(−) (Stratagene) to create plasmid pMC367. pMC367 was digested with *Eco*RV to remove 726 bp of the *PET122* coding sequence carrying 242 codons and religated with a *Bam*HI linker; then a *hisG::URA3::hisG* cassette (Alani *et al.* 1987) was ligated into the resulting *Bam*HI site to create plasmid pMC369. pMC369 was digested with *Spe*I and *Sal*I to cleave the *pet122*Δ*::URA3* insert from the plasmid backbone and was used to transform *S. kluyveri* GRY1175 (Table 1) to uracil prototrophy. PCR reactions were performed using genomic DNA of transformants as a template, with primers corresponding to the *PET122* region and *URA3*, to verify that the gene replacement occurred as expected leaving only the N-terminal 12 codons and the C-terminal 59 codons of *PET122*.

A 1.5-kb *Eco*RI fragment carrying the 5′ half of *K. lactis PET111* was subcloned from a *cox7*-complementing plasmid isolated from the *K. lactis* genomic library into pTZ19U (Bio-Rad, Richmond, CA) to generate the plasmid pKLN20. To disrupt *PET111* in pKLN20, a 1.4-kb fragment containing a kanamycin resistance cassette was obtained from plasmid pUG6 (Guldener *et al.* 1996) by digestion with *Xho*I and *Bgl*II, made blunt-ended using T4 DNA polymerase, and ligated to a 3.26-kb fragment generated by cleaving pKLN20 with *Apa*I and *Eco*RV. This disruption removed the 5′ 847 bp of *PET111* carrying the N-terminal 282 codons. A 1.8-kb *Eco*RI fragment from the resulting plasmid, pKLN22B, was used to transform *K. lactis*. An *atp2.1* mutant strain (CK56-7A; see Table 1) was used as a recipient for transformation because *atp* mutant strains recover better after transformation than wild type (G. D. Clark-Walker, unpublished results). Transformants

TABLE 2

Genes isolated in this study

Gene	Plasmid	GenBank accession no.
K. lactis COX3	pDP002	AF120716
K. lactis COX7	pCOX7-3-1-2	Sequence not determined
K. lactis PET111	pCOX7-3-1-2	AF120714
S. bayanus PET54	pMC311	AF023874
S. bayanus PET111	pNB92	AF056619
S. bayanus PET122	pMC347	AF026394
S. bayanus PET494	pMC346	AF056620
S. kluyveri COX2	pD1.1-2-12 (3′ end)	AF120715
S. kluyveri COX3	pS1.0-2-58 pD4.0-2-63	AF120717
S. kluyveri COX7	pSK28/4	Sequence not determined
S. kluyveri OXA1	pMC365	Sequence not determined
S. kluyveri PET111	pNB143	AF047833
S. kluyveri PET122	pMC367	AF026396
S. servazzii COX7	pSS1	Sequence not determined
S. servazzii OXA1	pMC355	AF026395
S. servazzii PET111	pSS1 (3′ end)	AF151694
S. servazzii PET122	pMC355	AF026395

resistant to G418 (200 μg/ml in YPD medium) were checked for stability of G418 resistance, and correct integration of the fragment at *PET111* was verified by Southern analysis. To isolate a strain carrying the *pet111* disruption and a wild-type *ATP2* gene, the *pet111* disruptant was crossed to a wild-type strain, the diploid was sporulated, and 15 asci were dissected. All spores were viable, and all tetrads showed 2:2 segregation of G418 resistance, marking the *pet111* disruption. All G418-resistant spores were respiratory deficient. Resistance to ethidium bromide (16 μg/ml in YPD medium), marking the *atp2.1* mutation, also segregated 2:2 and was unlinked to *pet111*Δ*::kan*^{r}. A strain carrying the *pet111* mutation but not the *atp2.1* mutation, CW64-1C (Table 1), was chosen for further study.

Isolation of mitochondria and immunological methods: Mitochondria were prepared from *S. cerevisiae* and *S. kluyveri* cells by differential centrifugation as described (Daum *et al.* 1982). Mitochondria were isolated from *K. lactis* as described for *S. cerevisiae* (Glick and Pon 1995), except that liquid cultures were grown in YPD, and crude mitochondria were purified on Nycodenz step gradients with 5% intervals. Wild-type *K. lactis* mitochondria floated at the 20%/25% interface of a 5% to 25% gradient, while *K. lactis pet111* mutant mitochondria floated at the 15%/20% interface of a 5% to 35% gradient.

Antibody against Cox2p was the mouse monoclonal CCO6 (a gift of T. L. Mason; Pinkham *et al.* 1994). Anti-Cox3p was the mouse monoclonal DA5 (Molecular Probes, Eugene, OR). Anti-Cox1p was the rabbit polyclonal SS7-T, obtained from G. Schatz. SDS-polyacrylamide gel electrophoresis and Western blotting were performed using standard techniques (Harlow and Lane 1988). Antigen-antibody complexes were visualized using horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (Gibco BRL, Bethesda, MD) secondary antibody and the enhanced chemiluminescence system (Amersham Life Science, Arlington Heights, IL). Immunoprecipitations were performed by standard techniques (Harlow and Lane 1988) except that goat anti-mouse IgG was added to the immunoprecipitation mixture before the addition of protein A-Sepharose.

***In vivo* labeling of mitochondrial translation products:** *In vivo* ^{35}S-labeling in the presence of cycloheximide was performed as described previously (Fox *et al.* 1991), except that *S. kluyveri* labelings were performed on exponential-phase rather than stationary-phase cells. SDS gel electrophoresis of labeled mitochondrial translation products was performed as described (Green-Willms *et al.* 1998).

RNA gel blot hybridization analysis: Total RNA was isolated from yeast strains grown to exponential phase in galactose-containing complete medium, using the hot-phenol extraction method (Ausubel *et al.* 1993). Equal amounts (10 μg) were subjected to electrophoresis, blotted to a filter, and probed with radioactively labeled probes produced by random-primed labeling (Boehringer Mannheim, Indianapolis). Blots were probed sequentially with labeled PCR products corresponding to the *S. kluyveri COX2* gene, the *S. kluyveri COX3* gene, and the *S. cerevisiae* mitochondrial 15S rRNA gene. mRNA abundance was quantitated using a Storm 840 Phosphoimager (Molecular Dynamics, Sunnyvale, CA) and the ImageQuant software package.

RESULTS

Isolation of genes from divergent budding yeasts, homologous to *S. cerevisiae* genes for mRNA-specific translational activators: *Complementation of S. cerevisiae mutations by functional homologs only from closely related species:* The phylogenetic relationships of the various yeasts used in this study are diagrammed in Figure 1. *S. bayanus* is very closely related to *S. cerevisiae*, based on analysis of ribosomal RNA sequences (Kurtzman and Robnett 1991, 1998; James *et al.* 1997). They are nearly identical phenotypically (Barnett *et al.* 1990) and have the same number of chromosomes, although their electrophoretic karyotypes are distinguishable (Naumov *et al.* 1992). Many genes are arranged in the same relative order and are similar enough to cross-hybridize (Ryu *et al.* 1996). The divergence of *S. servazzii* and *S. kluyveri* from *S. cerevisiae*, as well as from each other, is much

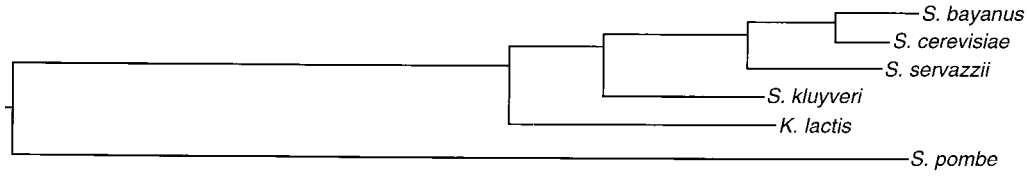


Figure 1.—Phylogenetic relationships of yeasts used in this study and the distantly related fission yeast *S. pombe*. Relative distances were calculated using the neighbor-joining method (Saitou and Nei 1987) to compare sequences of the D1/D2 domains of 26S rRNA (Kurtzman and Robnett 1998).

higher and comparable to that of *K. lactis* (Kurtzman and Robnett 1991, 1998; James *et al.* 1997). Each has distinct physiological characteristics and fewer chromosomes than *S. cerevisiae* (Vaughan-Martini *et al.* 1993).

To seek complementing functional homologs, we constructed libraries of *S. bayanus*, *S. servazzii*, and *S. kluyveri* genomic DNA in a high-copy plasmid vector carrying the *URA3* gene (materials and methods). Appropriate *S. cerevisiae* strains (materials and methods; Table 1) were transformed to Ura^+ with the libraries and transformants were screened for growth on nonfermentable carbon sources (Pet^+ phenotype). In cases where Pet^+ transformants were obtained, linkage to the *URA3* marker was tested in plasmid loss assays. Complementing plasmids were isolated from the yeast transformants, propagated in *E. coli*, and reintroduced into the same host strains to confirm complementation.

The *S. bayanus* library yielded complementing clones at a frequency of about 1 per 10,000 Ura^+ transformants when introduced into the *S. cerevisiae pet111*, *pet122*, and *pet494* mutants. *S. bayanus PET54* was isolated independently by cross-hybridization (materials and methods) and complemented when introduced into an *S. cerevisiae pet54* mutant. For all four genes, the *S. bayanus* homologs complemented as strongly as the corresponding wild-type *S. cerevisiae* genes.

The *S. servazzii* library yielded two clones that exhibited weak complementation, among 32,000 Ura^+ transformants of the *pet122* mutant. However, no Pet^+ colonies were found in 700,000 Ura^+ transformants of the *pet111* mutant strain, in more than 200,000 transformants of the *pet54* mutant strain, or in more than 130,000 transformants of the *pet494* mutant strain.

The *S. kluyveri* library failed to yield any complementing clones after screening of 170,000 Ura^+ transformants of the *pet111* mutant, 45,000 transformants of the *pet54* mutant, 131,000 transformants of the *pet122* mutant, and 51,000 transformants of the *pet494* mutant. This library did yield one *leu2* complementing clone out of 9,500 transformants, and four *oxa1* complementing clones (see below) out of 16,000 transformants, suggesting that the failure to complement translational activator mutations was not due to poor quality of the library. As shown below, direct tests with the *S. kluyveri* orthologs of *PET111* and *PET122*, isolated by a different approach, confirmed that they do not function in *S. cerevisiae*. These complementation results are summarized in Table 3.

Isolation of sequence homologs by complementation of mutations in neighboring S. cerevisiae genes: The *S. servazzii* DNA fragment with weak *pet122*-complementing activity contained a sequence homolog of *PET122* adjacent to a sequence homolog of the *OXA1* gene. This arrangement is similar to that in *S. cerevisiae*, where *PET122* is 215 bp away from *OXA1*. *OXA1* encodes a protein that is required for the assembly of cytochrome *c* oxidase and assembly or stability of ATP synthase and is functionally conserved in humans (Bonnefoy *et al.* 1994a,b; Altamura *et al.* 1996). This observation suggested that translational activator homologs might be isolated indirectly through cloning of genes from other yeasts by complementation of neighboring *S. cerevisiae* mutations, if synteny were preserved during evolutionary divergence

TABLE 3

Cross-complementation of *S. cerevisiae* mutations by genes from other yeasts

S. cerevisiae mutation	Species of origin of genomic clone			
	S. bayanus	*S. kluyveri*	*S. servazzii*	*K. lactis*
pet111	+++	−[a,b]	−[a]	−[b]
pet54	+++	−[a]	−[a]	n.t.
pet122	+++	−[a,b]	+	n.t.
pet494	+++	−[a]	−[a]	n.t.
cox7	n.t.	+	+	+
oxa1	n.t.	+	n.t.	−[a]

Ability of genomic clones from the indicated species to complement the respiratory growth defect of *S. cerevisiae* mutant strains. Complementation was assayed as growth on YPEG medium (see materials and methods). Plus signs indicate the strength of complementation, as measured by colony size: +++, like the wild-type *S. cerevisiae* gene; +, partial complementation; −, no complementation. n.t., not tested (we did not attempt to isolate a complementing clone from the indicated species). In the case of *S. servazzii OXA1*, we isolated a plasmid carrying the entire gene by virtue of its location next to *PET122*, but did not test its ability to complement the *S. cerevisiae oxa1* mutation. Negative results were of two types (below).

[a] Inability to isolate a complementing clone from a good-quality genomic library.

[b] Plasmid carrying the entire homologous gene from the indicated species failed to complement the corresponding *S. cerevisiae* mutation.

(Kuwabara and Shah 1994; Agnan *et al.* 1997). The only other *S. cerevisiae* specific translational activator gene that is closely linked to a highly conserved gene is *PET111*, which is situated 502 bp from the gene encoding subunit VII of cytochrome *c* oxidase, *COX7* (Calder and McEwen 1991). A Cox7p ortholog is present in the mammalian enzyme (Capaldi 1990), suggesting that the gene is likely to be present in other yeasts.

Null mutants in *cox7* and *oxa1* have a tight Pet^- phenotype (Calder and McEwen 1991; Bonnefoy *et al.* 1994a). We therefore transformed *cox7* and *oxa1* mutant strains (Table 1) with genomic libraries from *S. kluyveri* and *K. lactis* and screened as described above for Pet^+ transformants whose phenotype depended on plasmid-encoded genes. In addition, we transformed the *cox7* mutant with the *S. servazzii* library in an attempt to isolate a *PET111* homolog.

We obtained plasmids complementing *cox7* from the *S. servazzii*, *S. kluyveri*, and *K. lactis* (Mulder *et al.* 1994) genomic libraries, and sequencing revealed the presence of a *PET111* homolog in the genomic fragments from each of these species (Table 2). The *S. servazzii* clone contained only the 3′ end of the *PET111* gene, but the others carried complete coding sequences. We also obtained plasmids complementing *oxa1* from the *S. kluyveri* library, and they contained a complete *PET122* homolog (Table 2). Direct testing of complementation, by transformation of the corresponding *S. cerevisiae* mutant strains with *S. kluyveri PET111* and *PET122* and *K. lactis PET111*, demonstrated that none of these genes functions in *S. cerevisiae* (Table 3). We were unable to find any *oxa1* complementing plasmids from the *K. lactis* library among more than 1.3×10^6 transformants. Since the library appears to be good (it yielded four *COX7* clones among 25,000 transformants), and since the Oxa1p ortholog from humans functions in *S. cerevisiae* (Bonnefoy *et al.* 1994b), the most likely explanation for this result is that the *K. lactis OXA1* gene is not expressed in *S. cerevisiae*.

There are no characterized *S. cerevisiae* genes immediately adjacent to *PET54* or *PET494* suitable for cloning by complementation. However, we attempted to clone *PET494* from the *S. kluyveri* library by complementation of the linked gene *LYS9* (Müller and Fox 1984), which is located 6.6 kb from *PET494* in *S. cerevisiae*. We isolated six independent *S. kluyveri* genomic fragments that complemented the *lys9* strain TF112 (Table 1). Unfortunately, partial sequencing of these fragments revealed that *PET494* and *LYS9* are not syntenic in *S. kluyveri*. Instead, the *S. kluyveri LYS9* chromosomal region has sequences similar to at least three different *S. cerevisiae* chromosomes (materials and methods).

Sequence comparisons among specific translational activators from budding yeasts: Comparison of the proteins coded by orthologous genes of the sister species *S. cerevisiae* and *S. bayanus* reveals that they are all similar in sequence: 73% identity for Pet111p, 72% for Pet54p and Pet122p, and 76% for Pet494p. Sequence identity is evenly dispersed over the lengths of these protein pairs, with one notable exception. Residues 198–259 of *S. cerevisiae* Pet54p probably comprise an RNA recognition motif (RRM; reviewed in Burd and Dreyfuss 1994). This region of Pet54p has 27% identity and 50% similarity to the identified RRM (residues 143–216) of the *C. elegans fox-1* protein, a known RNA-binding protein involved in sex determination (Hodgkin *et al.* 1994). Although the match between Pet54p and the RRM consensus is weak enough that the motif is not identifiable by comparison with databases such as PROSITE (Bairoch *et al.* 1997) or BLOCKS (Henikoff and Henikoff 1994), it was identified in a search using a profile generated from more than 300 known RRMs (M. E. Cusick, personal communication). In the predicted RRM region, *S. bayanus* Pet54p is 90% identical to *S. cerevisiae* Pet54p, while the overall identity between the two proteins is 72%. This is not surprising given the ability of *S. bayanus* Pet54p to interact with the *S. cerevisiae COX3* mRNA 5′-UTL.

Comparisons of the complete Pet111p-homologous sequences from *S. kluyveri* and *K. lactis* reveal that they are highly diverged from that of *S. cerevisiae* and from each other: the *S. cerevisiae* and *S. kluyveri* proteins are 32% identical while the *S. cerevisiae* and *K. lactis* proteins are only 20% identical (Figure 2; Table 4). While we obtained the sequence of only 238 C-terminal residues of *S. servazzii* Pet111p, these residues exhibited a similar degree of sequence divergence (Table 4). Similarity between all the sequences is evenly distributed over their entire lengths and there are no highly conserved regions (Figure 2).

Comparisons of the Pet122p-homologous sequences from *S. servazzii* and *S. kluyveri* also revealed a high degree of divergence. The very weakly complementing *S. servazzii PET122* encodes a protein that is 27% identical to *S. cerevisiae* Pet122p and has a C-terminal extension of 31 residues relative to *S. cerevisiae* Pet122p. *S. kluyveri* Pet122p, which failed to complement, is 33% identical to *S. cerevisiae* Pet122p and has a C-terminal extension of 42 residues relative to it. Sequence identity is distributed evenly over the whole lengths of Pet122p sequences except for the C-terminal extensions, which do not resemble each other (not shown). The longest stretch of amino acid identity shared by all four Pet122p sequences is eight amino acids, corresponding to residues 145–152 of *S. cerevisiae* Pet122p.

Mitochondrially coded RNA targets of S. kluyveri and K. lactis translational activators: The *COX2*- and *COX3*-mRNA- specific translational activators interact functionally with targets in the 5′-UTLs of the mitochondrially coded mRNAs (Costanzo and Fox 1988, 1993; Mulero and Fox 1993a,b; Brown *et al.* 1994; Wiesenberger *et al.* 1995). In the case of the 54-nucleotide (nt) *COX2* 5′-UTL of *S. cerevisiae*, a central 32-nt region containing a stem-loop structure is necessary and par-

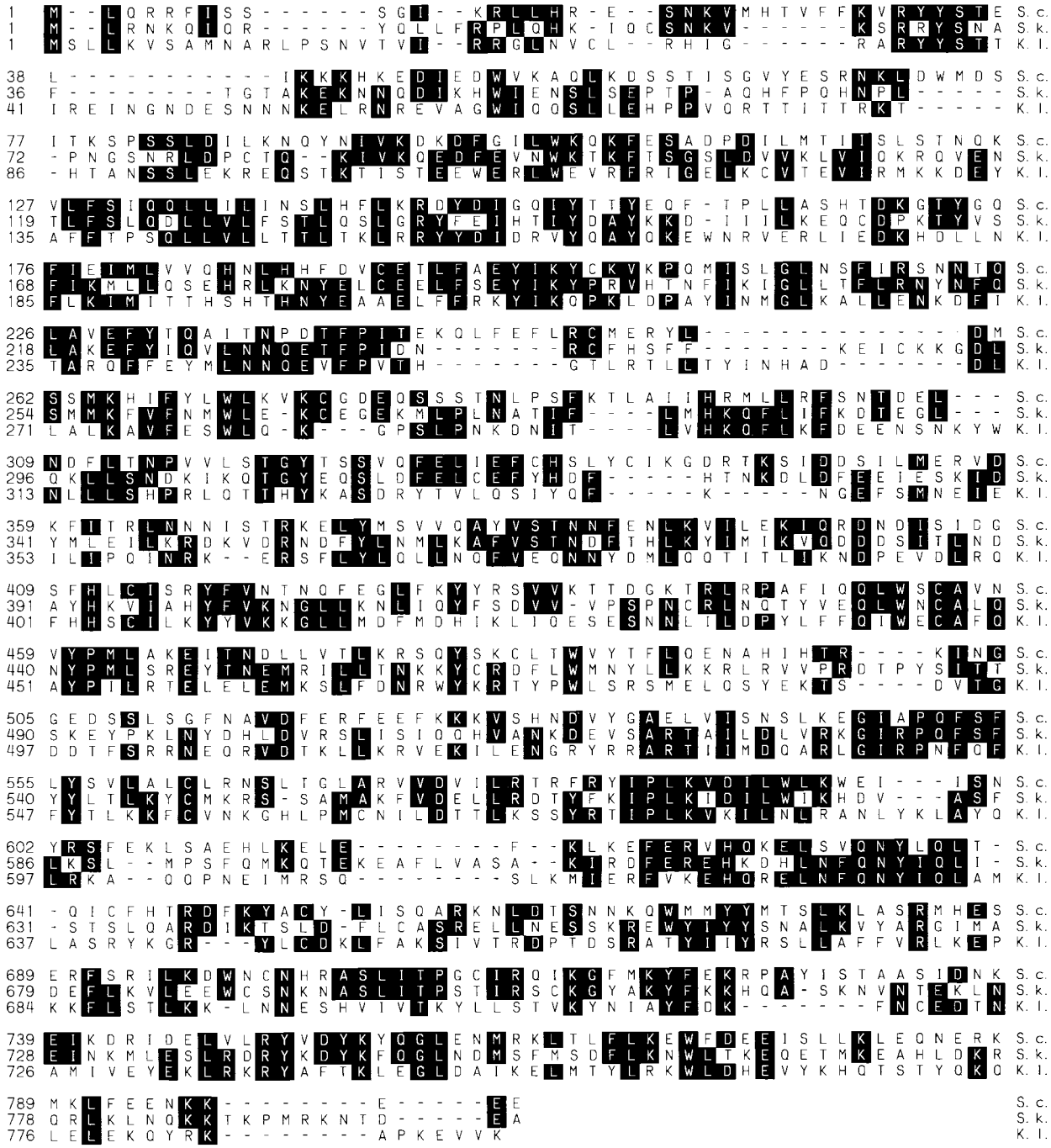


Figure 2.—Alignment of Pet111p orthologs from *S. cerevisiae* (S.c.), *S. kluyveri* (S.k.), and *K. lactis* (K.l.). Sequences were aligned using the Jotun-Hein method with the MegAlign program (materials and methods). Identical amino acids are shaded. Dashes represent gaps.

tially sufficient for Pet111p activation of translation (Dunstan *et al.* 1997). This region contains the sequence UCUAA, which has been found upstream of *COX2* in a number of budding yeasts, including *K. lactis* (Hardy and Clark-Walker 1990; Clark-Walker and Weiller 1994). UCUAA comprises part of the stem structure and is necessary for translation in *S. cerevisiae* (Dunstan *et al.* 1997). The nature of the target(s) in the 613-nt *COX3* 5′-UTL of *S. cerevisiae* is less well understood. However, a 151-nt region in its upstream half is necessary and, with modifications, partially sufficient for translational activation (Wiesenberger *et al.* 1995).

To allow further comparisons, we cloned and sequenced *COX2* and *COX3* from *S. kluyveri* and *COX3* from *K. lactis* (materials and methods and Table 2). These data revealed that Cox2p and Cox3p are far more highly conserved than the translational activators: *S. kluyveri* and *K. lactis* Cox2p share 89 and 86% amino

TABLE 4

Percentage identity between Pet111p and Pet122p orthologs from different budding yeasts

	S. bayanus (%)	*S. kluyveri* (%)	*S. servazzii* (%)	*K. lactis* (%)
		A. Pet111p		
S. cerevisiae	73	32	24[a]	20
S. bayanus		34	24[a]	23
S. kluyveri			25[a]	27
S. servazzii				21[a]
		B. Pet122p		
S. cerevisiae	80	33	27	
S. bayanus		31	25	
S. kluyveri			39	

Percentage identity over the entire lengths of the proteins (except where noted) as determined using BLAST analysis (Altschul *et al.* 1990).

[a] Based on the C-terminal 238 residues of *S. servazzii* Pet111p.

acid identity to *S. cerevisiae* Cox2p, respectively, and 91% identity to each other. *S. kluyveri* and *K. lactis* Cox3p have 85 and 82% identity to *S. cerevisiae* Cox3p, respectively, and 87% identity to each other.

Comparison of the *COX2* and *COX3* mRNA 5′-UTLs is complicated by the fact that the 5′ ends of the *S. kluyveri* and *K. lactis* mRNAs have not been experimentally determined. However, *S. cerevisiae*, *K. lactis*, and several other budding yeasts share the same mitochondrial promoter consensus sequence (TATAAGTAA) (Osinga *et al.* 1982; Clark-Walker *et al.* 1985; Ragnini and Frontali 1994; Biswas 1998), and thus *S. kluyveri* is likely to as well. Indeed, upstream of the *S. kluyveri COX2* gene there is a perfect match to the promoter consensus at positions −95 to −87, predicting an 88-nt 5′-UTL. The *K. lactis COX2* promoter appears to direct cotranscription of $tRNA^{Val}$ and the *COX2* mRNA from 330 nt upstream of the *COX2* initiation codon (Hardy and Clark-Walker 1990). In *S. cerevisiae*, $tRNA^{Val}$ is cotranscribed with the *COX3* mRNA, and 3′-processing of the tRNA generates the mRNA 5′- end (Wiesenberger *et al.* 1995). Similar events in *K. lactis* would produce a 226-nt *COX2* mRNA 5′-UTL.

In the *S. cerevisiae COX2* mRNA 5′-UTL, the downstream side of the 5-bp stem contains the first four bases of the conserved sequence UCUAA and ends at −20 relative to the initiation codon (Dunstan *et al.* 1997; Figure 3). The *S. kluyveri COX2* 5′-UTL also contains a putative stem-loop structure with a 4-bp stem whose downstream side contains the first three bases of the UCUAA sequence and ends at position −21 (Figure 3). A potential stem structure containing the conserved sequence UCUAA is also present in the *K. lactis COX2* 5′-UTL (Figure 3). The 4-bp stem could form in a position similar to those of *S. cerevisiae* and *S. kluyveri*, with the 3′ end of the stem at position −30. However, in this case, the UCUAA sequence would be in the upstream half of the stem. Other than the UCUAA element, there is no significant primary sequence conservation between the *COX2* 5′-UTLs of *S. cerevisiae*, *S. kluyveri*, and *K. lactis*.

The *S. kluyveri COX3* gene is apparently transcribed from a perfect match to the consensus mitochondrial promoter sequence at −331 to −323. A $tRNA^{Val}$ gene is located at positions −289 to −217. If the $tRNA^{Val}$ and *COX3* are cotranscribed and processed as in *S. cerevisiae*, then the *COX3* 5′-UTL would be 216 nt in length. Upstream of *K. lactis COX3* there is a consensus promoter sequence at −147 to −139, suggesting that its *COX3* 5′-UTL would be 140 nt in length. We could find no significant similarities between the *COX3* 5′-UTL sequences of *S. cerevisiae*, *S. kluyveri*, and *K. lactis* as detectable by analysis with the BLAST or MegAlign programs (materials and methods).

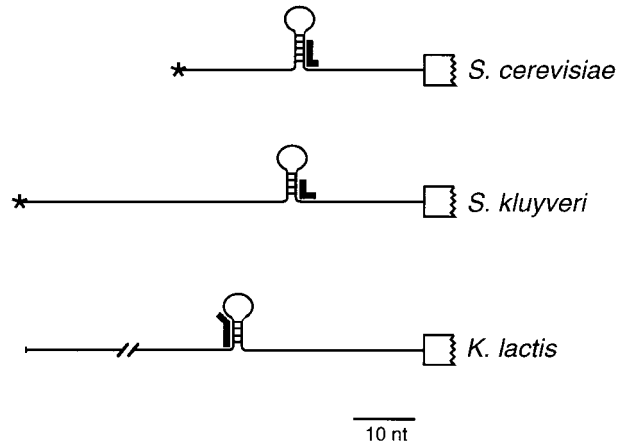


Figure 3.—A conserved sequence and secondary structure in the 5′-untranslated leaders of *COX2* mRNAs from *S. cerevisiae*, *S. kluyveri*, and *K. lactis*. Lines represent 5′-UTLs, while boxes represent the 5′ ends of the *COX2* coding sequences. Distances between the coding sequence and the predicted hairpin loop (19 nt in *S. cerevisiae*, 20 nt in *S. kluyveri*, 29 nt in *K. lactis*) are drawn to scale. Thick black line indicates the location of UCUAA (Hardy and Clark-Walker 1990) in a predicted stem loop (Dunstan *et al.* 1997) in each 5′-UTL. Asterisk at the 5′ end of *S. cerevisiae* and *S. kluyveri* 5′-UTLs indicates that they are known or predicted primary transcripts; the 5′ end of the *K. lactis COX2* mRNA is predicted to arise from a processing event.

Mutations in homologous genes of *S. kluyveri* and *K. lactis* demonstrate orthologous mRNA-specific translational activator functions: *Null mutations in S. kluyveri PET111 and PET122 and in K. lactis PET111 cause nonrespiratory phenotypes:* The highly diverged *PET111* and *PET122* homologs isolated from *S. kluyveri* and *K. lactis* failed to complement the corresponding mutations in *S. cerevisiae*, raising the question of whether they were truly orthologous in function. To answer this question, we constructed null mutations in these genetically tractable yeast species by deleting substantial portions of each coding region from their chromosomal DNA and replacing them with either the *S. cerevisiae URA3* gene or a kanamycin resistance cassette (materials and methods). These null mutations in *S. kluyveri PET111*, *K. lactis PET111*, or *S. kluyveri PET122* all prevented respiratory growth on nonfermentable carbon sources. In each case, transformation with the corresponding *S. cerevisiae* gene failed to restore respiratory growth of the null mutant (not shown).

S. kluyveri pet111 and pet122 mutants and a K. lactis pet111 mutant are specifically deficient in cytochrome c oxidase: To test whether the *S. kluyveri pet111* and *pet122* mutants affected cytochrome *c* oxidase we compared their whole-cell cytochrome absorption spectra to that of an isogenic wild-type *S. kluyveri* strain, as well as to the corresponding *S. cerevisiae* strains (Figure 4). The cytochrome aa_3 peak, corresponding to cytochrome *c* oxidase, was missing from the absorption spectrum of each mutant strain, while the other cytochrome peaks were unaffected. We have obtained a similar result for a *K. lactis pet111* null mutant (N. Bonnefoy and G. D. Clark-Walker, unpublished results). This indicates that the respiratory defects of the *S. kluyveri pet111* and *pet122* mutant strains and of the *K. lactis pet111* mutant are due to a specific cytochrome *c* oxidase deficiency, as is the case in *S. cerevisiae*.

pet111 and pet122 null mutations in S. kluyveri and K. lactis block translation of the COX2 and COX3 mRNAs, respectively: The fact that *pet111* and *pet122* null mutations blocked respiration in *S. kluyveri* and *K. lactis* and specifically affected cytochrome *c* oxidase in *S. kluyveri* suggested that they were orthologous to their *S. cerevisiae* counterparts. To test this possibility directly, we examined expression of the mitochondrial *COX2* and *COX3* genes in the mutant strains.

Accumulation of Cox2p and Cox3p in mitochondria from the *S. kluyveri* mutant strains was assayed by Western blotting using monoclonal antibodies against the *S. cerevisiae* proteins (Figure 5A). Cox2p and Cox3p from wild-type *S. kluyveri* mitochondria had approximately the same SDS-gel mobility as the corresponding *S. cerevisiae* proteins and cross-reacted well with the antibodies. The *S. kluyveri pet111* null mutant strain completely lacked Cox2p and had significantly reduced levels of Cox3p, while the *S. kluyveri pet122* mutant strain completely lacked Cox3p and had significantly reduced levels of Cox2p (Figure 5A). Thus the *pet111* and *pet122*

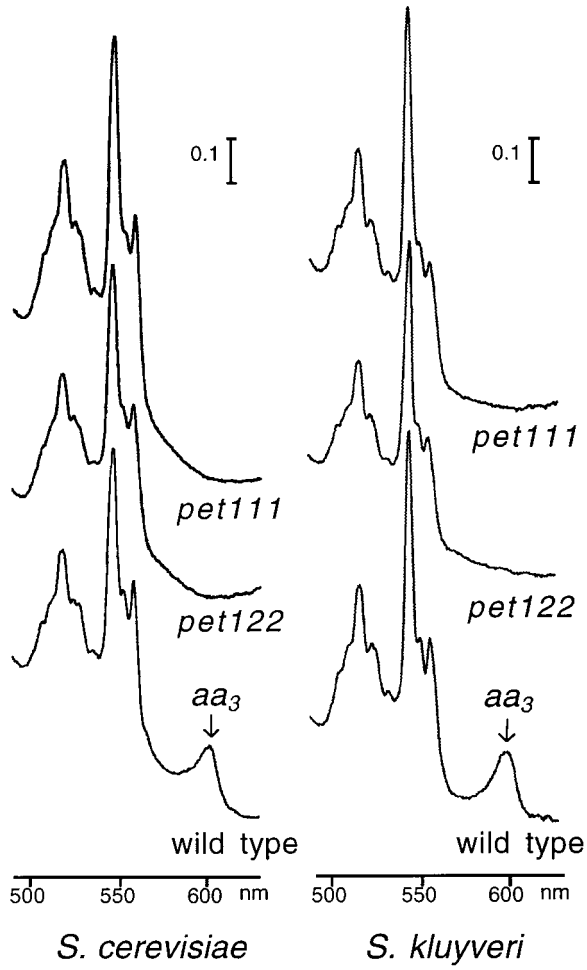


Figure 4.—Whole-cell absorption spectra of *S. cerevisiae* and *S. kluyveri* wild-type and *pet111* or *pet122* mutants. Low temperature absorption spectra of galactose-grown cells were recorded as described by Claisse *et al.* (1970). The absorption maximum expected for the alpha band of cytochromes *aa3* at 602 nm is indicated. The absorbance scale is indicated at the top right of each panel. The strains used were the following: *S. cerevisiae*: wild type, DL1; *pet122*, GW226; *pet111*, NB39-5D. *S. kluyveri*: wild type, GRY1175; *pet122*, MCC328; *pet111*, NB180.

null mutations in *S. kluyveri* specifically blocked accumulation of Cox2p and Cox3p, respectively, as they do in *S. cerevisiae*. The reduced accumulation of Cox3p in the *pet111* mutant and of Cox2p in the *pet122* mutant is probably due to degradation of the unassembled subunits. *S. cerevisiae* Cox2p and Cox3p are known to be unstable when cytochrome *c* oxidase assembly is blocked (Pearce and Sherman 1995; Glerum and Tzagoloff 1997; Lemaire *et al.* 1998).

The phenotype of the *K. lactis pet111* mutant was examined by Western blotting of mitochondrial proteins with antibodies against *S. cerevisiae* Cox1p and Cox2p (Figure 5B). (The available antibody against *S. cerevisiae* Cox3p failed to cross-react with *K. lactis* Cox3p.) *K. lactis* Cox1p and Cox2p comigrated with the homologous *S. cerevisiae* proteins (data not shown). The *K. lactis pet111* mutant mitochondria had no detectable Cox2p, but

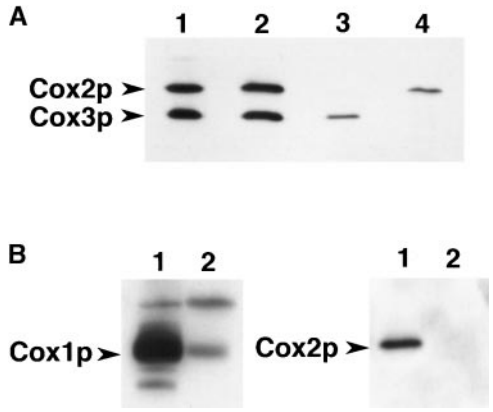


Figure 5.—Accumulation of mitochondrial translation products in *S. kluyveri* and *K. lactis* mutant strains. (A) A total of 20 μg per lane of mitochondrial protein from the indicated *S. cerevisiae* and *S. kluyveri* strains (materials and methods) were subjected to electrophoresis on a 16% SDS-polyacrylamide gel and Western blotted. The blot was probed with mouse monoclonal antibodies against *S. cerevisiae* Cox2p and Cox3p (materials and methods). Lane 1, wild-type *S. cerevisiae* (strain PTY11); lane 2, wild-type *S. kluyveri* (strain GRY1175); lane 3, *pet111* mutant *S. kluyveri* (strain NB180); lane 4, *pet122* mutant *S. kluyveri* (strain MCC328). (B) Left, 30 μg per lane of mitochondrial protein from the indicated *K. lactis* strains (materials and methods) were subjected to electrophoresis on a 12% SDS-polyacrylamide gel and blotted. The blot was probed with rabbit polyclonal antisera against *S. cerevisiae* Cox1p (materials and methods). Right, 10 μg per lane of the indicated mitochondrial proteins were treated as above and probed with mouse monoclonal antibody against *S. cerevisiae* Cox2p. Lane 1, wild-type *K. lactis* (strain KB101); lane 2, *pet111* mutant *K. lactis* (strain CW64-1C).

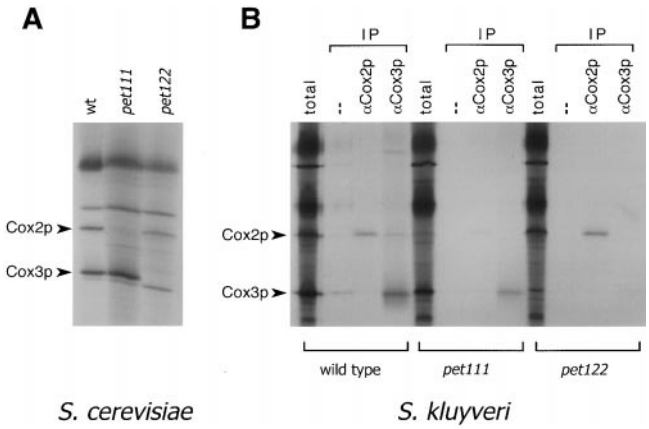


Figure 6.—Effects of *S. kluyveri pet111* and *pet122* mutations on mitochondrial protein synthesis. Mitochondrial translation products were ^{35}S-labeled *in vivo* in the presence of cycloheximide. Portions of each sample were solubilized in SDS and immunoprecipitated; immunoprecipitates and total labeled proteins were subjected to electrophoresis on SDS-16% polyacrylamide glycerol-containing gels and autoradiographed (see materials and methods). (A) *In vivo*-labeled total mitochondrial translation products from *S. cerevisiae* wild type (wt; strain DL1), *pet111* mutant (strain NB39-5D), and *pet122* mutant (strain TWM10-41). (B) *In vivo*-labeled total *S. kluyveri* mitochondrial translation products (total) or immunoprecipitated *S. kluyveri* mitochondrial translation products (IP). Immunoprecipitations were performed with no serum (––), with mouse monoclonal antibody against *S. cerevisiae* Cox2p (αCox2p), or with mouse monoclonal antibody against *S. cerevisiae* Cox3p (αCox3p). *S. kluyveri* strains used were the following: GRY1175, wild type; NB180, *pet111* mutant; and MCC328, *pet122* mutant.

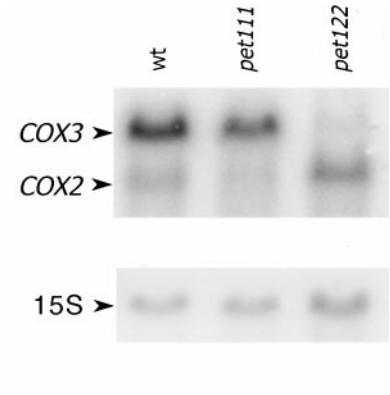
contained Cox1p, albeit at a reduced level relative to wild type. Thus, the *K. lactis pet111* mutation appears to specifically block accumulation of Cox2p. The reduced Cox1p level in the *pet111* mutant strain is presumably due to instability of the unassembled protein.

To test whether the *S. kluyveri pet111* and *pet122* mutations affected synthesis of Cox2p and Cox3p, we examined mitochondrial translation products labeled *in vivo* in the presence of cycloheximide by SDS-gel electrophoresis and autoradiography (Figure 6). Unfortunately, the pattern of mitochondrial translation products in wild-type *S. kluyveri* (Figure 6B) was neither as reproducible nor as clear as that in *S. cerevisiae* (Figure 6A). To positively identify bands corresponding to *S. kluyveri* Cox2p and Cox3p, we immunoprecipitated each protein from *in vivo*-labeled mitochondria using monoclonal anti-Cox2p and anti-Cox3p antibodies before gel electrophoresis (Figure 6B). This analysis showed that synthesis of Cox2p was greatly reduced in the *S. kluyveri pet111* mutant while synthesis of Cox3p was nearly normal. Conversely, no synthesis of Cox3p was detectable in the *pet122* mutant but Cox2p was synthesized at wild-type levels (Figure 6B). These data suggested that *S. kluyveri* Pet111p and Pet122p are required for translation of Cox2p and Cox3p, respectively. This analysis could not be carried out in *K. lactis* since it is resistant to cycloheximide (Dehoux *et al.* 1993).

Finally, we compared steady-state levels of the *COX2* and *COX3* mRNAs in wild-type *S. kluyveri* to the *pet111* and *pet122* mutants by Northern hybridization (Figure 7). The mRNA levels were normalized to levels of the mitochondrial 15S ribosomal RNA. In the *pet111* mutant, both the *COX2* and *COX3* mRNA levels were reduced to ~50% of wild type. In the *pet122* mutant, the *COX2* mRNA level was slightly reduced, while the *COX3* mRNA level was reduced to ~7% of wild type. These results are consistent with a role for *S. kluyveri* Pet111p and Pet122p in translational activation. They apparently also affect mRNA stability. In *S. cerevisiae*, *pet111* mutations reduce the levels of *COX2* mRNA to 10–30% of wild-type levels (Poutre and Fox 1987), while *pet122* mutations reduce the level of the *COX3* mRNA to ~50% of wild type (Wiesenberger and Fox 1997).

DISCUSSION

Mitochondrial gene expression systems have diverged to an extraordinary degree during the evolution of eukaryotes (Gray *et al.* 1999). In this study, we have begun to explore the extent to which the mRNA-specific trans-



COX3	1.00	0.64	0.07
COX2	1.00	0.56	0.85

Figure 7.—Abundance of the mitochondrial *COX2* and *COX3* mRNAs in *S. kluyveri* translational activator mutant strains. Total RNA from *S. kluyveri* wild type (wt, GRY1175), *pet111* mutant (NB180), and *pet122* mutant (MCC328) was subjected to electrophoresis, blotted to a membrane, and probed with radioactively labeled *S. kluyveri COX2* or *COX3* genes or with the *S. cerevisiae* mitochondrial 15S rRNA gene (see materials and methods). Table at bottom shows the level of each mRNA, normalized to the level of 15S rRNA, in the mutant strains.

lational activation system uncovered in *S. cerevisiae* may be conserved in other organisms. We found that among budding yeasts, both the nuclearly coded activator proteins and their mitochondrially coded mRNA 5′-UTL targets have diverged extensively in sequence. However, the correspondences between activator protein orthologs and the mitochondrial translation products they control have been conserved.

Among *S. bayanus* and *S. kluyveri*, an assessment of divergence from *S. cerevisiae* is hampered by the fact that only a small set of gene sequences is available (<20 complete sequences at present), and it is biased in favor of those that function in *S. cerevisiae*. Nevertheless, the orthologs from *S. bayanus* and *S. kluyveri* are all more diverged from the *S. cerevisiae* translational activators than any other known protein from those species. (No protein-coding sequences from the nuclear genome of *S. servazzii* are currently available, except those reported in this article.)

A set of *K. lactis* random partial sequences representing 296 genes has recently been generated and compared to *S. cerevisiae* (Ozier-Kalogeropoulos *et al.* 1998). Overall, the sequences in the random set have a mean amino acid identity of 63.6% to their *S. cerevisiae* counterparts (Ozier-Kalogeropoulos *et al.* 1998), considerably higher than the 20% identity observed between *K. lactis* and *S. cerevisiae* Pet111p. Eight of the genes identified by Ozier-Kalogeropoulos *et al.* encode proteins with similarity to *S. cerevisiae* proteins known to be involved in mitochondrial biogenesis, and the amino acid identity of these sequence fragments to the *S. cerevisiae* proteins ranges from 77 to 42%. Interestingly, the least conserved of these genes encodes a potential Pet54p ortholog, with 42% identity over 57 residues to *S. cerevisiae* Pet54p (Ozier-Kalogeropoulos *et al.* 1998). Thus, mRNA-specific mitochondrial translational activators appear to be among the most rapidly diverging proteins in budding yeasts, despite their conserved functions.

In some cases, rapid protein divergence driven by positive Darwinian selection has been detectable through an elevated ratio of missense to silent nucleotide substitutions (Whitfield *et al.* 1993; Lee *et al.* 1995; Sutton and Wilkinson 1997). Much more often, however, rapid divergence appears to reflect the absence of positive or negative selection. In Drosophila species, a large number of proteins exhibit very rapid neutral divergence (Schmid and Tautz 1997). Our analysis of substitutions in the genes reported here is consistent with rapid neutral evolution of the translational activator proteins. In this context it is interesting to note that even Pet111p and Pet122p amino acids that can mutate to suppress mutations in their target mRNA 5′-UTLs or in other proteins in the *S. cerevisiae* genetic system (Costanzo and Fox 1993; Mulero and Fox 1993a; Brown *et al.* 1994) are not highly conserved in the other species.

The mitochondrially coded targets of the mRNA-specific translational activators in the *COX2* and *COX3* mRNA 5′-UTLs also appear to have evolved rapidly. This contrasts sharply with the highly conserved mitochondrially coded protein sequences, which are known to evolve more slowly in yeasts than the nuclearly encoded cytochrome *c* (Clark-Walker 1991). Even the lengths (known or predicted) of the 5′-UTLs are quite different from each other: the *COX2* mRNA 5′-UTLs in *S. cerevisiae*, *S. kluyveri*, and *K. lactis* are 54, 88, and 226 nt, respectively, while the *COX3* mRNA 5′-UTLs in the same species are 613, 216, and 140 nt. The only significant conserved sequence element among any of the 5′-UTLs is the UCUAA pentanucleotide previously noted in the *COX2* 5′-UTLs of *S. cerevisiae*, *K. lactis*, *Hansenula saturnus*, and *Torulopsis (Candida) glabrata* (Hardy and Clark-Walker 1990); we found the same element in *S. kluyveri*. However, while it is clear that other sequences in the *COX2* 5′-UTL are important functionally in *S. cerevisiae* (Dunstan *et al.* 1997), these are not recognizably conserved in the other yeasts.

The correspondences of translational activator proteins to their target mRNAs are conserved in the species studied here. The phenotypes of *S. kluyveri pet111* and *pet122* null mutants and of a *K. lactis pet111* null mutant show that these genes are truly orthologous to their *S. cerevisiae* counterparts. The weak functional complementation of the *S. cerevisiae pet122* mutation by *S. servazzii PET122* indicates conservation of orthologs in that species as well. Thus, the proteins and their RNA targets are coevolving.

Experimentally, the activator dependence of mitochondrial mRNAs can be altered without eliminating translation by *in vivo* expression of chimeric mRNAs containing 5′-UTLs and coding sequences derived from different genes (Müller *et al.* 1984; Costanzo and Fox 1986, 1988; Poutre and Fox 1987; Rödel and Fox 1987; Mulero and Fox 1993b; Manthey and McEwen 1995). The conservation of the mRNA specificity during the evolution of budding yeasts—despite this fact and despite the sequence divergence of the system's components—suggests that the mRNA specificity has adaptive value in promoting efficient synthesis of respiratory complexes. The untranslated regions of the *COX2* and *COX3* mRNAs play a role in correctly localizing translation of Cox2p and Cox3p (Sanchirico *et al.* 1998). Thus, it seems plausible that the membrane-bound mRNA-specific translational activators aid in assembly of the respiratory chain by topologically ordering the synthesis of key components on the inner membrane surface.

mRNA-specific translational activation may be a general feature of fungal mitochondrial gene expression. The nuclearly coded *Neurospora crassa* gene *cya-5* is required at a post-transcriptional step in mitochondrial expression of Cox1p (Nargang *et al.* 1978; Coffin *et al.* 1997). Interestingly, its protein product has significant sequence similarity to Pet309p, a membrane-bound protein specifically required for *COX1* mRNA translation in *S. cerevisiae* (Manthey and McEwen 1995; Manthey *et al.* 1998), strongly suggesting that the CYA-5 protein and Pet309p are orthologous (Coffin *et al.* 1997). Possible *PET309* orthologs are also present in the currently available genomic sequences of *C. albicans* and *Schizosaccharomyces pombe*, two fungi for which nearly complete sequence data are available. Interestingly, the soluble maize chloroplast translational activator protein CRP1 exhibits sequence similarity to Pet309p (Fisk *et al.* 1999). There are no significant matches to Pet111p, Pet54p, Pet122p, or Pet494p in the available *C. albicans* and *S. pombe* sequences (see materials and methods). However, given the high divergence we find for these proteins among more closely related species, it seems possible that orthologs are present in those species but are difficult to detect by sequence similarity alone.

There is little information on how translation initiation occurs in animal systems. Animal mitochondrial mRNAs typically lack 5′-UTLs (Attardi and Schatz 1988). Thus, if translational activation occurs in animal mitochondria, there must be significant differences in mechanism compared to fungi. There are no predicted sequences significantly similar to yeast translational activators in the *C. elegans* genome (materials and methods; Consortium 1998), except for similarity between Pet54p and the *fox-1* gene product (Hodgkin *et al.* 1994), which is confined to the RRM domain. Thus, mitochondrial translational activators could represent a possible target for antifungal drugs.

Estimates of the conservation of gene order between *K. lactis* and *S. cerevisiae* range from 50% (Ozier-Kalogeropoulos *et al.* 1998) to ~74% (Keogh *et al.* 1998). While there are not enough data available from *S. servazzii* or *S. kluyveri* to make accurate predictions, our results suggest that the approach of cloning conserved genes syntenic to highly diverged genes of interest (Kuwabara and Shah 1994; Agnan *et al.* 1997) will often be fruitful for genes of these yeasts.

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