On the mitochondrial ribosome synthesis and genetic interactions between the nucleus and mitochondria in Saccharomyces cerevisiae

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(Degree)
博士（学術）

(Date of Degree)
1991-03-31

(Date of Publication)
2015-03-31

(Resource Type)
doctoral thesis

(Report Number)
甲0979

(JaLCDOI)
https://doi.org/10.11501/3057163

(URL)
https://hdl.handle.net/20.500.14094/D1000979

博 士 論 文

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平成３年１月

神戸大学大学院自然科学研究科

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＜パン酵母 (*Saccharomyces cerevisiae*) のミトコンドリアリボソーム合成における核とミトコンドリアの遺伝的相互作用＞

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On the Mitochondrial Ribosome Synthesis and Genetic Interactions between the Nucleus and Mitochondria in *Saccharomyces cerevisiae*

A dissertation for partial fulfillment of a Doctoral Degree

at the Department of Science and Technology

of the Postgraduate School of Kobe University

January, 1991

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Acknowledgements

I am grateful to Prof. Katsumi Isono for his guidance, encouragement, and useful suggestions throughout the whole of this work, to Dr. Madoka Kitakawa and Mr. Yasuhiko Matsushita for help, stimulating discussion and for providing with clones containing *MRP-L8*, *MRP-L20*, *YMR31*, and *YMR44*, to Mr. Seiji Tanaka for help, discussion and providing with the filters prepared from OFAGE, and to Dr. Setsuko Isono and all members of the laboratory of Genetics, Department of Biology, Kobe University for their help, discussion and friendship. Thanks are also due to Drs. Akira Ishihama and Nobuyuki Fujita of the National Institute of Genetics for their help in amino acid sequencing, to Prof. Akio Toh'e of University of Tokyo for providing plasmid YIp1, and to Drs. Hanns-Rüdiger Graack and Lutz Grohmann of the Max-Planck-Institut für Molekulare Genetik in Berlin, Germany, for the N-terminal sequencing of YmL33 protein.

Summary

The mitochondrial ribosome of yeast (*Saccharomyces cerevisiae*) consists of two RNA molecules and as many as 70 proteins (Faye and Sor, 1977) or even more (Graack, 1990). These components are encoded by both nuclear and mitochondrial genes. The two ribosomal RNAs are encoded in the mitochondrial DNA, while all except one of the ribosomal proteins are encoded by nuclear genes. They are, therefore, translated on cytoplasmic ribosomes and then transported into mitochondria (Tzagoloff and Myers, 1986). Thus, the biogenesis of mitochondrial ribosomes requires that genes in both mitochondria and nucleus must be transcribed and resultant messengers are translated in a concerted manner. The endosymbiont hypothesis predicts that the mitochondrion is a descendent of a prokaryotic symbiont in the eukaryotic cell (Schwartz and Dayhoff, 1978; Gray and Doolittle, 1982). Several lines of evidence for this theory have accumulated, a notable example of which is that the secondary structure of mitochondrial ribosomal RNAs from several organisms resembles that of *E. coli* ribosomal RNA (Tell and Fox, 1988).

It is interesting to investigate the structural characteristics of the genes encoding mitochondrial ribosomal proteins from an evolutionary point of view and to establish how their genes are organized and regulated in the nucleus and mitochondria. To ask the questions, I have undertaken cloning and characterization of several nuclear genes for mitochondrial ribosomal proteins by using synthetic oligonucleotide mixtures corresponding to the N-terminal amino acid sequences.

The *YMR26* gene was cloned by hybridization with synthetic oligonucleotide

mixtures corresponding to the N-terminal amino acid sequence of YMR26 protein. The nucleotide sequence of a cloned segment containing this gene showed the presence of an open reading frame capable of encoding a basic protein (net charge = +21) of 18.5 kDa with 158 amino acid residues. The deduced amino acid sequence showed no significant similarity to any known ribosomal proteins of prokaryotic or eukaryotic origins. In addition, the gene for YmL33 protein was cloned as well: in this case, the yeast genomic DNA corresponding to part of the YmL33-coding region was amplified by polymerase chain reaction (PCR) using two kinds of oligonucleotide mixtures synthesized on the basis of the N-terminal amino acid sequence of protein YmL33. By nucleotide sequencing of the cloned gene, termed *MRP-L33*, it was found to encode a basic protein (net charge = +10) of 11 kDa with 99 amino acid residues. The N-terminal two-thirds of the deduced amino acid sequence showed a significant degree of sequence similarity to ribosomal protein L30 of *E. coli*. In addition, the C-terminal one-third showed sequence similarity, though to a lesser extent, to a yeast cytoplasmic ribosomal protein termed L16. Comparison of the amino acid sequence with that deduced from nucleotide sequence indicates that both YMR26 and YmL33 proteins lack a leader sequence.

By hybridization with the yeast chromosomes separated by pulse-field gel electrophoresis, the *YMR26* was found to reside on either chromosome VII or XV and the *MRP-L33* gene on either chromosome XIII or XVI. Insertional gene disruption experiments indicate that both YMR26 and YmL33 proteins are essential for the mitochondrial function. Moreover, the inactivation of the two genes led to instability of the mitochondrial genome, resulting in the creation of ρ^- or ρ^o derivatives.

The expression level of the *YMR26* and *MRP-L33* genes was monitored upon catabolite repression in strains with various mitochondrial genetic backgrounds. In ρ^+ cells, the transcription of these genes was more repressed in a medium with glucose than in those will either galactose or nonfermentable carbon source (glycerol). However, in ρ^o cells, their transcription appeared not to be repressed even with a high concentration of glucose. Further studies were performed on the degree of coordination in the expression of the nuclear encoded mitochondrial ribosomal genes after the introduction of extra copies of *YMR26*, *MRP-L33*, *MRP-L8*, *MRP-L20*, *YMR31* or *YMR44* gene. Increases in their dosage were accompanied by increases in the amount of their mRNA, indicating that their transcription is not strictly coupled with each other.

Introduction

1. Cell and subcellular organelles

1-1. Gene expression in the subcellular organelles

All living organisms from bacteria to animals and plants are composed of cells. The cells are categorized into two types, prokaryotic cells and eukaryotic cells. Prokaryotic cells including bacteria are relatively simple, and eukaryotic cells, especially of higher animals and plants, are more complex and are different in their morphology and functions. A prokaryotic cell possesses plasma membrane which encloses the single cytoplasmic compartment containing DNA, RNA, proteins and their complexes with lipids and carbohydrates. In contrast, a eukaryotic cell has a membrane-bound nucleus which contains most of DNA molecules. In addition, other membrane-bound structures are present in the eukaryotic cytoplasm. Of such subcellular structures, mitochondria and chloroplasts occupy a unique position because of their possession of DNA molecules and all the machinery for transcription and translation of the genetic information. Thus, a eukaryotic cell contains two to three (in case of plants) genetic information systems.

Even though the size of mitochondrial DNA varies from approximately 16 kb in animals to more than 100 kb in plants (Wallace, 1982), all fungal and animal mitochondrial DNA studied to date code for two rRNAs, a variable but complete set of tRNAs, and mRNAs for subunits 1, 2 and 3 of cytochrome oxidase, the cytochrome b, and subunits 6 and 8 of ATPase complex (Tzagoloff and Myers, 1986). These genes are probably also present in the genomes of plant mitochondria. In fact, rRNAs (Stern et al., 1982), several subunits of cytochrome

oxidase, and cytochrome b (Fox and Leaver, 1981) have been confirmed to be present in plant mitochondria. By the recent nucleotide sequencing of chloroplast DNA (Ohyama et al., 1986; Hiratsuka et al., 1989), it was found that the chloroplast DNA codes for rRNAs, tRNAs, RNA polymerase and several ribosomal proteins and proteins involved in photosynthesis. However, there are several hundred proteins in chloroplasts and mitochondria, and the proteins encoded in their own genomes are not enough. Recently, several nuclear genes have been found to code for the proteins of mitochondria and chloroplasts. For examples in yeast, the genes for RNA polymerase (Kelly et al., 1986), aminoacyl-tRNA synthetases (Myers and Tzagoloff, 1985; Pape et al., 1985; Natsoulis et al., 1986), and cytochrome c (Tzagoloff and Myers, 1986) have been cloned from the nuclear DNA. Therefore, many proteins involved in the biogenesis of organelles are dependent on the expression of genes located in the nuclear DNA. The nuclear-encoded proteins are synthesized in the cytoplasm and then imported into organelles.

In addition to nuclear encoded organellar proteins, there are protein complexes whose subunits are encoded in nuclear genes as well as in organellar genes. The yeast mitochondrial ATPase complex, for example, contains ten defined subunits: of these, seven are encoded in nuclear genes and three are encoded in mitochondrial genes (Dujon, 1981). In the case of chloroplast ribulose bisphosphate carboxylase-oxygenase (Rubisco), the genes for eight small subunits are located in the nuclear genome and the eight large subunits are encoded in chloroplast genes (Smith and Ellis, 1979). To produce an active form of these enzymes, therefore, the genes in both organelles and the nucleus must be expressed in a concerted manner. It is interesting to ask how the expression of genes encoded in the two genomes is coordinated and how the nuclear-encoded

proteins are targeted efficiently and unambiguously to organelles.

The examples described above clearly indicate the genetic contribution of both organellar and nuclear genes for the organellar biogenesis, which in turn implies the existence of mechanisms for coordinate expression of genes encoding proteins and RNAs in the two genomes. Recent studies in *S. cerevisiae* have identified some of the molecular mechanisms by which heme and catabolites regulate the expression of nuclear genes for mitochondrial proteins. "Upstream activator sequence (UAS)" have been identified on the 5'-side of the TATA boxes of the genes for iso-1-cytochrome c (Guarente and Mason, 1983) and iso-2-cytochrome c (Pfeifer et al., 1987). These UAS's resemble enhancer sequences of higher eukaryotes and are conceived to mediate the stimulation of transcription of both genes by heme or its inhibition by catabolites. *Trans*-acting factors encoded in *HAP1* (Pfeifer et al., 1987), *HAP2* and *HAP3* (Forsburg and Guarente, 1988) also regulate the synthesis of subunits of cytochrome c oxidase (Pinkham et al., 1987). Interaction between such *trans*-acting factors and enhancer-like *cis*-elements affects the expression of nuclear genes encoding mitochondrial proteins and may be one of the fundamental mechanisms by which the expression of nuclear and mitochondrial genes is coordinated. However, virtually nothing is known about how the organellar genetic system influences nuclear genes. Schatz and Mason (1974) showed that the export of polypeptides or RNA from mitochondria have been negative. However, Parikh et al. (1987) found that the transcription of some yeast nuclear genes responded to the absence or damage of mitochondrial DNA. The mechanism of this response is unclear, but it needs not depend on the export of macromolecules from mitochondria.

The nuclear-encoded proteins must be accurately targeted to transport them

from the cytoplasm, their site of synthesis, to their site of functional residence, organelles. These proteins involve sorting signals that allow them to be specifically identified and then delivered to their correct organellar destination. Many proteins are synthesized as longer precursors with transient amino-terminal amino acid extensions (Schatz and Butow, 1983). This presequence is processed from the protein during or after import into organelles, but pre-processed precursors are not imported into mitochondria *in vitro* (Gasser et al., 1982). In addition, only a presequence is found to be sufficient to deliver a protein into organelle. For example, the presequence of yeast cytochrome c oxidase subunit IV was fused to the cytosolic protein dehydrofolate reductase, and the resulting hybrid protein was transported into the mitochondrial matrix *in vitro* (Hurt et al., 1984). On the other hand, it was also clearly demonstrated that some mitochondrial proteins such as cytochrome c (Zimmermann et al., 1979a) and ADP/ATP translocator protein (Zimmermann et al., 1979b) were not synthesized as precursors and yet they were efficiently imported into mitochondria. Furthermore, non-amino-terminal (carboxyl terminal) regions of nuclear-encoded proteins have been shown to contain specific transport signals (Pfanner et al., 1987a; Pfanner et al., 1987b). Recently, several components involved in the transporting process were identified and their structure and function characterized (Pfanner and Neupert, 1990). Among these, the existence of two distinct receptors were investigated to function specifically for proteins with or without presequence (Söllner et al., 1989; Söllner et al., 1990).

1-2. The origin of subcellular organelles

There are two hypotheses concerning the origin of mitochondria and

chloroplasts: one is that they arose by the compartmentalization of DNA within the cytoplasm of an evolving protoeukaryote; the other is that they arose from free-living forms that established symbiotic relationships with host cells (Schwartz and Dayhoff, 1978; Gray and Doolittle, 1982). A large number of data supports the latter endosymbiont hypothesis. From rRNA sequence comparison, it is quite clear that nuclear and organellar genomes are derived from different lineages: organellar rRNA sequences are prokaryotic in character and quite different from cytoplasmic rRNA sequence (Gray et al., 1984). In addition, the secondary structure of the mitochondrial rRNAs from several organisms resembles that of *E coli* rRNA (Tell and Fox, 1988). Some features of gene expression in organelles resemble that of prokaryotes rather than that of eukaryotes. First, the protein synthesis of mitochondria and chloroplasts is sensitive to bacterial inhibitors such as chloramphenicol, streptomycin, erythromycin and tetracycline. Conversely, cycloheximide, which inhibits cytoplasmic protein synthesis in eukaryotes, does not affect the protein synthesis in mitochondria and chloroplasts. Secondly, mitochondria and chloroplasts use a formylated $tRNA^{Met}$ as initiator of translation (Gray and Doolittle, 1982) as in bacterial translation. Thirdly, chloroplast ribosomes are able to use bacterial tRNAs in protein synthesis: it is even possible to make functional hybrid ribosomes by combining a chloroplast small subunit with an *E. coli* large subunit (Alberts et al., 1989). Finally, the organellar DNA does not complex with histones like prokaryotic DNA.

If the endosymbiont hypothesis is correct, then, most of the genes for organellar proteins must have been transferred from the organellar genome to the nucleus during the course of evolution. In this connection, it should be noted that evidence of gene transfer has been accumulating (Baldauf and Palmer, 1990),

suggesting that the process is still continuing. The plant *tufA* encodes the chloroplast protein elongation factor Tu (EF-Tu) and is located in chloroplasts in green algae. In higher land plant, however, the gene is found in the nuclear DNA. One charophycean alga, *Coleochaete orbicularis*, was shown to contain the *tufA* gene encoded in both nucleus and chloroplasts but the chloroplast gene-encoded protein is non-functional (Baldauf et al., 1990). Another example is the gene for subunit 9 of ATPase complex. In higher eukaryotes, this subunit is encoded by a nuclear gene. This is also true in *N. crassa*, although in this organism a copy of the gene is conserved in the mitochondrial DNA as well (van den Boogaart et al., 1982). The nuclear gene appears to be expressed while the mitochondrial gene may be a pseudogene (Tzagoloff and Myers, 1986). The corresponding gene is encoded in the mitochondrial DNA in yeast.

2. Yeast mitochondrial ribosome

Ribosomes are present in all prokaryotic and eukaryotic cells. In contrast to bacterial cells, eukaryotic cells contain two distinctly different types of ribosomes: one in the cytoplasm and the other in mitochondria and/or chloroplasts. The former type of ribosome is larger in size with a sedimentation coefficient of 80S, while the latter has a coefficient of 70S or smaller. The ribosomes of mitochondria and chloroplasts are interesting because the genes for the constituents of organellar ribosomes are present in both genetic systems (Tzagoloff and Myers, 1986).

In the case of the mitochondrial ribosome of *Saccharomyces cerevisiae*, the 15S and 21S rRNAs and one ribosomal protein termed VAR1are encoded in the mitochondrial DNA (Tzagoloff and Myers, 1986). The remaining approximately 70

ribosomal proteins are encoded by nuclear genes, translated on cytoplasmic ribosomes, and imported into the mitochondria. The biogenesis of the mitochondrial ribosome, therefore, depends on the activity of two physically separated sets of genes in a concerted manner. The genes for the mitochondrial encoded components have been identified and sequenced, but relatively little is known about the genes for the nuclear-encoded ribosomal proteins. Recently, several nuclear genes for mitochondrial ribosomal proteins have been cloned and characterized in yeast and in other organisms. It has been shown that some of the mitochondrial ribosomal proteins of yeast show significant sequence similarity to those of *E. coli*. For example, mitochondrial ribosomal proteins MRP2, MRP7 and YmL8 are considerably similar to *E. coli* ribosomal protein S14, L27 and L17, respectively (Myers et al., 1987; Fearon and Mason, 1988; Kitakawa et al., 1990). On the other hand, there are other mitochondrial ribosomal proteins of yeast characterized so far which show no such sequence similarity (Grohmann et al., 1989; Matsushita et al., 1989; Partaledis and Mason, 1988).

These data suggest that there are apparently three classes of mitochondrial ribosomal proteins in yeast. One is those originated from the original prokaryote, the putative mitochondrial ancestor, and still contains several conserved primary sequences due most likely to some structural and/or functional constraints. Another class of proteins is those which are derived from the original prokaryote but have undergone extensive amino acid changes during the evolution of mitochondria so that their sequence similarity to *E. coli* ribosomal proteins can no longer be traced. A third class of proteins is those which are derived from preexisting nuclear genes that were originally unrelated to mitochondria and were newly adopted for mitochondrial ribosomes. However, it is, at least at present,

difficult to distinguish between the second and third class of mitochondrial ribosomal proteins if they do exist. Like the nuclear encoded mitochondrial proteins, the transcription of mitochondrial ribosomal protein genes has been shown to be affected by catabolites such as glucose (Myers et al., 1987; Paretaledis and Mason, 1988). The level of transcription in cells growing in medium with nonfermentable carbon sources is significantly higher than that in cells growing with a fermentable carbon source such as glucose.

To investigate the structural characteristics of the genes encoding mitochondrial ribosomal proteins and to establish how their genes are organized and regulated in the nucleus and mitochondria, I have begun to identify, clone and characterize additional nuclear genes for the components of mitochondrial ribosome. In this study, I describe the isolation and molecular characterization of two nuclear genes designated *YMR26* and *MRP-L33*. The *YMR26* gene was cloned by hybridization with oligonucleotide mixtures corresponding to the N-terminal amino acid sequence of the YMR26 protein. The gene was found to encode a basic protein of 18.5 kDa and to exist in single copy on either chromosome VII or XV. The *MRP-L33* gene codes for a large subunit protein, YmL33, and was cloned after amplification of DNA by polymerase chain reaction (PCR) using two kinds of oligonucleotide mixtures synthesized on the basis of the N-terminal amino acid sequence. The *MRP-L33* gene encodes a basic protein of 11 kDa and is located as a single copy gene on either chromosome XIII or XVI. In addition, the N-terminal region of YmL33 protein shows a high degree of sequence similarity to *E. coli* ribosomal protein L30. These two proteins have no presequence and are essential for the mitochondrial function. The transcription of the *YMR26* and *MRP-L33* genes respond to catabolite repression. Their

expression was not linked to each other and to the expression of the genes for other mitochondrial ribosomal proteins.

Almost all genes for mitochondrial ribosomal protein are found to be essential for the mitochondrial function. However, two genes-*MRP13* and *YMR31* have been found to be non-essential, because their gene disruptants grew normally on nonfermentable carbon sources (glycerol) (Paretaledis and Mason, 1988; Matsushita, 1991). Therefore, it is interesting to investigate whether the cells carrying both *MRP13* and *YMR31* genes disrupted are able to grow on nonfermentable carbon source or not. Necessary strain construction was performed and this point was examined. It was found that cells with two disrupted genes were quite normal. Significance of these findings are discussed in terms of cooperative gene expression between mitochondria and the nucleus.

Materials and Methods

1. Strains and vectors

1-1. Strains

E. coli	HB101	F-, *leu, thi, lacY, str^r, hsdR, hsdM, EndoI-, recA*
	XL-1blue	*endA1, hsdR17, supE44, thi-1, recA, gyrA, relA,* Δ*(lac)* F' (*proAB, lacI^{q}ZΔM15,* Tn10)
S. cerevisiae	AB972 ρ^o	α, *trp1*
	D13-1A	a, *trp1, his3, gal1, suc2, cup1, mal*
	DC04	α, cir^o, *ade1, leu2*
	DC-5	a, *leu2-3, leu2-112, his3, can1*
	DC-5 ρ^o	a ρ^o derivative of DC-5
	DC-K12	a/α, DC-5xK12-2A *leu2-3, leu2-112*
	K12-2A	α, *leu2-3, leu2-112, ura3*
	KWK01	a derivative of D13-1AxDC04 α, *ade1, leu2, his3*
	KWK02	a derivative of D13-1AxDC04 a, *ade1, leu2, his3, trp1*
	KWK03	a derivative of D13-1AxDC04 a, *ade1, his3, trp1*
	YM078	a derivative of K12-2AxKWK03 α, *ura3, leu2, his3, trp1*
	07173	a/α, wild type

1-2. Vectors

Bacterial	pBR322	Amp^r, Tet^r
	pUC118	Amp^r

	pUC119	Amp^r
Shuttle	YEp13	Amp^r, Tet^r, *LEU2*
	YEpH	a derivative of YEp13, Amp^r, *HIS3*
	Ylp1	Amp^r, *HIS3*

2. Media

2-1. Media for bacteria

LT	1 % tryptone, 0.5 % yeast extract, 0.5 % NaCl, 25 μg/ml Thymidine
AN	1 x minimal A solution, 0.5 % glucose, if necessary, add amino acid and base (amino acid; 60 μg/ml, thiamine; 10 μg/ml, thymidine; 20 μg/ml)

10 x minimal A stock solution was prepared by dissolving as follows:

$(NH_4)_2SO_4$	50.00 g
K_2HPO_4 (or $K_2PO_4 \cdot 3H_2O$)	350.00 g (458.50 g)
KH_2PO_4	150.00 g
Na_3·citrate·$3H_2O$ (Na_3·citrate·$2H_2O$)	25.00 g (23.50 g)
$MgSO_4 \cdot 7H_2O$	1.02 g
H_2O	to 500 ml

2-2. Media for yeast (Sherman et al., 1986)

YPD	2 % peptone, 1 % yeast extract, 2 % glucose
YPG	2 % peptone, 1 % yeast extract, 3 % glycerol
YPGal	2 % peptone, 1 % yeast extract, 2 % galactose
SD	0.17 % yeast nitrogen base without amino acid, 2 % glucose if necessary, add amino acid and base (final con. ade; 20 μg/ml, his; 20 μg/ml, leu; 60 μg/ml, trp; 40 μg/ml, ura; 20 μg/ml)
SDG	0.17 % yeast nitrogen base, 3 % glycerol, 0.1 % glucose

Sporulation medium: 1 % potassium acetate, 0.1 % yeast extract, 0.05 % glucose

All media were solidified, when necessary, with 2 % agar.

3. Analysis of proteins

3-1. Isolation of yeast mitochondrial ribosomes

(1) Isolation of yeast mitochondria (Schatz, 1967; Guérin et al., 1979)

1. 200 g cells of commercial baker's yeast were washed with H_2O three times and with breaking buffer two times.

   Breaking buffer: 10 mM Tris-HCl (pH 7.0), 0.65 M Sorbitol, 0.1 M EDTA (pH 8.0), after autoclave 0.025 % BSA and 4 drops of 5 N KOH per 1 l were added.

2. Cells were resuspended in 140 ml of breaking buffer and disrupted mechanically with glass beads (0.5 mm diameter) in a Dyno Mill.
3. Disrupted cell suspension was centrifuged in JA-10 rotor at 4,700 rpm for 10 min in a Beckman J2-21 centrifuge. The supernatant was then centrifuged in a JA-10 rotor at 10,000 rpm for 26 min at 4°C.
4. The resultant pellet and brown supernatant were cleared by differential centrifugation for 10 min each: twice at 3,500 and 12,000 rpm in JA-17 rotor of Beckman J2-21 centrifuge.
5. After centrifugation at 15,000 rpm for 10 min, approximately 2 g wet weight of mitochondrial pellet was obtained from 200 g wet weight yeast cells.

(2) Puromycin treatment of yeast mitochondria

1. Mitochondrial pellet was suspended in 1.5 ml of mitochondrial suspension buffer.

Mitochondrial suspension buffer	50 mM Tris-HCl (pH 7.4), 0.1 M EDTA (pH 8.0), 0.5 M Sorbitol

2. 60 mg of BSA, 167 μl of 0.3 M ATP (pH 6.7), 0.5 ml of 2 mg/ml puromycin, and 10 ml puromycin incubation medium were added into mitochondrial suspension.

Puromycin incubation medium	40 mM Tris-HCl (pH 8.0), 300 mM KCl, 20 mM KH_2PO_4, 20 mM $MgCl_2$, 10 mM α-ketoglutaric acid,0.6 % BSA

Distilled water was added to fill up to 20 ml, mixed and stored at 27°C for 10 min.

3. The suspension was centrifuged at 12,000 rpm for 10 min in a JA-17 rotor and supernatant was discarded.

(3) Solubilization with Triton X-100 and sucrose gradient centrifugation (Lambowitz, 1979)

1. The mitochondrial pellet was suspended in 4.5 ml HKCTSH 100/50.

10x HKCT 100/50	1 M KCl, 0.5 M $CaCl_2 \cdot 2H_2O$, 0.25 M Tris-HCl (pH 7.5)
HKCTSH 100/50	20 ml of 10x HKCT 100/50, 84 μl of 2-mercaptoethanol, 180 ml of H_2O

2. Mitochondria were solubilized by incubating with 2 % Triton X-100 and cleared by a centrifugation at 12,000 rpm for 15 min.
3. The supernatant was overlaid on a 10 %-30 % sucrose gradient and centrifuged at 2°C and 19,000 rpm for 16 hrs. in a SRP-28SA rotor of the Hitachi 80P centrifuge.
4. Fractions containing ribosomes were identified by measuring absorbance at 260 nm and combined.
5. Ribosomes were then precipitated with 10 % PEG6000 on ice for overnight.

3-2. Two-dimensional gel electrophoresis

(1) Extraction of ribosomal proteins

1. 3 volumes of extraction solution were added into ribosomal solution and mixed for 1hr with stirring in the cold room.

   Extraction solution 68 mM $MgCl_2$, 68 % acetic acid

2. The solution was centrifuged at 15,000 rpm for 10 min at 4°C in a JA-17 rotor. The supernatant was dialyzed against 2 % acetic acid and lyophilized.

(2) First-dimensional gel electrophoresis

1. Gel mixture was prepared by dissolving as follows:

Acrylamide	4.00 g
Bis-acrylamide	0.10 g
Bis-Tris	1.19 g
Urea	38.0 g
EDTA	0.20 g
H_2O	to 100 ml

   adjusted pH to 5.0 with acetic acid.

2. 35 μl of TEMED and 100 μl of 7 % APS were ed into 10 ml ice-cold gel mixture, mixed and added into gel tubes (upto 10 cm of 0.6 x 11 cm glass tubes). 4 M urea was overlaid.

3. The ribosomal protein samples were dissolved in 100 μl of sample-loading buffer.

   Sample-loading buffer — 36 g urea, 1 ml of DTT, 10 ml of 10 x upper buffer, upto 100 ml with H_2O, and 3 drops of 0.5 mg/ml of basic fushsin per 20 ml

   10 x Upper buffer (pH 4.0) — 20.9 g Bis-Tris and 45 ml of acetic acid per 1 l

   10 x lower buffer (pH 5.0) — 175.7 g K-acetate and 49 ml of acetic acid per 1 l

4. After eliminating the urea, the gel was placed in the electrophoresis chamber.

Dissolved samples prepared as above were overlaid.

5. In the cold room, electrophoresis was started at a constant current of 1~2 mA for 30 min. After the dye migrated into the gel completely, the current was increased to 4 mA.
6. After electrophoresis, the gels were taken out by carefully inserting a syringe needle and by introducing 80 % glycerol.

(3) Second-dimensional gel electrophoresis (Mets and Bogorad, 1974)

1. First-dimension gels were placed along the top of second dimension gel slabs immediately after electrophoresis.

Second slab gel	0.143 M Bis-Tris (pH 6.75 with HCl), 10 % (w/v) acrylamide,0.25 % (w/v) bis-acrylamide. Per 35 ml, 25 µl TEMED and 60 µl of 10 % APS were added.
Upper buffer	0.07 M MES, 0.07 M Bis-Tris, 3.5 mM SDS. 0.05 ml of 80 % thioglycolic acid per 400 ml buffer
Lower buffer	0.028 M Bis-Tris (pH 6.75 with HCl)

2. The electrophoresis was carried out at a constant current of 25 mA/gel.
3. When the gel was stained, staining was performed by soaking into the staining solution for 15 min and destaining were carried out overnight in the destaining solution.

Staining solution	0.5 % Coomasie Brilliant Blue R-250, 20 % acetic acid and 50 % methanol
Destaining solution	10 % methanol, 7.5 % acetic acid

3-3. Electroblotting (Matsudaira, 1987) and **Amino acid sequencing**

1. After electrophoresis, gels were soaked in the transfer buffer for 5 min.

Transfer buffer 10 mM CAPS (pH 11.0), 10 % methanol

2. A sheet of PVDF (polyvinylidene difluoride) membrane was rinsed with 100 % methanol and stored in the transfer buffer.
3. The gel was sandwiched between the PVDF membrane and several sheets of blotting paper, placed into a blotting apparatus and the proteins were electroeluted for 1 hr at 90 V in the transfer buffer.
4. The PVDF membrane was washed in deionized H_2O for 5 min, stained for 5 min, and destained for 10 min.

   Staining solution 0.1 % CBB, 50 % methanol

   Destaining solution 50 % methanol, 10 % acetic acid
5. The membrane was rinsed in deionized H_2O for 5 to 10 min and air-dried.
6. Each protein spot was cut out with a clean razor blade, centered onto a Teflon seal and placed in a cartridge block of the model 477A protein sequencer (Applied Biosystem) for N-terminal amino acid sequencing.

4. Preparation and Analysis of yeast DNA

4-1. Isolation of chromosomal DNA

(1) Large scale preparation (Niederacher and Entian, 1987)

1. 4 ml of a preculture was transferred to 200 ml of YPD medium, and incubated at 28°C overnight until OD_{600} = 5 ~ 10.
2. The cells were harvested, washed with TE, and resuspended in 10 ml of SCE solution.

   SCE solution 1 M sorbitol, 100 mM trisodium citrate (pH 7.0 with 10 N NaOH), 60 mM EDTA

   TE 10 mM Tris-HCl, 1 mM EDTA

3. 160 µl of 2-mercaptoehanol and 3.5 mg Zymolyase 100-T were added and incubated at 37°C for 2 hrs. Spheroplast formation was monitored by removing 50 µl of the mixture into 200 µl of 1 M sorbitol and 200 µl of 10 % SDS.
4. 10 ml of SDS solution were added and mixed by inverting the tubes and stored at 65°C for 15 min.

   SDS solution 50 mM Tris-HCl (pH8.0), 10 mM EDTA, 2 % SDS
5. The mixture was cooled down to room temperature. 4 ml of 5 M NaCl was added and stored on ice for 1 hr.
6. The mixture was centrifuged and the supernatant was discarded. The pellet was resuspended in 10 ml of TES buffer, 100 µg of proteinase K was added and incubated at 37°C overnight.

   TES solution 10 mM Tris-HCl (pH 8.0), 5 mM EDTA, 0.5 % SDS
7. The mixture was centrifuged at 15,000 rpm for 10 min at 4°C. The supernatant was extracted twice with phenol/chloroform (1:1) and once with chloroform.
8. RNase A was added (final; 1 mg/ml) and incubated at 37°C for 30 min. Phenol/chloroform and chloroform extraction was repeated.
9. 2 volumes of ethanol were added, the chromosomal DNA was wound up and washed with 70 % ethanol.
10. The DNA was dissolved in 500 µl TE.

(2) Rapid isolation (Hoffman and Winston, 1987)

1. 10 ml of YPD medium was inoculated with a single colony and incubated at 30°C overnight (approximate to full growth).
2. The cells were harvested, washed with H_2O and transferred into a 1.5 ml sampling tube.

3. The cells were collected by 5 sec centrifugation and the supernatant was decanted.
4. The tube was vortexed briefly. 0.2 ml of extraction solution, 0.2 ml of phenol/chloroform and 0.3 g glass beads (0.5 mm diameter) were added and vortexed for 5 min.

   Extraction solution — 2 % Triton X-100, 1 % SDS, 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA
5. 0.2 ml of TE was added and centrifuged for 5 min.
6. 1 ml of ethanol was added to the aqueous layer, mixed by inverting and centrifuged for 2 min.
7. The pellet was resuspended in 0.4 ml of TE containing 30 μg of RNase A and stored at 37°C for 5 min.
8. 40 μl of 3 M sodium acetate and 1 ml of ethanol were added and mixed. After centrifugation for 2 min, DNA pellet was resuspended in 50 μl of TE.

4-2. Isolation of plasmid DNA (Hoffman and Winston, 1987)

1. A small overnight culture (ca. 2 ml) in SD medium to maintain selection for the plasmid was prepared.
2. The cell culture was transferred into a 1.5 ml sampling tube and the cells were harvested by centrifugation.
3. The supernatant was decanted and vortexed. 0.2 ml of extraction buffer (see 4-1), 0.2 ml of phenol/chloroform and 0.3 g glass beads were added and vortexed for 2 min.
4. After centrifugation for 5 min, 5 μl to 20 μl of aqueous layer containing plasmid DNA were used to transform *E. coli* XL-1 or HB101.

4-3. Synthesis of oligonucleotide

1. Oligonucleotide mixtures were synthesized using a model 381A DNA synthesizer (Applied Biosystem).
2. After synthesis, the synthetic oligonucleotide was cleaved from column with aqueous ammonia.
3. The oligonucleotide was deprotected by incubation at 55°C for 18 hrs or at 65°C for 2 hrs.
4. The oligonucleotide was dried by speed vacuum and resuspended in 200 μl of 0.01 M TEAA.
5. The oligonucleotide was purified with reverse phase HPLC, taken off trityl with 80 % acetic acid and suspended in 100 μl of TE.

4-4. Labeling of probe

(1) End-labeling of oligonucleotide

1. End-labeling reaction was carried out at 37°C for 30 min in a mixture shown below. 0.8 μl of 0.5 M EDTA (pH 7.5) was added to stop the reaction.

10x reaction buffer	2 μl
1 M DTT	2 μl
oligonucleotide	17 pmol
T4 polynucleotide kinase (10 U/μl)	1 μl
[γ-^{32}P]ATP	1 μl
H_2O	to 20 μl

10x reaction buffer 0.5 M Tris-HCl (pH 8.0), 0.1 M $MgCl_2$

2. The labeled oligonucleotides were separated from unincorporated nucleotide triphosphate by Quiagen-tip 5 (Diagen).
3. Quiagen-tip 5 was equilibrated with 400 μl of sol-oligo before samples were applied.

Sol-oligo 200 mM NaCl, 15 % ethanol, 50 mM MOPS (pH 7.0)

4. The tip was washed with 500 μl of sol. B. This step was repeated once more.

Sol. B 750 mM NaCl, 15 % ethanol, 50 mM MOPS (pH 7.0)

5. The oligonucleotides were eluted by washing the tip with sol. F two times.

Sol. F 1500 mM NaCl, 15 % ethanol, 50 mM MOPS (pH 7.5)

(2) Labeling using random primer (Feinberg and Vogelstain, 1983)

1. DNA fragment was heated to 95°C~100°C for 2 min, cooled down immediately to 0°C in an ice bath. 5 μl of OLB, 1 μl of BSA (10 mg/ml), 1 μl of [α-^{32}P]dATP, 1 μl of Klenow fragment and H_2O (volume upto 25 μl) were added.

OLB mix A : B : C = 100 : 250 : 150

A 1 ml of sol. O, 18 μl of 2-mercaptoethanol, 5 μl of dNTP (0.1 M dCTP, dGTP, dTTP)

Sol. O 1.25 M Tris-HCl (pH 8.0), 0.125 M $MgCl_2$

B 2 M HEPES (pH 6.6)

C Hexa deoxy-ribonucleotide primer

2. The mixture was incubated at 37°C for 2 hrs. or at 30°C overnight.

3. The labeled DNA was separated from unincorporated nucleotide triphosphate by Quiagen-tip 5 as described above. Sol. A was used instead of sol-oligo.

Sol. A 400 mM NaCl, 15 % ethanol, 50 mM MOPS (pH 7.0)

4-5. Hybridization

(1) Hybridization with end-labeled probe (Wood et al., 1985)

1. Prehybridization was carried out at 37°C for 4 hrs or more.

Prehybridization solution 6x SSC, 50 mM Na_2HPO_4/NaH_2PO_4, 5x DH, 0.1

mg/ml salmon sperm DNA

20 x SSC	175.3 g of NaCl, 88.2 g of sodium citrate, to 1000 ml of H_2O (adjust pH 7.0 with NaOH)
50 x DH	5 g Ficoll, 5 g polyvinylpyrrolidone, 5 g BSA, to 500 ml of H_2O

2. 10 % sodium dextran sulfate and labeled probe were added to the hybridization solution that is same to prehybridization solution. Hybridization was carried out overnight at 37°C.
3. The filter was washed with ice-cold 6x SSC five times and rinsed with ice-cold wash solution I twice.

   Wash solution I 50 M Tris-HCl (pH 8.0), 2 mM EDTA (pH 8.0), 0.1 %SDS
4. The filter was washed in wash solution II at 52°C for 20 min. This step was repeated once more.

   Wash solution II wash solution I containing 3 M Me_4NCl
5. The filter was rinsed with ice-cold 6x SSC.

(2) Hybridization with labeled fragment

The standard protocols (Casey and Davidson, 1977) were used.

(3) Colony hybridization

For the screening of colonies by hybridization, colonies were transferred to nitrocellulose filter and treated according to the method of Grunstein and Wallis (1979).

4-6. Polymerase chain reaction (Saiki et al., 1988)

1. The reaction mixture contained 1.0 μM primer, 1 ng/assay template, 2.5 U/assay

Taq DNA polymerase, 200 μM dNTP mixture, 1x buffer.

10x buffer 500mM KCl, 100 mM Tris-HCl (pH 8.3), 15 mM $MgCl_2$, 0.1 % (w/v) gelatin

100 μl of mineral oil was overlaid to avoid evaporation.

2. The mixture was subjected to repeated cycles of 1 min at 94°C (denaturation), 2 min at 53°C (annealing) and 9.9 min at 72°C (extension) in a Perkin-Elmer Cetus Thermalcycler.

4-7. OFAGE (Carle et al., 1986)

1. An appropriate concentration (~ 1.5 %) of agarose gel solution in 0.5x TBE buffer was prepared to make a 9 cm square horizontal gel.

   TBE 0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA

2. The gel was allowed to solidify at room temperature, then the comb was carefully removed. The sample gel block was then placed into the well.
3. The separation gel was placed into the chamber for OFAGE (Nippon Eido) filled with 1.5 l of 0.5x TBE buffer.
4. The switching device was connected to a power supply and the electrophoresis apparatus under which magnetic stirrer was set.
5. The cooling water flow was connected between the chamber and a water bath (Mini-80, Taiyo), and the electrophoresis was started under the conditions listed below. During the run, the stirring was continued to keep the buffer homogeneous. The time of electrophoresis was usually 16 hours.
6. After the run, the gel was stained with ethidium bromide (0.5 μg/ml) and destained overnight to decrease the background level. The separated chromosomal DNA bands were then transferred to filter (Southern, 1975).

<Time intervals for OFAGE>

Pulse time	Approximate size of DNA molecule to be separated:	
50 seconds	50-2000 kb	(1.5 % agarose, 180 mA, 0.5x TBE)
50 seconds	200->2000 kb	(1.2 % agarose, 180 mA, 0.5x TBE)
40 seconds	<50-1000 kb	(1.5 % agarose, 160-180 mA, 0.5x TBE)
30 seconds	<50-650 kb	(1.5 % agarose, 160-180 mA, 0.5x TBE)

5. Preparation and analysis of RNA

5-1. Isolation of total RNA (Sprague et al., 1983)

1. 40 ml of culture (ρ^+; OD_{600}=1~3, ρ^o; OD_{600}=0.7~2) were prepared.
2. The cells were harvested and transferred into 2 ml sampling tube.
3. The cells were resuspended in 0.2 ml extraction buffer. 20 μl of 10 % SDS, 0.2 ml of phenol/chloroform and 0.3 g glass beads were added. The mixture was vigorously vortexed for 60 sec and immediately cooled in ice bath for 30 sec. This step was repeated three times.

   Extraction buffer 0.2 M Tris-HCl (pH 7.6), 10 mM EDTA, 0.5 M NaCl
4. After centrifugation, the aqueous layer was extracted with phenol/chloroform twice and then with chloroform.
5. 2 volumes of ethanol were added and the mixture was stored at -20°C for 1 hr or more.
6. The tube was centrifuged at 15,000 rpm for 5 min at 0°C, washed with 70 % ethanol and dried in a desiccator.
7. The pellet was resuspended in 0.6 ml LETS buffer.

   LETS buffer 10 mM Tris-HCl (pH 7.6), 10 mM EDTA, 0.1 M LiCl, 0.2 % SDS
8. 0.4 ml of 5 M LiCl was added and stored on ice for 1 hr or more.

9. The tube was centrifuged at 15,000 rpm for 5 min at 0°C, washed with 70 % ethanol and dried. The RNA pellet was dissolved in 200 µl of TE.

5-2. Electrophoresis and blotting

1. The gel tray and electrophoretic chamber were treated with 0.2 N NaOH.
2. 1.2 g agarose was dissolved in 85 ml of 1x MOPS, to which 5.4 ml of 37 % formaldehyde were added.

   10x MOPS 0.2 M MOPS (pH 7.0), 50 mM sodium acetate, 10 mM EDTA
3. 4.5 µl of total RNA (20 µg), 2.0 µl of 10x MOPS, 3.5 µl of formaldehyde, 10.0 µl of deionzed formamide were mixed and boiled at 90°C for 2 min. 2 µl of loading buffer were then added. The sample mixtures were loaded in each lane.

   Loading buffer 50 % glycerol, 1 mM EDTA, 0.4 % BPB, 0.4 % XCFF

   The electrophoresis was performed at 100 V for 1 hr.
4. The separated RNA was transferred to nitrocellulose filter using a vacuum blotter (Sanplatec) for 2 hrs with 1 M ammonium acetate.

5-3. Primer extension (Fujita and Ishihama, 1987)

1. The oligonucleotides for primer (27 pmol) was end-labeled with [γ-^{32}P]ATP.
2. 100 µg total RNA, end-labeled primer, 2 µl of 5 mg/ml tRNA were mixed and precipitated with ethanol.
3. The pellet was resuspended in 30 µl of hybridization buffer and incubated at 75°C for 10 min. The tube was cooled down gradually to 30°C and precipitated with ethanol.

   Hybridization buffer 40 mM PIPES (pH 6.54), 400 mM NaCl, 1 mM

EDTA(pH 7.5), 80 % formamide

4. The pellet was resuspended in 20 µl of 1x RT buffer.

1x RT buffer	12.8 µl of 5x RT buffer, 6 µl of 4 mM dNTP mixture, 45.2 µl of H_2O
5x RT buffer	250 mM Tris-HCl (pH 8.3 at 42°C), 50 mM $MgCl_2$, 50 mM DTT, 300 mM NaCl

5. 0.6 µl of reverse transcriptase (RAV-2) was added and the mixture was incubated at 42°C for 1 hr.
6. 150 µl of 0.15 N NaOH and 5 mM EDTA were added and the mixture kept at 75°C for 20 min.
7. After ethanol precipitation, the pellet was resuspended in 6 µl H_2O. 4 µl of sequence stop solution were then added and 2.5 µl were loaded in sequence gel.

6. Yeast Genetics

6-1. Transformation (Ito et al., 1983)

1. 2 ml of overnight culture was inoculated into 20 ml YPD and incubated at 30°C until OD_{600}=4~8.
2. The cells were harvested and washed with TE. After centrifugation, the cells were resuspended in 1 ml of Li-acetate solution and stored at 30°C for 1 hr.

   Li-acetate solution — 0.1 M lithium acetate and 15 % glycerol in TE
3. DNA (no more than 15 µl) was added to 0.3 ml of Li-acetate-treated cells and the mixture was left standing for 30 min with occasional agitation.
4. 0.3 ml of 70 % PEG 4000 was added and the mixture was incubated at 30°C for 1 hr and then at 42°or 5 min.

5. The cells were washed with H_2O twice and resuspended in 0.2 ml SD medium.
6. The cells were spread into appropriate selection plate.

6-2. Mating and random spore analysis (Ausubel et al.)

(1) Mating and sporulation

1. The cells were mixed from each haploid parent were mixed on agar plate and incubated overnight at 30°C.
2. The mating mixture was streaked onto agar plate for the diploid phenotype selection.
3. The diploid cells were grown to OD_{600} of 2.5~3.0 in YPD medium.
4. The cells from 3 ml culture were washed with H_2O two times.
5. The cells were resuspended in 3 ml sporulation medium and incubated for 2~3 days at 30°C.

(2) Random spore analysis

1. 1 ml of sporulated culture was centrifuged at 3,000 rpm for 5 min. The pellet was resuspended in 5 ml H_2O. 0.1 ml of 1 mg/ml Zymolyase 100-T and 10 μl 2-mercaptoethanol were added and the mixture was incubated overnight at 30°C with gentle shaking.
2. 5 ml of 1.5 % NP-40 were added to the Zymolyase-treated cells and the mixture was stored on ice for 15 min.
3. The mixture was sonicated for 30 sec at 50 % to 75 % full power and set on ice for 2 min. This step was repeated twice.
4. After centrifugation, the spores were resuspended in 10 ml of 1.5 % NP-40 by

vigorous vortexing. This step was repeated twice.

5. The spores were resuspended in 2 ml H_2O. 0.1 ml of appropriate dilution (10^3 spores/ml) was spread onto YPD plate and incubated for 2 days at 30°C. Spores of interest were screened by replica-plating.

7. Miscellaneous methods

The standard procedures for transformation of *E. coli* cells, plasmid preparation and *in vitro* DNA manipulation were used (Maniatis et al., 1982). For the deletions of plasmids p26BP9 and pL33, an enzyme kit containing Exonuclease III, Mungbean nuclease, *E. coli* DNA polymerase klenow fragment and T4 DNA ligase was purchased from Takara Shuzo and used according to the procedure of Henikoff (1984). Nucleotide sequencing was performed according to Sanger et al. (1977) using T7 DNA polymerase (Pharmacia). Nucleotide sequence data were stored and analyzed using computer programs DATBAS, NUCDAT (Isono, 1984) and AACOMP (Isono, unpublished). The National Biomedical Research Foundation (NBRF) protein data (Release no. 18.0) was used for homology search.

For quantitation of copy numbers and mRNA levels, the autoradiograms were traced by using Dual-wavelength flying-spot scanner CS-9000 (Shimadzu). The area of peak in each lane was normalized by dividing by the area of the actin peak.

Table 1. Abbreviations

Amp	Ampicillin
APS	Ammonium Persulfate
BPB	Bromophenol blue
BSA	Bovine Serum Albumin
CAPS	3-Cyclohexylaminopropanesulfonic acid
CBB	Coomassie Brilliant Blue R-250
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid, disodium salt
HEPES	N-2-Hydroxylethylpiperazine-N'-2-ethanesulfonic acid
MES	2-(N-Morpholino)ethanesulfonic acid, monohydrate
MOPS	Morpholinopropanesulfonic acid
NP-40	Nonidet P-40
NTP	Nucleoside triphosphate
OFAGE	Orthogonal field alteration gel electrophoresis
PEG	Polyethylene Glycol
PIPES	Piperazine-N,N'-bis(2-ethanesulfonic acid)
PVDF	Polyvinylidene difluoride
SDS	Sodium dodecyl sulfate
TEAA	Triethylamine acetate
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tet	Tetracycline
XCFF	Xylene cyanol FF
YEp	Yeast episomal plasmid
YIp	Yeast integrating plasmid

Results

1. Isolation of ribosomes from yeast mitochondria

Total mitochondrial ribosomal proteins were prepared from commercial baker's yeast cells according to the conditions determined by Matsushita (1988) as described in Materials and Methods. Then, the proteins of mitochondrial ribosomes were separated by two-dimensional polyacrylamide gel electrophoresis (Fig. 1). The electrophoretic patterns of the mitochondrial ribosomal proteins from laboratory strains of *S. cerevisiae* such as DC-5 were almost identical to each other (data not shown) and to that shown in Fig. 1. Therefore, I decided to use the amino acid sequence data obtained from the mitochondrial proteins of commercial baker's yeast for cloning of the corresponding genes.

The ribosomal proteins of mitochondria isolated from commercial baker's yeast cells were purified by two-dimensional SDS-PAGE (Mets and Bogorad, 1974), blotted to PVDF membrane, detected by staining with Coomasie blue and sequenced directly (Matsudaira, 1987). Fourteen ribosomal proteins which were well separated from one another on two-dimensional gels were determined for their amino acid sequence (Fig. 2). Among these proteins, YMR6 and YMR24 proteins were found to be identical to MRP7 and YmL8 proteins, respectively (Fearon and Mason, 1988; Kitakawa et al., 1990). The amino-terminal amino acid sequence of the protein: identical to MRP7 agreed with the deduced amino acid sequence from the *MRP7* nucleotide sequence starting at the Ala at the 28th position which was predicted to be the case from the nucleotide sequence (Fearon and Mason, 1988) This suggested, therefore, the existence of a 27 amino acid residue-long leader sequence. The similarity of this protein to *E. coli* ribosomal

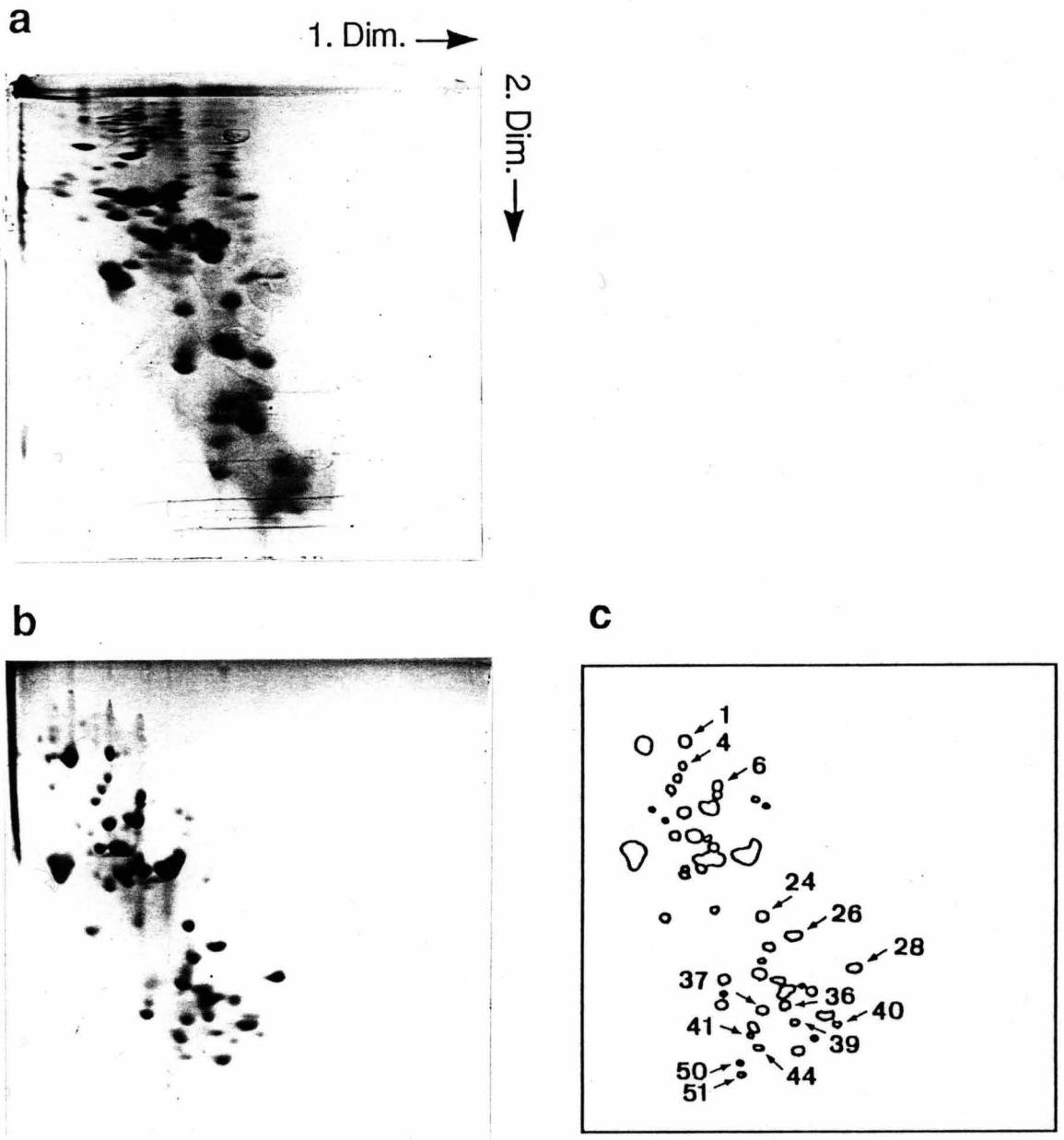


Fig. 1 Two-dimensional gel electropherograms of the mitochondrial proteins from baker's yeast cells. Electrophoresis was performed in the first dimension (horizontal) and in the second dimension (vertical) essentially as described by Geyl et al. (1981) in **a** and as described by Mets and Bogorad (1974) in **b**. **c.** Schematic diagram of the protein pattern shown in panel **b**. The only spots for which amino acid sequence was determined (Fig. 2) are numbered.

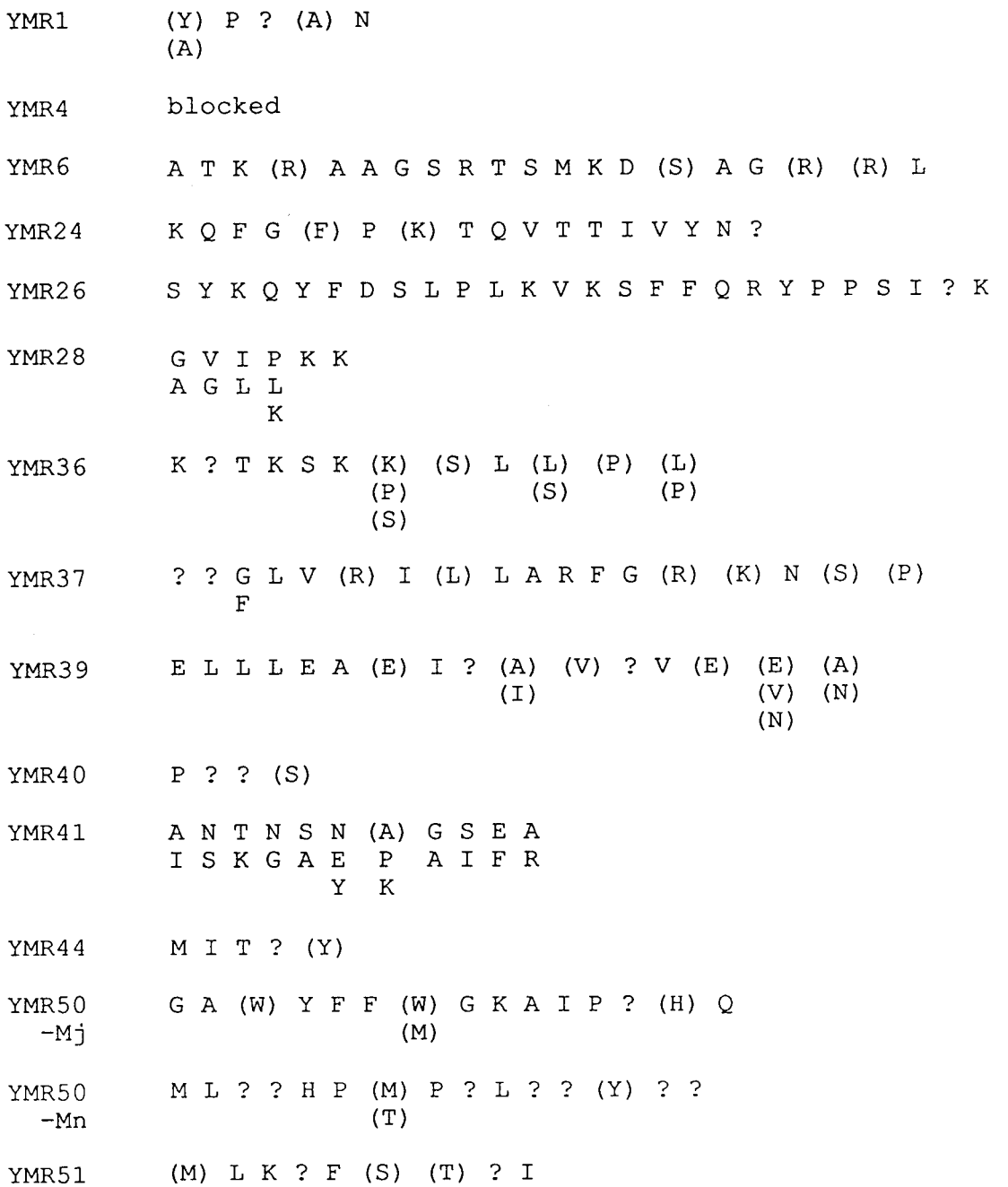


Fig. 2 N-terminal amino acid sequences of ribosomal proteins from yeast mitochondria. Amino acid residues in parentheses are slightly ambiguous and question marks indicate that the residues at the corresponding positions were notdeterminable. The amino acid sequence of YMR50 was divided into two sequences, i.e. a major peak (Mj) and a minor peak (Mn).

protein L27 starts at the 28th amino acid residue. In other word, protein MRP7 appears to have acquired a 27 residue-long leader sequence attached to a "pre-mitochondrial" ribosomal protein resembling *E. coli* L27.

2. Analysis of the *YMR26* gene

2-1. Cloning of the *YMR26* gene

The amino acid sequence of protein YMR26 was determined for the N-terminal 26 residues as shown in Figs. 2 and 3. An oligonucleotide mixture (32 mixture) was accordingly synthesized for a region near the N-terminus of this protein which contained amino acid residues with least degenerate codons (Fig. 3). It was end-labelled with [γ-^{32}P]ATP and used as a probe to hybridize the genomic DNA prepared from strain DC-5 ρ^o. ρ^o strain is chosen to minimize the contamination of mitochondrial DNA. As shown in Fig. 3, a single band was observed when the DNA was digested with *Bam* HI, *Hin* dIII or *Pst* I. It was found that the digestion with *Pst* I yielded a fragment of 4.4 kb that was shortest among the used restriction enzymes. Thus, *Pst* I fragments of this size were purified, ligated with pBR322 and used to transform *E. coli* strain HB101 to Amp^r. Transformants were tested by colony hybridization with [γ-^{32}P]ATP labelled oligonucleotide mixture as a probe. A positive recombinant was obtained and double strand nucleotide sequencing of the clone was performed using the oligonucleotide mixture as a primer. The deduced amino acid sequence from the nucleotide sequence data was consistent with the amino acid sequence of protein YMR26 from Lys at the 14th position (data not shown). Therefore, this clone was concluded to encode the protein YMR26. This plasmid was termed pYM-26 and analyzed further.

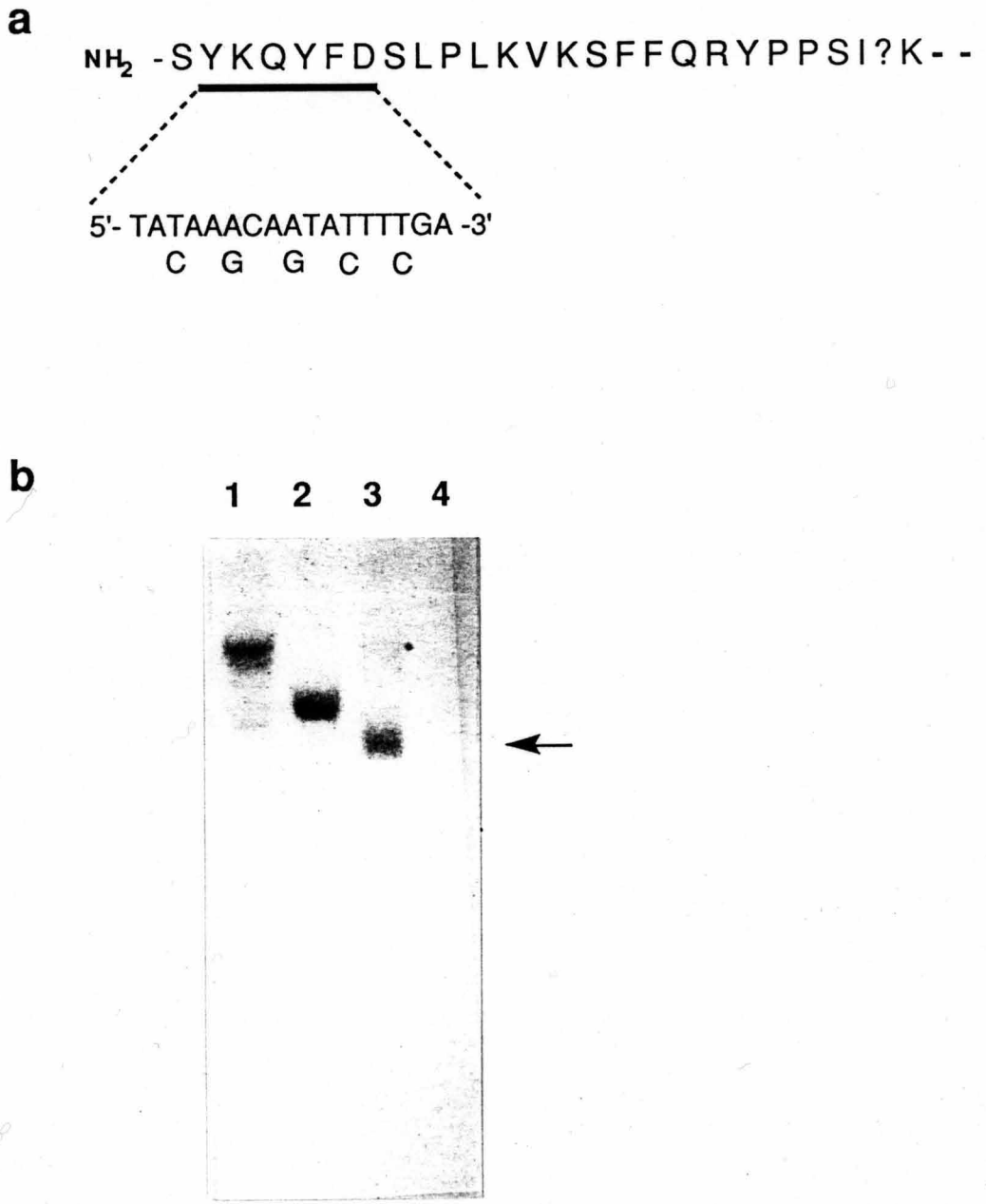


Fig. 3 Southern hybridization with a YMR26 probe. **a.** A stretch (underline) of the N-terminal amino acid sequence of protein YMR26 was chosen and an oligonucleotide mixture (32 mix.) was synthesized as shown. **b.** The oligonucleotide mixture was end-labeled with [γ-^{32}P]ATP and used as a probe in Southern hybridization with 10 μg of the genomic DNA from DC-5 ρ^o cells digested with *Bam* HI (lane 1), *Hin* dIII (lane 2) or *Pst* I (lane 3). Arrow points to the 4.4 kb *Pst* I fragment used for cloning of the *YMR26* gene. *Hin* dIII digested λ DNA was loaded in lane 4 as visual size markers.

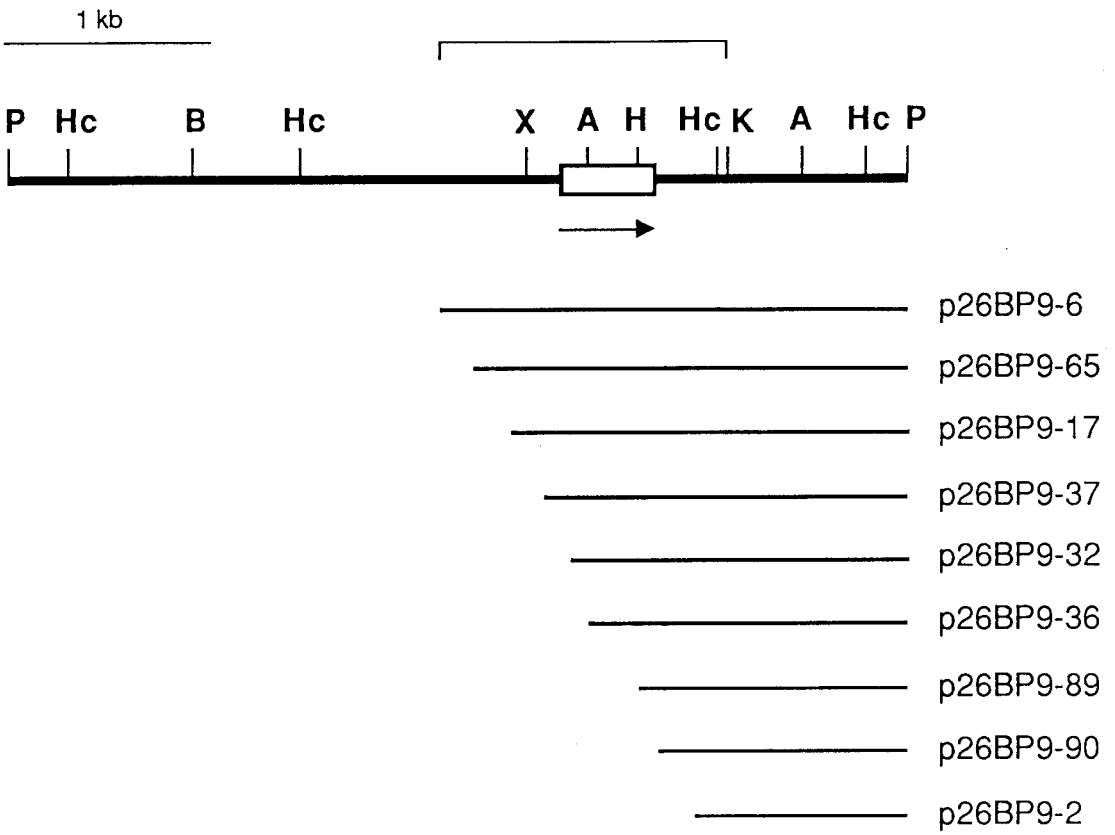


Fig. 4 The restriction map of the 4.4 kb *Pst* I fragment in plasmid pYM-26. The open box represents the coding region of the *YMR26* gene and its direction of transcription determined by polymerase chain reaction (see text) is indicated by the horizontal arrow. The sequenced region shown in Fig. 5 is bracketed. The deletion series of p26BP9 is shown below the restriction map (see text). Abbreviations for restriction enzyme sites are: **A** for *Acc* I, **B** for *Bam* HI, **H** for *Hin* dIII, **Hc** for *Hin* cII, **K** for *Kpn* I, **P** for *Pst* I, and **X** for *Xba* I. No site was found for *Eco* RI, *Sac* I and *Sma* I.

A detailed restriction map analysis was performed with pYM-26 as shown in Fig. 4. The oligonucleotide probe was found to hybridize with a 3.4 kb *Bam* HI-*Pst* I fragment (data not shown). Accordingly, the 3.4 kb *Bam* HI-*Pst* I fragment was subcloned into pUC118 and pUC119 and named p26BP8 and p26BP9, respectively. To determine the location of the *YMR26* gene and its direction of transcription, the two plasmids (p26BP8, p26BP9) were subjected to amplification by polymerase chain reaction using a universal sequencing primer (M4) and the oligonucleotide mixture (Fig. 3). An amplified fragment appeared only when p26BP8 was used as template and its size was estimated to be 1.7 kb (data not shown). Therefore, it was concluded that the *YMR26* gene is located at 1.7 kb from the *Pst* I site and the direction of its transcription is from the *Bam* HI to the *Pst* I site.

2-2. Nucleotide sequence of *YMR26*

To determine the nucleotide sequence of *YMR26*, a series of deletion of plasmid p26BP9 was constructed with Exonuclease III and Mungbean nuclease as outlined in Fig. 4 (Henikoff, 1984). Then, the nucleotide sequence of a central 1.4 kb region of p26BP9 (Fig. 4) containing the *YMR26* gene was determined by the dideoxy chain termination method (Sanger et al., 1977). The sequence data obtained were stored and analyzed using computer programs DATBAS and NUCDAT (Isono, 1984). The complete nucleotide sequence of the region is shown in Fig. 5. An open reading frame was found which was capable of encoding a basic protein (net charge=+21) containing 158 amino acid residues with a calculated molecular weight of 18,454. The molecular weight and the net charge are consistent with those estimated from the mobility of YMR26 protein in SDS PAGE (data not shown). In addition, the deduced amino acid sequence from

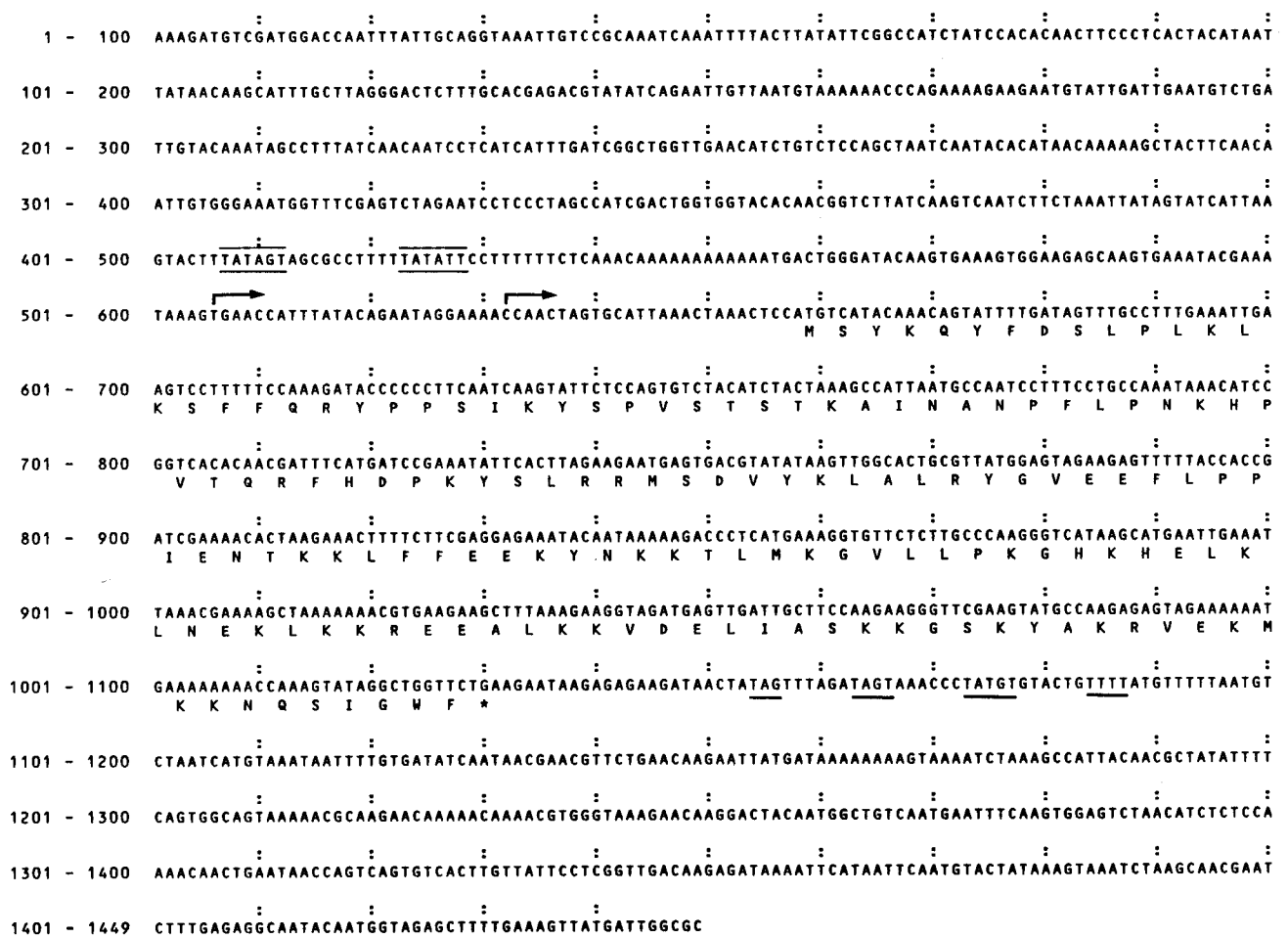


Fig. 5 Nucleotide sequence of the *YMR26* gene and its flanking region. The sequence was determined using the deletion series of p26BP9 (see Fig. 4) as described in the text and numbered arbitrarily. Bent arrows indicate the initiation sites of the *YMR26* transcripts which were determined by the primer extension experiments as described in Materials and Methods section. The predicted amino acid translation of the coding region of the gene is shown in single letter codes. Potential TATA-sequence are flanked by horizontal lines and putative transcription termination signals are underlined.

Ser at the second position agreed with the amino acid sequence of YMR26 protein (shown in Figs. 2 and 3). Thus, it was concluded that the open reading frame encodes the YMR26 protein. Comparison of the amino acid sequence of YMR26 protein and its gene also indicated that YMR26 protein contains no leader sequence. Search by the computer for sequences similar to the predicted amino acid sequence of YMR26 protein in the National Biomedical Research Foundation (NBRF) protein sequence database (Release no. 18.0) failed to indicate the presence of another protein with a significant degree of similarity.

The 5' end of the *YMR26* transcript was determined by the primer extension method (data not shown). Most cellular mRNAs have 5' noncoding sequence in the range of 40 to 80 nucleotides (Kozak, 1983). The primer extension analysis indicated that the *YMR26* transcript contains an untranslated region of approximately 50 nucleotides long. The 5' untranslated region of *YMR26* was AT-rich (69 %). These features have been found in the 5' regions of various transcripts in the yeasts and slime molds (Kozak, 1983). Two potential TATA-sequences were found at 130 bp to 140 bp upstream of the AUG initiation codon. A typical yeast transcriptional termination sequence, TAG...TATGT...TTT (Zaret and Sherman, 1982; Birnstiel et al., 1985), was found at the 3' end of *YMR26*. However, a polyadenylation signal in yeast, ATAAATAAA/G (Bennetzen and Hall, 1982), was not found.

2-3. Chromosomal location and copy number of the *YMR26* gene

To determine the copy number of the *YMR26* gene in the yeast genome, the genomic DNA of strain DC-5 ρ^o was digested with restriction enzymes *Bam* HI, *Hin* dIII or *Pst* I, electrophoresed and hybridized with a 1.2 kb *Eco* RI-*Hin* cII fragment

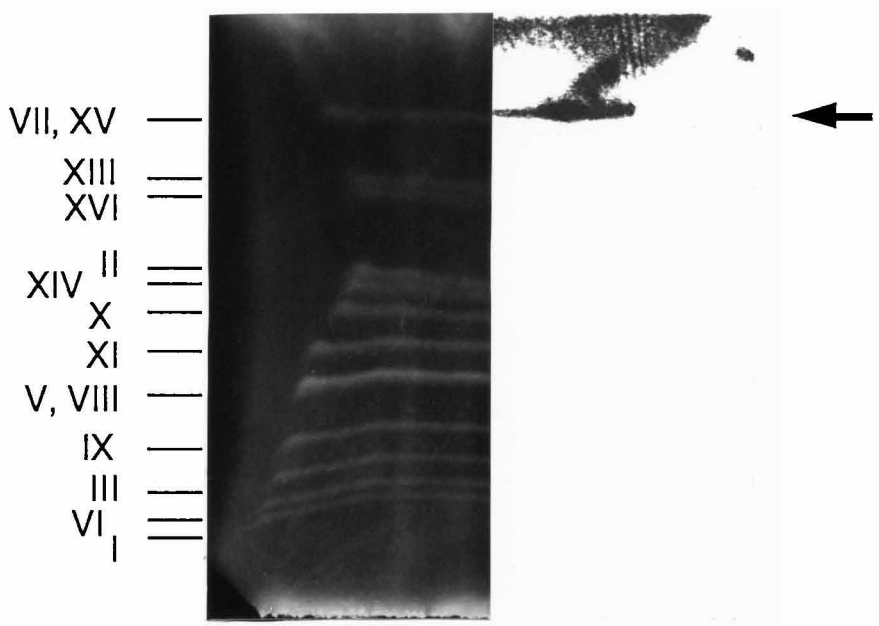


Fig. 6 Chromosomal location of the *YMR26* gene. The chromosomal DNA of strain DC-5 ρ^o was separated by pulse-field electrophoresis. After electrophoresis, the gel was stained with ethidium bromide (0.5 μg/ml) to visualize the DNA bands (left panel). The numbers of chromosomes are indicated at left. The DNA bands were transferred to nitrocellulose membrane and hybridized with a 1.2 kb *Eco* RI-*Hin* cII fragment of plasmid p26BP9-65 shown in Fig. 4 (right panel). Arrow points to the DNA band corresponding to chromosome VII or XV.

labeled with [α-^{32}P]dATP of p26BP9-65 which was one of the derivatives in the deletion series of p26BP9 (see Fig. 7). A single band was observed upon digestion with either *Bam* HI or *Pst* I, while two bands were observed upon digestion with *Hin* dIII (data not shown). Because there was a *Hin* dIII site between the *Bam* HI and *Pst* I site on pYM-26 (Fig. 4) and a single band was observed from hybridization using oligonucleotides (Fig. 3), I interpreted the data to indicate that the *YMR26* gene exists as a single copy gene in the yeast genome. However, the possibility can not be ruled out that the gene and its flanking regions including the sites for the three restriction enzymes are duplicated, although it is not likely the case. The same [α-^{32}P]dATP-labelled fragment used above was hybridized with the yeast chromosomes separated by OFAGE to determine the location of *YMR26* gene. As shown in Fig. 6, the *YMR26* gene was found to hybridized only with one band, corresponding to either chromosome VII or XV. Thus, it was concluded that the *YMR26* gene exists as a single copy gene on either chromosome VII or XV.

2-4. Gene disruption analysis

To determine whether the product of the *YMR26* gene is essential for the mitochondrial function in yeast cells, a gene disruption was performed. For this purpose, a disrupted allele was constructed as outlined in Fig. 7. In order to remove the downstream *Acc* I site, a 1.2 kb *Eco* RI-*Hin* cII fragment of plasmid p26BP9-65 (Fig. 4) which is a deletion derivative of p26BP9 was subcloned into pUC118 and named pEHc65 (Fig. 7). The 2.9 kb *Bgl* II fragment containing the *LEU2* gene was purified from plasmid YEp13 and treated with Klenow fragment for making the ends blunt. The resultant fragment was inserted into the coding region of *YMR26* at the *Acc* I site at nucleotide position 647 (shown in Fig. 5) which was

treated with Klenow fragment. The 4.4 kb *Pvu* II fragment of a resultant plasmid was used to replace the corresponding genomic region by integrative transformation. Leu+ transformants of both the haploid strain DC-5 and diploid strain DC-K12 were isolated and examined for their ability to grow on a nonfermentable carbon source (glycerol). The haploid Leu+ transformants were found to be respiration deficient and the diploid transformants were respiration competent. Southern blot analysis of the genomic DNA from representative haploid transformants confirmed that recombination had been directed to the putative *YMR26* locus (Fig. 7). These results indicate, therefore, that the *YMR26* gene is essential for the mitochondrial function in yeast, and further support that the *YMR26* gene is present in a single copy in the yeast genome.

The inactivation of genes whose functions are required for mitochondrial protein synthesis has been shown to lead not only to respiratory deficiency but also to instability of the mitochondrial genome (Myers et al., 1987). Therefore, if the mitochondrial protein synthesis is necessary for the maintenance of the mitochondrial DNA and *YMR26* provides an essential function in mitochondrial ribosome, then the haploid Leu+ transformants (*ymr26::LEU2*) are expected to be ρ^o or ρ^-. This possibility was tested genetically by crossing *ymr26::LEU2* mutants to a ρ^o *YMR26* strain (AB972) and examining for the respiration ability of resultant diploid derivatives. The diploids derived from this cross were all respiration-deficient. This result supports the hypothesis that mitochondrial protein synthesis is necessary for the maintenance of wild type mitochondrial DNA as reported by Myers et al. (1987).

To confirm further that respiration deficiency in the Leu+ transformants described above was indeed due to the disruption of the *YMR26* gene,

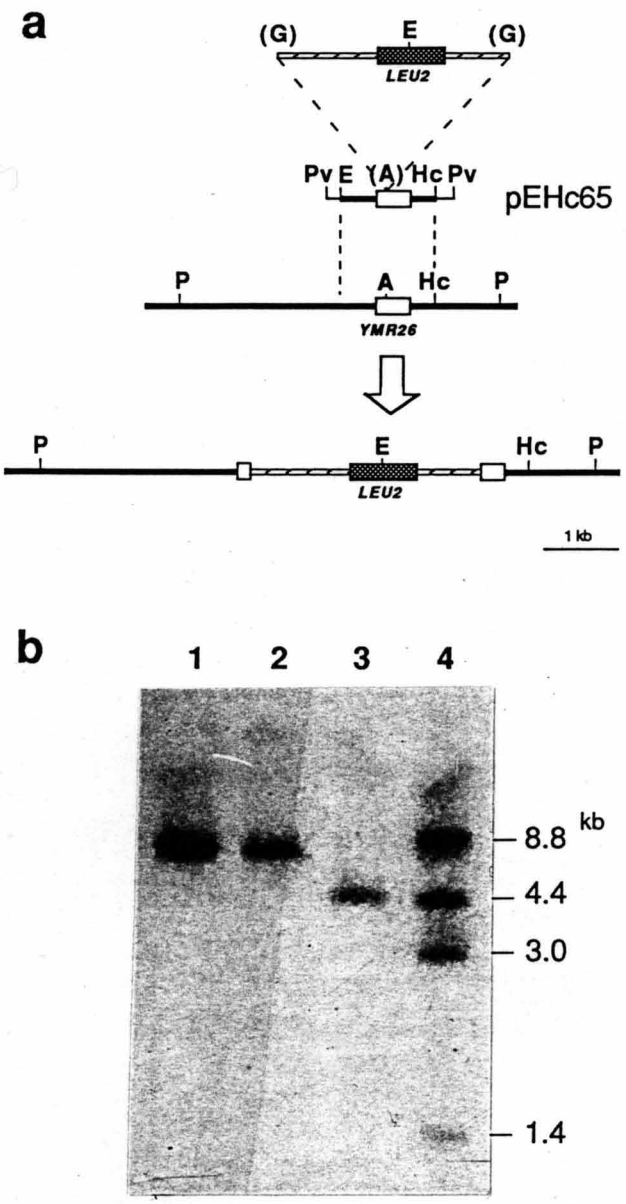


Fig. 7 Gene disruption analysis of *YMR26*. **a.** A 1.2kb *Eco* RI-*Hin* cII fragment of plasmid p26BP9-65, a deletion derivative of p26BP9, which contained the *YMR26* gene (open box) was subcloned into pUC118 and named pEHc65 (only the relevant portion is shown). A 2.96 kb *Bgl* II fragment containing the *LEU2* gene (hatched box) was ligated into the *Acc* I site of pEHc65. A 4.4 kb *Pvu* II fragment of the resultant plasmid in which the *YMR26* gene was disrupted was then used for transformation. As a result, the chromosomal *YMR26* gene was expected to be disrupted as indicated. **b.** The disruption of *YMR26* was confirmed by Southern hybridization analysis of the DNA from two Leu+ transformants (lanes 1, 2) and DC-5 (undisrupted control: lane 3) digested with *Pst* I, using as a probe the 1.2kb *Eco* RI-*Hin* cII fragment of plasmid p26BP9-65. As size markers, a mixture of DNA of plasmid pYM-26 digested with *Eco* RI, *Pst* I or *Hin* dII-*Pst* I was used (lane4). Abbreviations for restriction enzymes are: **A** for *Acc* I, **E** for *Eco* RI, **Hc** for *Hin* cII, **P** for *Pst* I, and **Pv** for *Pvu* II. The sites in parentheses indicate those treated with Klenow fragment.

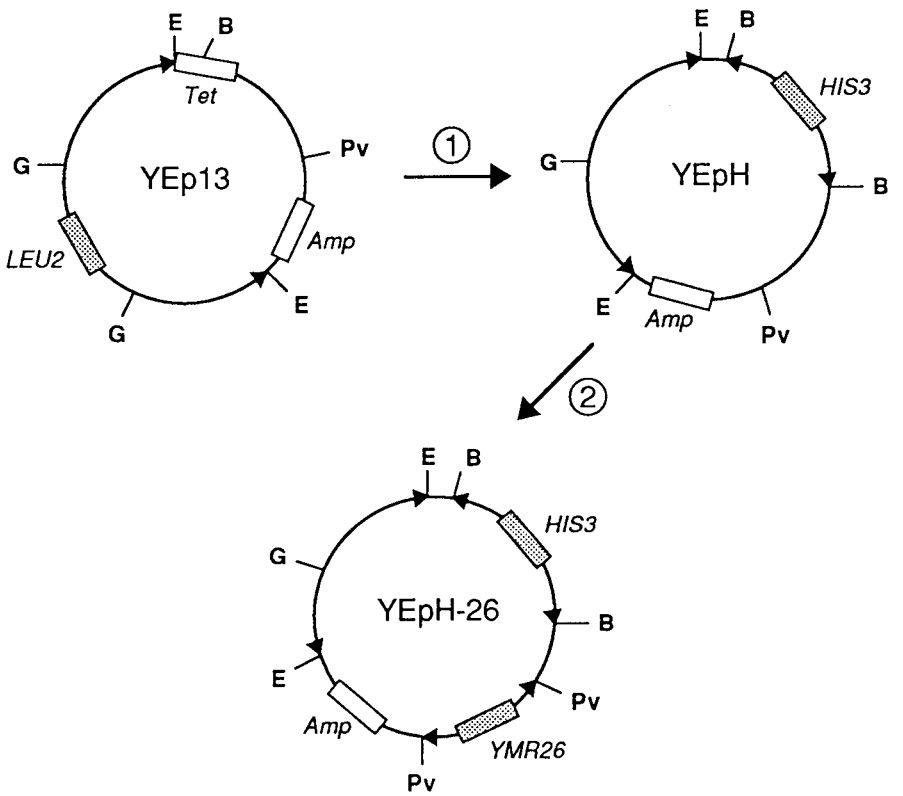


Fig. 8 Construction of plasmid YEpH-26 for complementation analysis. **Step 1**: Plasmid YEp13 was digested with *Bgl* II and self-ligated to remove *LEU2*. A 1.75 kb *Bam* HI fragment containing *HIS3* was then ligated into the *Bam* HI site of this plasmid to yield YEpH. **Step 2**: The 1.4 kb *Pvu* II fragment of pEHc65 (see Fig. 7) containing the *YMR26* gene was ligated into the *Pvu* II site of YEpH to yield YEpH-26. Abbreviations for restriction enzymes are; **B** for *Bam* HI, **E** for *Eco* RI, **G** for *Bgl* II, and **Pv** for *Pvu* II.

complementation analysis was carried out by transformation of the wild type *YMR26* gene into the *ymr26::LEU2* mutant. For this purpose, plasmids YEpH and YEpH-26 were constructed as shown in Fig. 8 and used to transform *ymr26::LEU2* mutant. His+ transformants were selected and examined for the presence of plasmid. Because *ymr26::LEU2* mutant was either ρ^o or ρ^- as described above, the His+ transformants were mated with a ρ^+ strain, KWK01, in order to supply wild type mitochondria. After sporulation, haploid cells were selected for Leu+, His+ and Ade. The haploid derivatives thus obtained were examined for their respiration ability. Cells harboring plasmid YEpH were respiration deficient, but cells harboring plasmid YEpH-26 which contained the wild type *YMR26* gene were respiration competent. Thus, these results clearly indicate that the respiration deficiency in the Leu+ transformants is due to the disruption of the *YMR26* gene.

2-5. Northern blot analysis

To investigate the expression of the *YMR26* gene, the transcription of *YMR26* was compared in ρ^+ and ρ^o cells growing under various degrees of catabolite repression. Total RNAs were prepared from early log phase cultures of DC-5 ρ^+ and DC-5 ρ^o grown in YP medium supplemented with either 6 % glucose, 2 % glycerol or 2 % galactose. Northern blot analysis (Fig. 9) showed that the level of *YMR26* transcript was repressed by growth in glucose compared with the derepressed levels of cells grown in glycerol or galactose. However, the transcription of *YMR26* in DC-5 ρ^o responded to glucose repression only slightly. This effect was seen most clearly in the direct comparison of the transcript levels for the two strains grown in glucose (lanes 3 and 5, Fig. 9). The effect was also seen as the difference in the ratio of derepressed (galactose) to repressed (glucose)

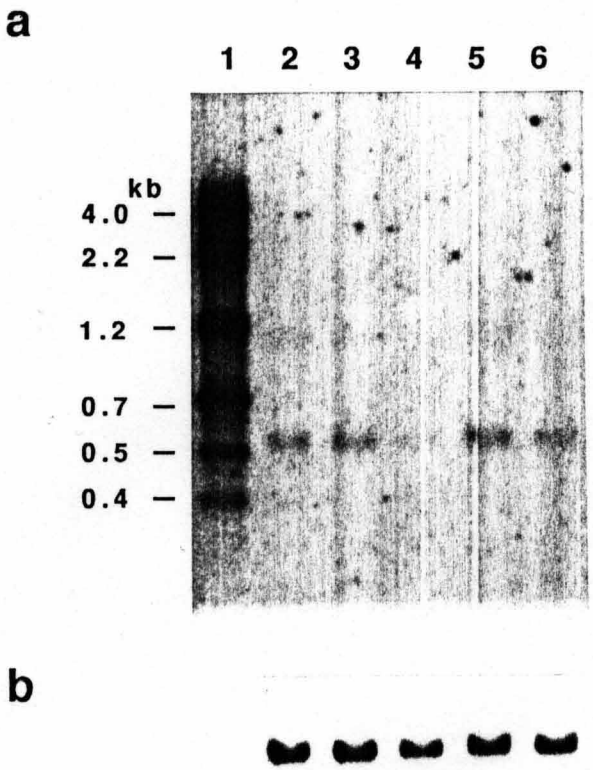


Fig. 9 Northern hybridization with the *YMR26*-specific probe. **a.** Total RNAs were prepared from early log phase cells of DC-5 ρ+ (lanes 2, 3 and 4) and DC-5 ρ⁰ (lanes 5, 6) grown in YP medium containing either 2 % glycerol (lane 2), 2 % galactose (lanes 3, 5) or 6 % glucose (lanes 4, 6). 20 µg RNA per lane were electrophoresed in a formaldehyde-agarose gel, transferred to nitrocellulose filter, and hybridized with the 1.2 kb *Eco* RI-*Hin* cII fragment of plasmid p26BP9-65 (Fig. 6). A mixture of various digestions of p26BP9-65 and pEHc65 DNA was used as size markers (lane 1). **b.** To calibrate the amounts of loaded RNA, the same filter used in **a** was hybridized with *ACT1* gene.

transcript levels for the two strains. In DC-5 ρ+, transcript levels were repressed 4 fold by growth in glucose in comparison with cells grown in either galactose or glycerol. In DC-5 ρ°, however, transcription appeared to be largely derepressed even in the presence of high concentration of glucose. The expression of the yeast actin gene is not affected by catabolite repression (Szekely and Montgomery, 1984) and serves as an internal control for the amounts of RNA for each sample on the blot. Thus, *YMR26*, like many other genes for mitochondrial proteins, was found to respond transcriptionally to catabolite repression when mitochondria are functional.

3. Analysis of the gene for YmL33

3-1. Cloning for the *MRP-L33* gene for YmL33

The large subunit mitochondrial ribosomal protein YmL33 was isolated from the purified mitochondria of strain 07173 (Graack et al., 1988). As shown in Fig. 10, the amino acid sequence of protein YmL33 was determined for N-terminal 43 residues and the data were provided by H.-R. Graack and L. Grohmann (personal communication). Two stretches of the sequence were chosen for the synthesis of oligonucleotide mixtures as shown in Fig. 10. The oligonucleotide mixtures were used as primers to amplify the region of yeast genomic DNA flanked by them using the polymerase chain reaction (PCR) method. A 110 bp-long band was observed to be amplified which coincided well with the expected size (114 bp) deduced from the amino acid sequence. This PCR product was treated with T4 DNA polymerase and then cloned into the *Sma* I site of pUC118. By nucleotide sequencing of the cloned stretch, it was found that the predicted amino acid sequence matched perfectly the N-terminal amino acid sequence of protein YmL33.

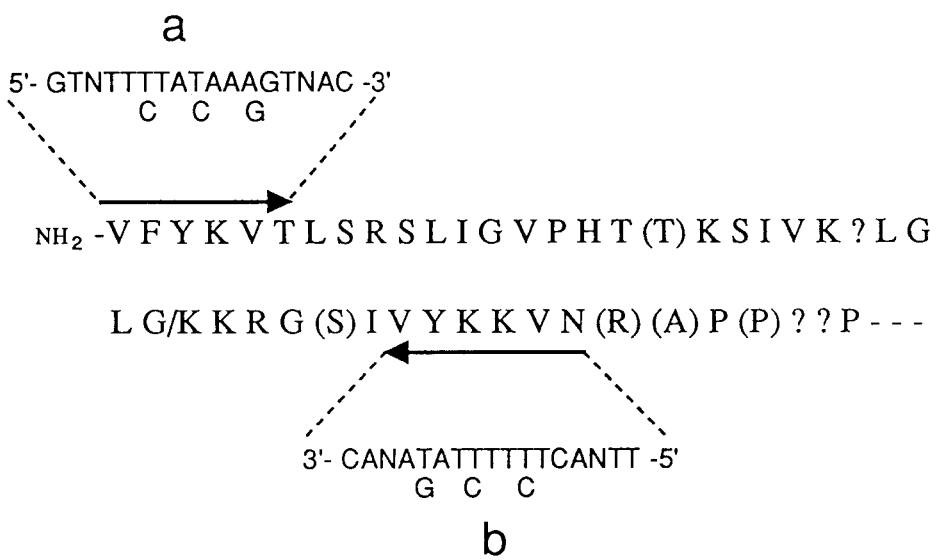


Fig. 10 N-terminal amino acid sequence of mitochondrial ribosomal protein YmL33. Amino acid residues in parentheses are slightly ambiguous and question marks indicate that the residues at the corresponding positions were undeterminable. Two stretches of the sequence indicated by horizontal arrows were chosen and oligonucleotide mixtures **a** and **b** were synthesized for PCR-amplification of the segment inbetween.

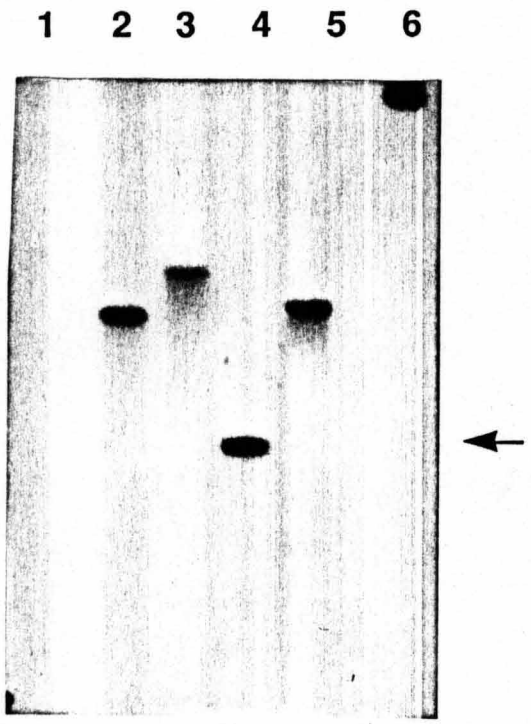


Fig. 11 Southern hybridization with the PCR-amplified segment delivered from *MRP-L33* gene (see text). The PCR-product was labeled with [α-^{32}P]dATP and used as a probe in Southern hybridization with 2 µg of the genomic DNA from DC-5 ρ^o cells digested with *Eco* RI (lane 2), *Bam* HI (lane 3), *Hin* dIII (lane 4) or *Pst* I (lane 5). Arrow points to the 1.9 kb *Hin* dIII fragment used for cloning of the *MRP-L33* gene. *Hin* dIII digested λ DNA was loaded in lanes 1 and 6 as visual size markers.

Thus, the PCR-amplified segment of this clone was concluded to be derived from the gene for YmL33 protein. The fragment was isolated after *Eco* RI and *Bam* HI digestion and then used as a probe to clone the nuclear gene for YmL33. As shown in Fig. 11, a single band was observed with the DNA from DC-5 ρ^o digested with *Eco*RI, *Bam* HI, *Hin* dIII or *Pst* I when the PCR-amplified fragment mentioned above was used as a probe. Digestion with *Hin* dIII was found to yield a shortest fragment of 1.9 kb among the restriction enzymes used. Therefore, approximately 1.8 kb to 2.0 kb *Hin* dIII fragments of the genomic DNA from strain DC-5 ρ^o were purified, ligated with pUC119 and used to transform *E. coli* strain XL-1 blue to Amp^r. By colony hybridization, two positive clones were obtained. These clones were analyzed by double strand sequencing using oligonucleotide mixture **a** (Fig. 10) as a primer. The deduced amino acid sequence agreed with the amino acid sequence of protein YmL33 shown in Fig. 10. Therefore, the clones were concluded to contain the gene for YmL33 which was named *MRP-L33*. One of the clones was designated as pL33 and analyzed further.

A detailed restriction map analysis was performed with pL33 as shown in Fig. 12. To determine the location of the *MRP-L33* gene, the plasmid pL33 was subjected to amplify by polymerase chain reaction using the oligonucleotide mixture **a** (Fig. 10) and a universal sequencing primer (M4 or RV). An amplified fragment appeared only when RV primer was used as primer and its size was estimated to be 1 kb (data not shown). Therefore, it was concluded that the *MRP-L33* gene is located at 1 kb from the right-handed *Hin* dIII site and the transcription is directed to the right-handed *Hin* dIII site as shown in Fig. 12.

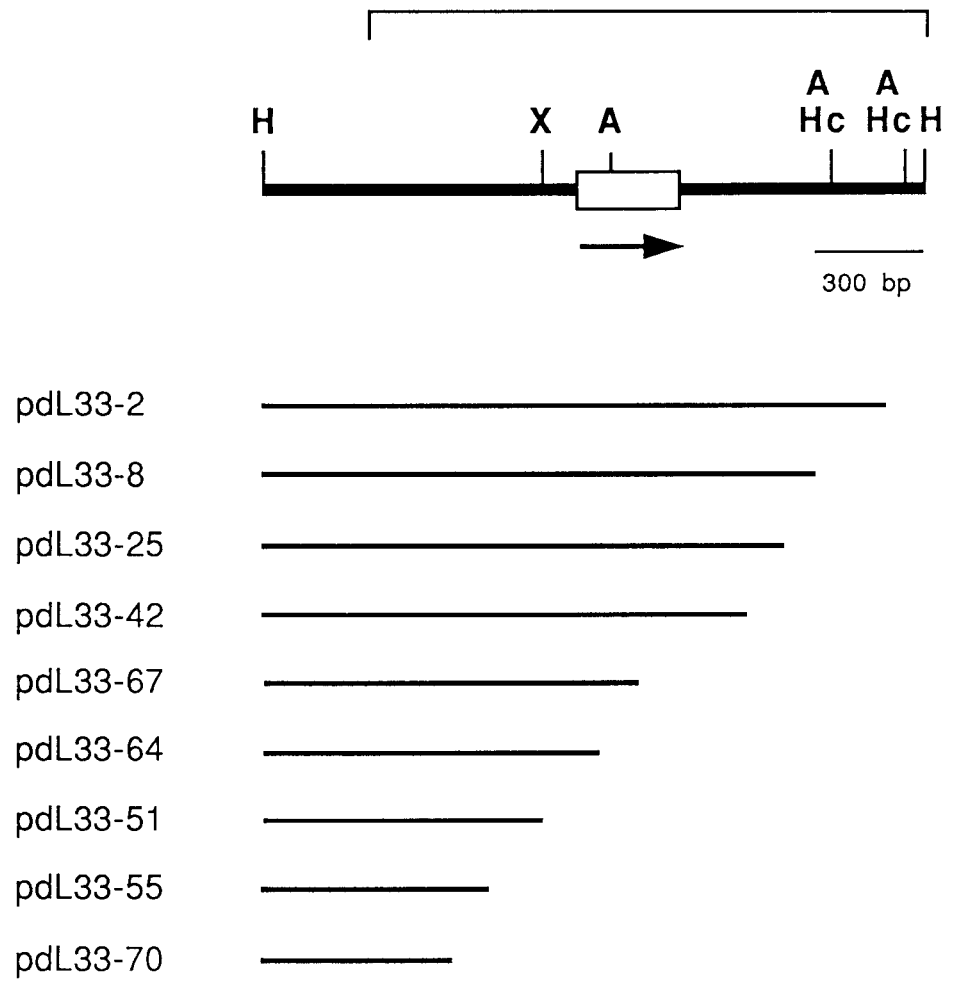


Fig. 12 The restriction map of plasmid pL33. Open box represents the coding region of *MRP-L33* gene. Arrow indicates the direction of transcription determined by polymerase chain reaction (see text). The sequenced region shown in Fig. 13 is bracketed. Abbreviations for restriction enzyme sites are: **A** for *Acc* I, **H** for *Hin* dIII, **Hc** for *Hin* cII, and **X** for *Xba* I. No site was found for *Bam* HI, *Cla* I, *Eco* RI, *Kpn* I, *Pst* I, *Pvu* II, *Sac* I and *Sma* I. Various deletions of pL33 are shown below the restriction map (see text).

3-2. The nucleotide sequence of *MRP-L33*

To determine the nucleotide sequence of the *MRP-L33* gene, I constructed a series of deletions of plasmid pL33 (Fig. 12) and determined their nucleotide sequence by the dideoxy chain termination method. The sequence data obtained were stored and analyzed using computer programs DATBAS and NUCDAT (Isono, 1984). A complete nucleotide sequence of the gene for YmL33 and its flanking regions is shown in Fig. 13. An open reading frame was found which is capable of encoding a basic protein (net charge = +10) containing 99 amino acid residues with a calculated molecular weight of 11,012. This value is consistent with the value 11 kDa estimated for protein YmL33 by SDS-PAGE (data not shown). The amino acid sequence deduced from the nucleotide sequence agreed with the amino acid sequence of protein YmL33 except for the N-terminal Met. This indicates that protein YmL33 contains no leader sequence. A comparison of the amino acid sequence of YmL33 with the NBRF database revealed that the N-terminal half of this protein had a high degree of sequence similarity to *E. coli* ribosomal protein L30 (EL30) (Cerretti et al., 1983) and its *Bacillus stearothermophilus* homologue BL30 (Kimura, 1984). The degree of amino acid identity between YmL33 and EL30 and BL30 was 39.6 % and 45.6 %, respectively, as shown in Fig. 14. In addition, the C-terminal region of YmL33 showed sequence similarity to the N-terminal sequence of yeast cytoplasmic ribosomal protein L16 with 26.2 % identity (Teem et al., 1984).

In addition to an open reading frame corresponding to the structural gene *MRP-L33*, several additional open reading frames were found to be present in its vicinity. Two of them are in the opposite direction and are situated upstream and downstream of *MRP-L33*, respectively. Whether they are indeed genes and if so

Fig. 13 Nucleotide sequence of the *MRP-L33* gene and its flanking region. The nucleotide sequence was determined using the deletion series of pL33 (see Fig. 12 and text). Nucleotides are numbered arbitrarily. The predicted amino acid translation of the coding region of the *MRP-L33* gene is shown in single letter codes. The right-handed *Hin* dIII site shown in Fig. 12 is located at the end of sequence as underlined.

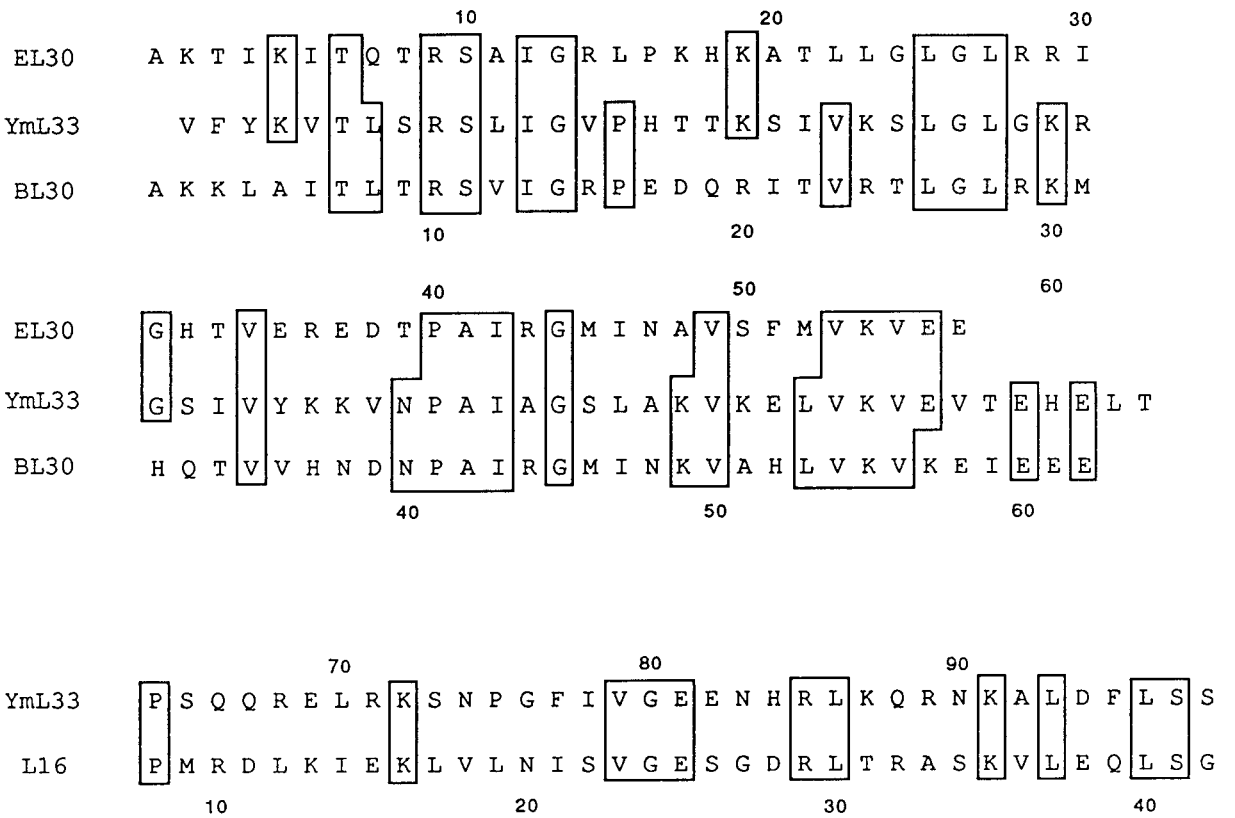


Fig. 14 Similarity of YmL33 to other ribosomal proteins. EL30 is a large subunit ribosomal protein from *E. coli* and BL30 is its homologue of *B. stearothermophilus*. L16 is a cytoplasmic ribosomal protein from *S. cerevisiae*. Identical residues are boxed. The residue numbers above the amino acid sequence are for YmL33 and those below the sequence are for the proteins in comparison.

what their functions are remain to be investigated further.

3-3. Chromosomal location and copy number of the *MRP-L33* gene

To determine the chromosomal location of the *MRP-L33* gene, a 1.1 kb *Hin* dIII-*Xba* I fragment (Fig. 12) was used to hybridize with the yeast chromosomes separated by OFAGE. As shown in Fig. 15, the resultant hybridization signal revealed the *MRP-L33* is located either on chromosome XIII or XVI. These two chromosomes co-migrated at the same position under the conditions employed. To estimate the copy number of *MRP-L33* in the yeast genome, the genomic DNA of strain DC-5 ρ^o was digested with *Bam* HI, *Hin* dIII or *Pst* I, electrophoresed, transferred and hybridized with the same fragment used above. A single band was observed in all cases (data not shown), indicating that the *MRP-L33* gene most likely exists as a single copy gene in the yeast genome. The result of hybridization with labelled PCR-product (Fig. 11) suggested the *MRP-L33* was a single copy gene. However, as described already for *YMR26* the possibility can not be ruled out that the gene and its flanking regions including the sites for three restriction enzymes used are multiplicated, although it seems not so likely.

3-4. Gene disruption analysis

To determine whether the product of *MRP-L33* is essential for the mitochondrial function in yeast cells, a gene disruption experiment was performed. For this purpose, a disrupted allele was constructed according to the scheme shown in Fig. 16. The 1.75 kb *Bam* HI fragment containing the *HIS3* gene was purified from YIp1 (Scherer and Davis, 1979) and treated with Klenow fragment for making the restricted ends blunt. Then, the resultant fragment was inserted into

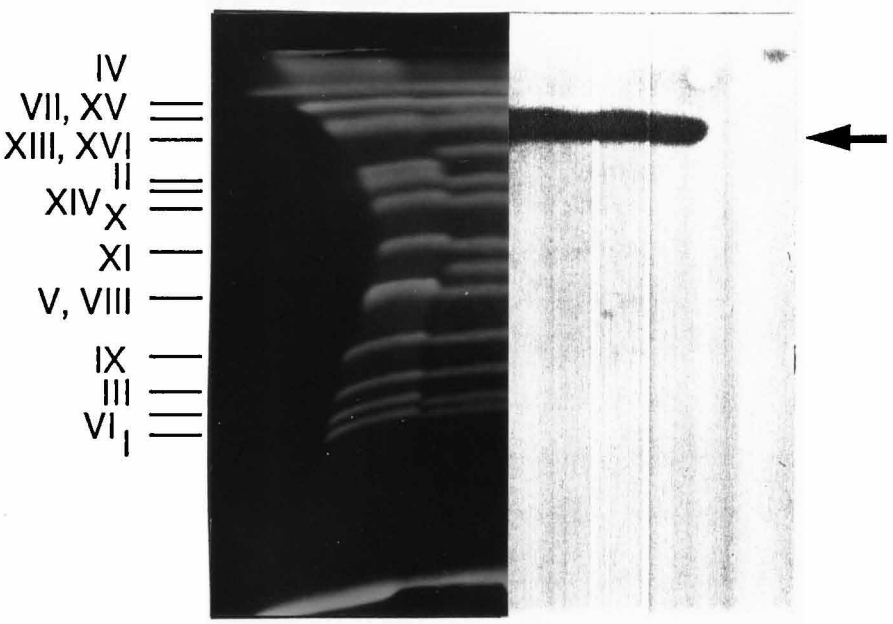


Fig. 15 Chromosomal location of the *MRP-L33* gene. *Left panel.* The chromosomal DNAs of strain DC-5 ρ^o (left) and strain YAT408 ρ^o (right) were separated by pulse-field electrophoresis. After electrophoresis, the gel was stained with ethidium bromide (0.5 μg/ml) to visualize the DNA bands. The numbers of chromosomes are indicated at left. *Right panel.* The chromosomal DNA bands were transferred to nitrocellulose membrane and hybridized with a 1.1 kb *Hin*dIII-*Xba* I fragment of plasmid pL33 shown in Fig. 12. Arrow points to the DNA band corresponding to chromosome XIII or XVI.

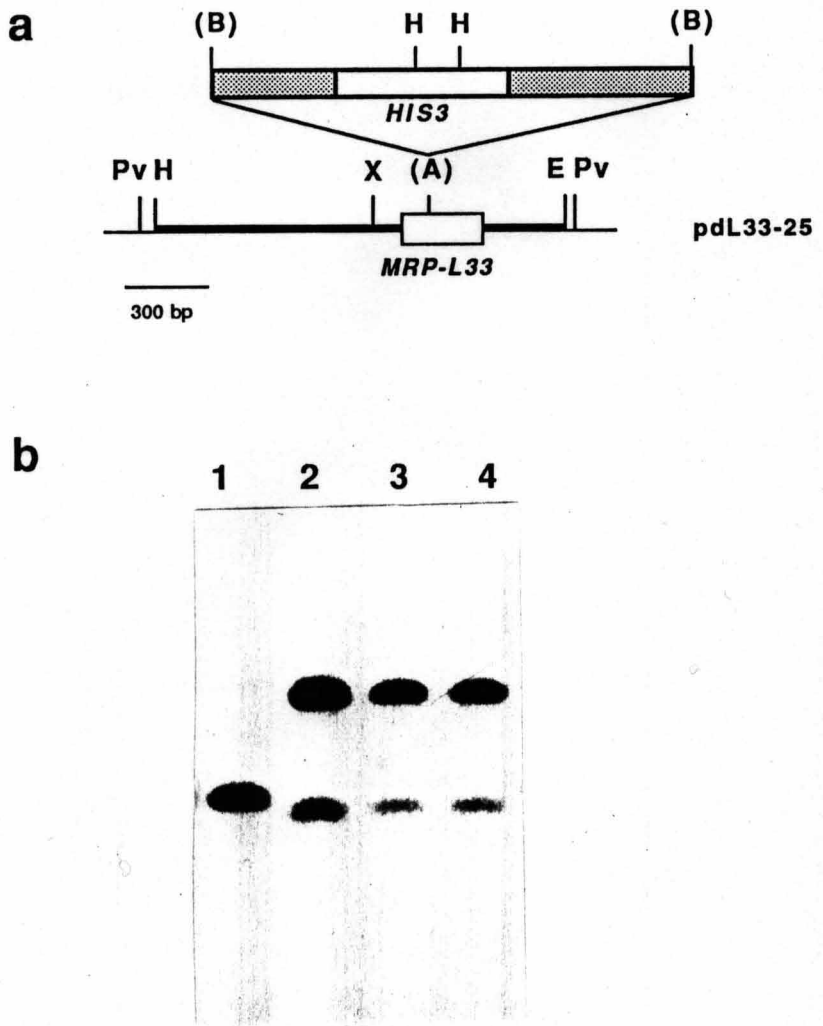


Fig. 16 Gene disruption analysis of *MRP-L33*. **a.** Plasmid pdL33-25, a deletion derivative of pL33 (see Fig. 12), was digested with *Acc* I and treated with Klenow fragment to make the restriction ends blunt. A 1.75 kb *Bam* HI fragment containing the *HIS3* gene (arrow) was purified from YIp1, treated with Klenow fragment to make the ends blunt and ligated with pretreated pdL33-25. A 3.3 kb *Pvu* II fragment of the resultant plasmid in which the *MRP-L33* gene was disrupted with the *HIS3* containing segment was then used to transform DC-5 to His+. **b.** Disruption of *MRP-L33* was confirmed by Southern hybridization after digestion with *Hin* dIII and *Xba* I of the chromosomal DNA from DC-5 as undisrupted control (lane 1) and from three His+ transformants (lanes 2, 3 and 4). Probe used was the 1.1 kb *Hin* dIII-*Xba* I fragment of plasmid pL33 shown in Fig. 12. Abbreviations for restriction enzymes are: **A** for *Acc* I, **B** for *Bam* HI, **E** for *Eco* RI, **H** for *Hin* dIII, **Pv** for *Pvu* II, and **X** for *Xba* I. The sites in parentheses indicate those treaed with Klenow fragment.

the coding region of *MRP-L33* at the *Acc* I site on the plasmid pdL33-25 which was a deletion derivative of pL33. A linearized fragment of the resultant plasmid carrying the *HIS3* insert was then excised by digestion with *Pvu* II and used to replace the genomic gene by homologous recombination. His+ transformants were selected and examined for their ability to grow on non-fermentable carbon source (glycerol). The His+ transformants were found to be respiration deficient. As shown in Fig. 16, Southern blot analysis of the genomic DNA of several representative transformants verified that the recombination had indeed taken place at the *MRP-L33* locus. These results, therefore, indicate that *MRP-L33* gene is essential for the mitochondrial function in yeast.

To investigate the stability of the mitochondrial genome when the *MRP-L33* gene is inactivated, the disrupted mutant, *mrp-l33::HIS3*, was mated with a *MRP-L33* strain which was ρ^o (AB972). The diploids generated were all respiration deficient. This result can be interpreted to indicate that the *mrp-l33::HIS3* disruptant was either ρ^- or ρ^o. The observation by Myers et al. (1987) that the mitochondrial protein synthesis is necessary for the maintenance of wild type mitochondrial DNA was also true for *MRP-L33* in addition to YMR26 described already.

3-5. Northern blot analysis

To investigate whether the expression of *MRP-L33* would be affected by different carbon sources, it was compared in ρ^+ and ρ^o cells growing on a fermentable or nonfermentable carbon source. Total RNAs were prepared from early log-phase cultures of DC-5 ρ^+ and DC-5 ρ^o growing in YP medium supplemented with either 6 % glucose, 2 % glycerol or 2 % galactose. Equal

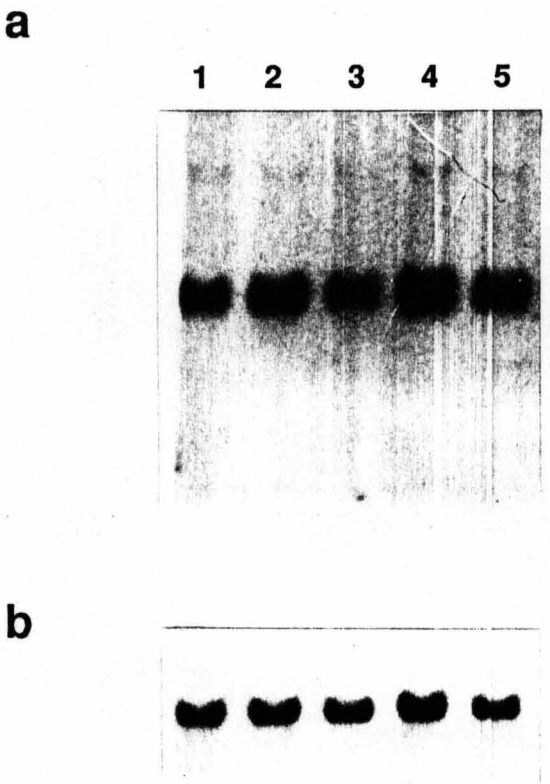


Fig. 17 Northern hybridization with an *MRP-L33* specific probe. **a.** Total RNAs were prepared from early log phase cells of DC-5 ρ^+ (lanes 1, 2 and 3) and DC-5 ρ^o (lanes 4 and 5) grown in YP medium containing either 2 % glycerol (lane1), 2 % galactose (lanes2 and 4) or 6 % glucose (lanes 3 and 5). 20 µg RNA per lane were electrophoresed in a formaldehyde-agarose gel, transferred to nitrocellulose filter, and hybridized with the 1.1 kb *Hin* dIII-*Xba* I fragment of plasmid pL33 shown in Fig. 12. **b.** To calibrate the amounts of loaded RNA, the same filter used in **a** was hybridized with *ACT1* gene.

amounts of RNA were blotted onto nitrocellulose membrane after agarose gel electrophoresis and hybridized with a 1.1 kb *Hin* dIII-*Xba* I fragment of pL33. The result of Northern blot analysis was shown in Fig. 17. When the gene was in the ρ^+ background, the amount of *MRP-L33* transcripts in the cells growing in galactose medium was more abundant than that in cells grown in glucose medium. In contrast, when the gene was in the ρ^o background, its transcription was at a high level even in glucose medium. Thus, the transcription of *MRP-L33*, like many other mitochondrial ribosomal protein genes, is repressed by glucose.

4. Effect of gene dosage on their expression

Balanced production of ribosomal components is a characteristic observed in prokaryotes (Lindahl and Zengel, 1986) as well as in the cytoplasm of yeast (Warner, 1989). To investigate whether the transcription of the genes for mitochondrial ribosomal proteins is affected when their gene dosage is changed and whether their expression is coordinated with each other, extra copies of the *YMR26*, *MRP-L33*, and some other genes for mitochondrial ribosomal protein including YmL8, YmL20, YMR31 and YMR44, were introduced into yeast cells and their effects were analyzed. For this purpose, multicopy plasmids were constructed. The plasmids pL8EP containing *MRP-L8* gene and pL20EP containing *MRP-L20* were obtained from M. Kitakawa and have been described (Kitakawa et al., 1990). pM1-XbaI containing *YMR31* gene and pN4Hc4.5 containing *YMR44* gene were provided by Y. Matsushita and have been described (Matsushita et al., 1989). As shown in Fig. 18 each fragment containing the gene as described above was ligated into the *Pvu* II site of YEp13. The resultant plasmids were then used to introduced into YM078 cells. Leu+ transformants were

selected and examined for the presence of plasmid. In the next step, total RNAs and chromosomal DNA were prepared from early log-phase cultures grown in YPD medium. Northern hybridization (Fig. 19) was performed with each gene as shown in Fig 18 and Southern hybridization (Fig. 20) was carried out with the 485 bp *Eco* RI-*Cla* I fragment of YEp13 containing *LEU2* gene as a probe. After Northern hybridization, each filter was used to hybridize with the 1.0 kb *Xho* I-*Hin* dIII fragment of pYA301 (Gallwitz and Seidel, 1980) containing *ACT1* gene as an internal control. The data were analyzed and summarized in Fig. 20. The YEp13 vector was reported to be maintained at approximately 20~50 copies per cell in yeast (Ausubel et al.). Therefore, the dosage of each gene was elevated 8 to 40 fold. The amounts of each transcript in cells harboring YEp13 with respective inserts were approximately 5 to 20-fold higher than in cells harboring YEp13 without an insert.

The above experiments revealed some features of the control of the expression of mitochondrial ribosomal protein genes. In the case of *YMR31*, the copy number of the plasmid carrying the gene is far smaller than that of the vector alone (Fig. 21), suggesting a selection against excess copies of the mitochondrial ribosomal protein gene. In the case of *YMR26* and *YMR44*, the amount of their mRNA is less than expected from the copy number of the genes. In the case of *MRP-L8*, *MRP-L20* and *MRP-L33*, increases in gene dosage were paralleled by increases in the amount of mRNA. Therefore, mRNA levels of these genes are apparently not subjected to autogenous feedback regulation that might have been used to balance the accumulation of the product of one gene to the other mitochondrial ribosomal proteins as was observed in *E. coli* (Lindahl and Zengel, 1986). In addition, overexpression of one gene does not affect the synthesis of

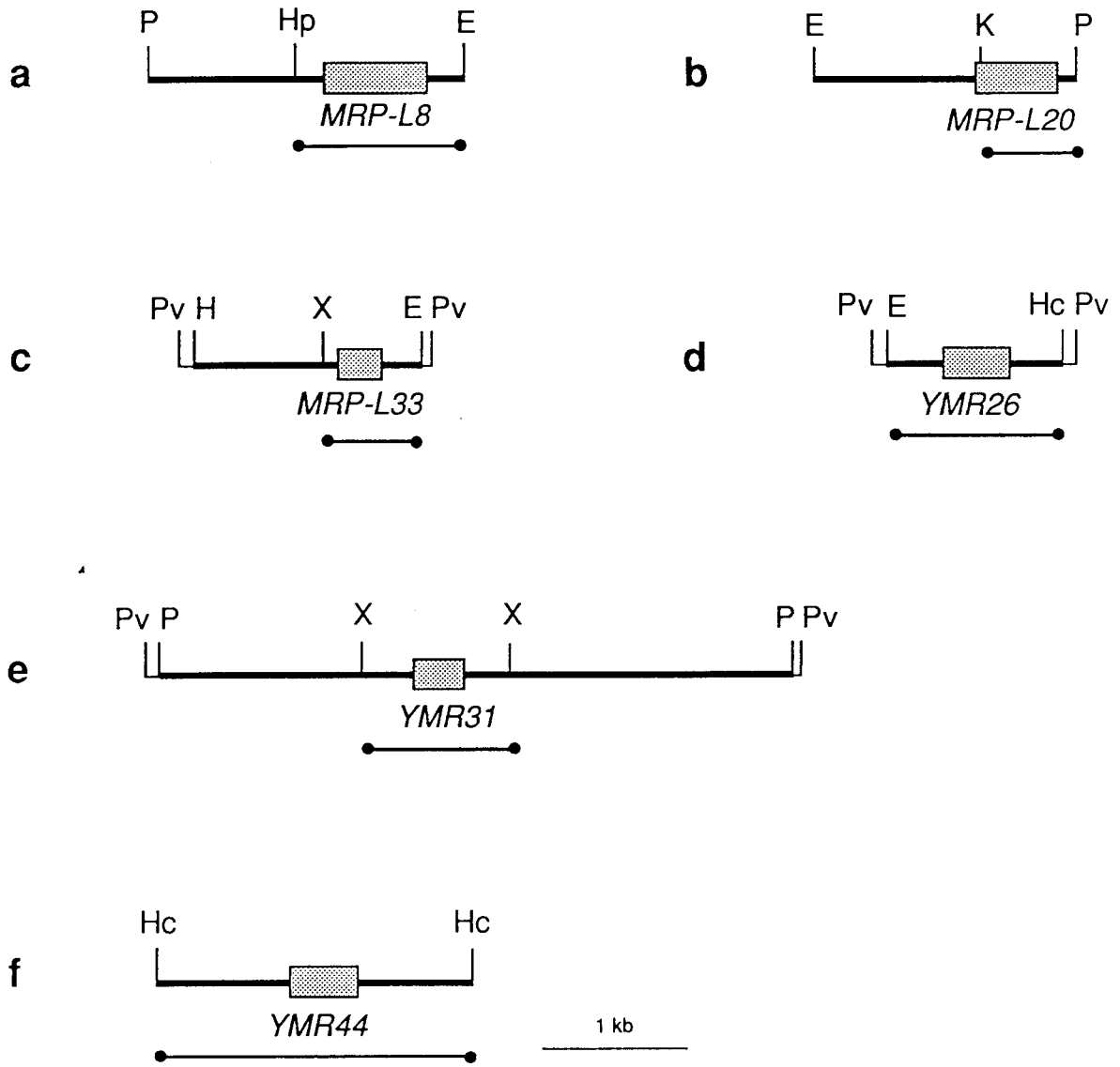


Fig. 18 Fragments inserted into multicopy plasmid YEp13. **a, b, c, d, e,** and **f** show the fragments containing *MRP-L8*, *MRP-L20*, *MRP-L33*, *YMR26*, *YMR31* and *YMR44*, respectively. *Eco* RI-*Pst* I fragments in **a** and **b** were treated with Klenow fragment to make the ends blunt. These fragments were ligated into BAP-treated *Pvu* II site of YEp13 and used to transform YM078 to LEU+. The shaded boxes represent the coding region of respective genes. Abbreviations for restriction enzymes are: **E** for *Eco* RI, **Hc** for *Hin* cII, **Hp** for *Hpa* I, **K** for *Kpn* I, **P** for *Pst* I, **Pv** for *Pvu* II, and **X** for *Xba* I. Bars with circular ends show the probes used for hybridizations shown in Figs. 19 and 20.

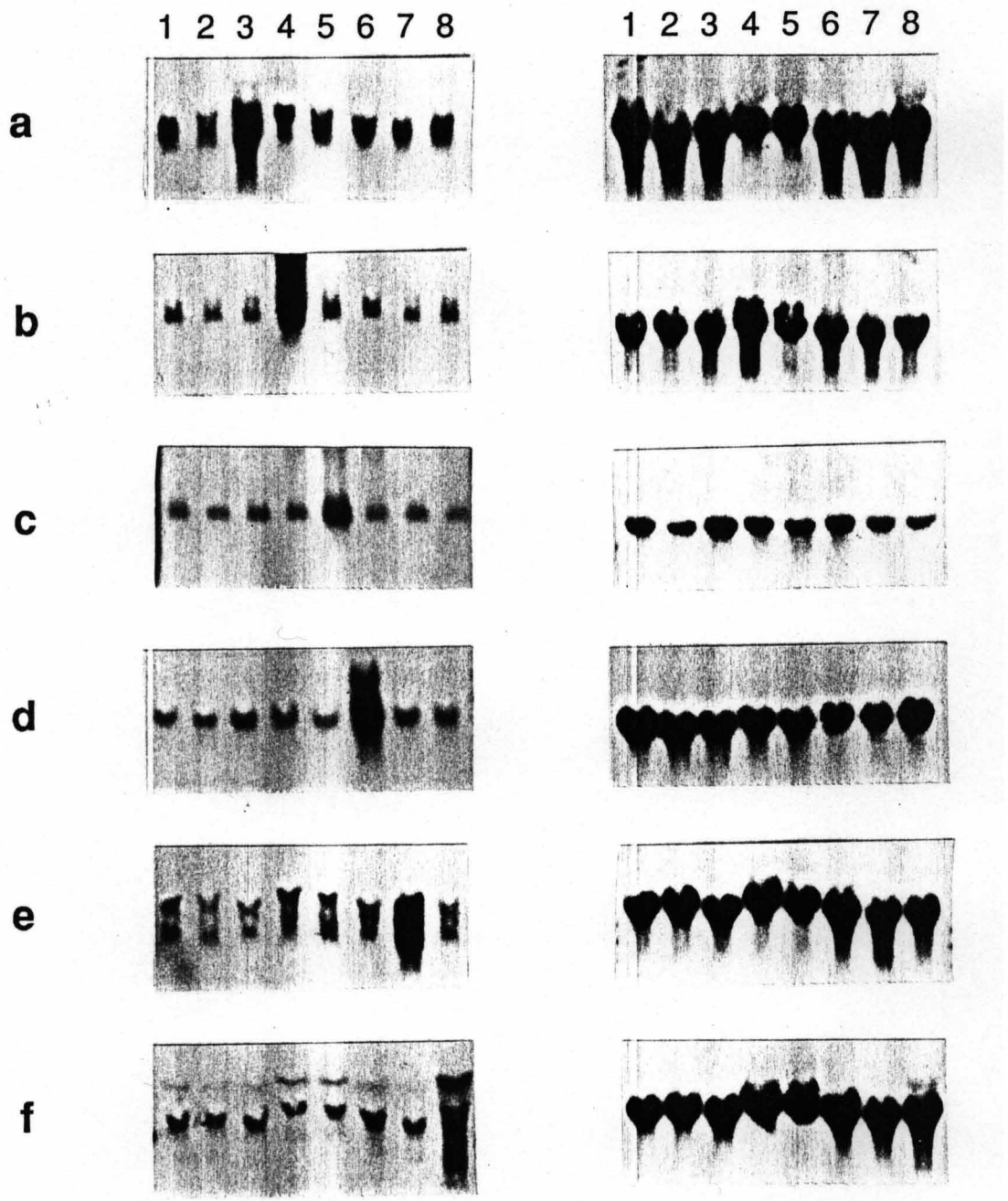
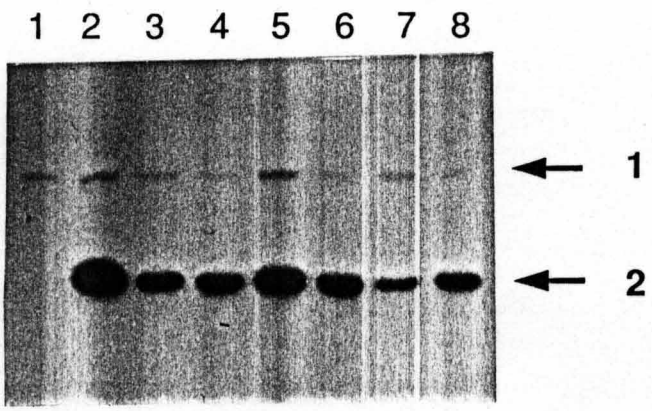


Fig. 19 Effects on the transcription of increasing gene dosage. *Left panels.* Total RNAs were prepared from early log phase cultures of YM078 (no plasmid control, lane 1), YM078 strains with YEp13 (lane 2), YEp13-L8 (lane 3), YEp13-L20 (Lane 4), YEp13-L33 (lane 5), YEp13-YMR26 (lane 6), YEp13-YMR31 (lane 7), or YEp13-YMR44 (lane 8) grown in YPD medium. 20 μg of resultant RNA per lane were loaded. Hybridization was performed with fragment of **a, b, c, d, e,** or **f** shown in Fig. 18. The *YMR31* transcript is the upper one as determined by Matsushita (1991), and the lower one is the transcript of opposite strand of *YMR31* gene. *Right panels.* The same filters were rehybridized with *ACT1* gene for internal control. The differences of relative electropherotic migration of RNA molecules between each lane shown in some panels are likely to due to the purity of each sample.

a



b

Strain	Copy number (a)	Increase in the RNA level (b)
no plasmid	1	-
+ YEp13	21.1	-
+ YEp13-L8	15.4	12.0 (±3.5)
+ YEp13-L20	28.4	16.1 (± 6.4)
+ YEp13-L33	13.6	6.6 (±1.6)
+ YEp13-YMR26	37.4	8.3 (±3.8)
+ YEp13-YMR31	8.4	15.6 (±3.1)
+ YEp13-YMR44	28.2	5.4 (±0.3)

Fig. 20 Quantitation by Southern hybridizationof the effects of increased gene dosage . **a.** The 485 bp *Eco* RI-*Cla* I fragment of YEp13 was labeled with [α-^{32}P]dATP and used as a probe in Southern hybridization of the genomic DNA from early log phase cultures of YM078 (no plasmid control, lane 1), YM078 strains with YEp13 (lane 2), YEp13-L8 (lane 3), YEp13-L20 (Lane 4), YEp13-L33 (lane 5), YEp13-YMR26 (lane 6), YEp13-YMR31 (lane 7), or YEp13-YMR44 (lane 8) grown in YPD medium. The DNA was digested with *Eco* RI. Arrow **1** represents the genomic *LEU2* gene and arrow **2** indicates the *LEU2* gene of YEp13. **b.** Values are calculated from the area of hybridizing bands shown in **a** and Fig. 19 traced with a densitometer. (a): the ratio of plasmid to chromosomal *LEU2*, (b): the average ratio of transcript from cells containing extra copies of the gene to that of cells without plasmid or with YEp13.

other mitochondrial ribosomal proteins. This indicates also that the expression of *MRP-L8*, *MRP-L20*, *MRP-L33*, *YMR26*, *YMR31* and *YMR44* is not linked to each other.

5. Effects of simultaneous disruption of *YMR31* and *MRP13* genes

As described already, almost all genes for mitochondrial ribosomal proteins including YMR26 and YmL33 are essential for mitochondrial function. However, two of the nuclear genes for mitochondrial ribosomal protein so far characterized, i.e. *MRP13* and *YMR31*, are nonessential (Partaledis and Mason, 1988; Matsushita, 1991). To investigate whether cells carrying both of the two genes disrupted are able to grow on nonfermentable carbons or not, a double gene disruption experiment was performed.

For this purpose, disrupted alleles of the two genes were constructed as outlined in Fig. 21. First, to clone the *MRP13* gene, an 17mer oligonucleotide (5'-^{4}TTCAGAAGTACAGTTTG^{20}-3') corresponding to the nucleotide sequence determined by Partaledis and Mason (1988) was synthesized. It was end-labelled with [γ-^{32}P]ATP and used as a probe to hybridize the genomic DNA of DC-5 ρ^{o} strain. It was found that the digestion with *Bgl* II yielded a shortest fragment of 2.2 kb among the restriction enzymes used. This result coincided well with the result of Partaledis and Mason (1988). Therefore, *Bgl* II fragments of approximately 2.2 kb were purified, ligated with the *Bam* HI site of pUC118 and used to transform *E. coli* strain XL-1 blue to Amp^{r}. Transformants were tested by colony hybridization with the oligonucleotide as a probe and three positive recombinants were isolated. They were examined further by nucleotide sequencing. Their nucleotide sequence agreed with the sequence determined by Partaledis and Mason (1988).

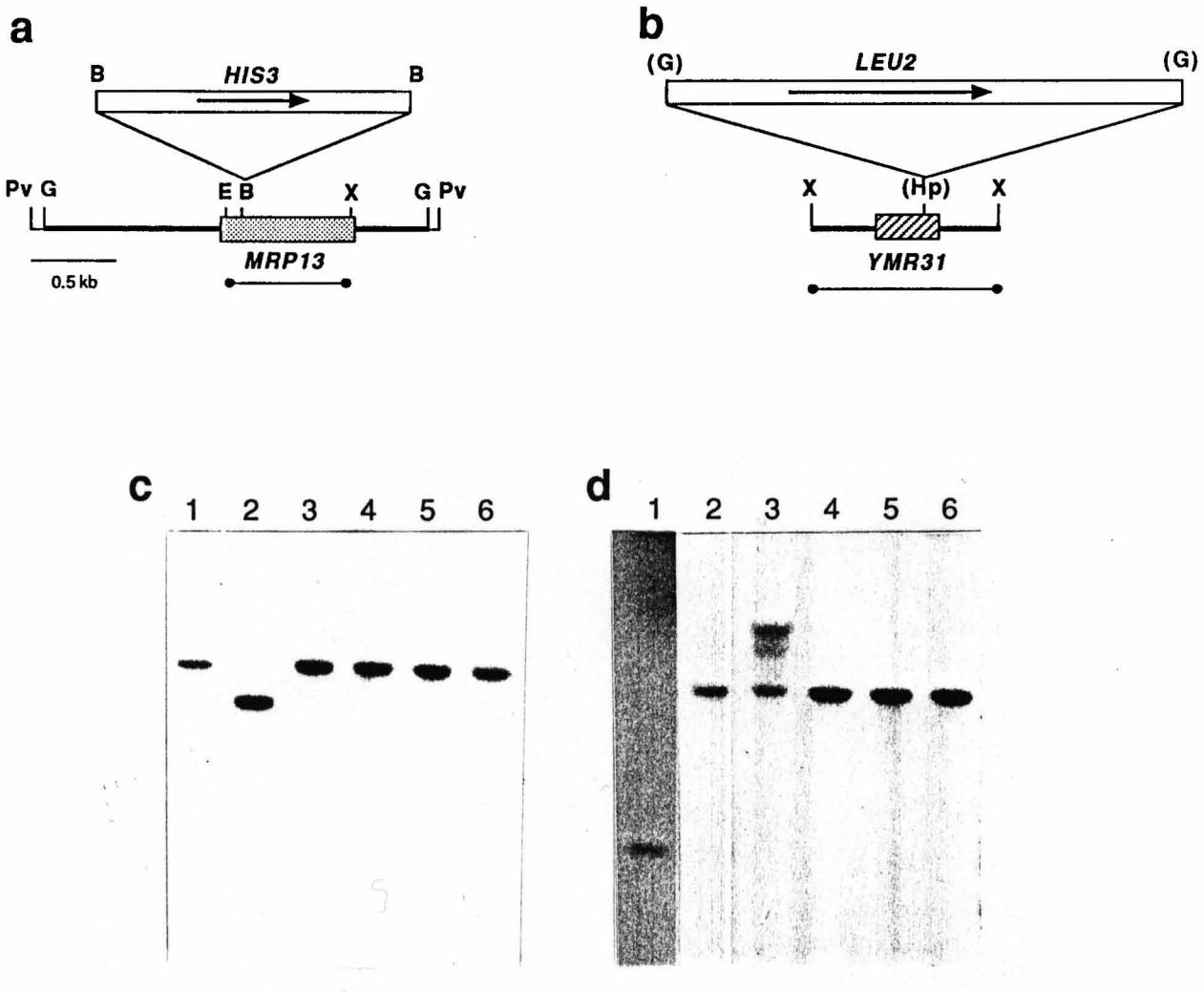


Fig. 21 Double gene disruption analysis. **a.** A 1.75 kb *Bam* HI fragment containing *HIS3* gene was ligated into the *Bam* HI site of pMRP13 (see text). The 4.1 kb *Pvu* II fragment of the resultant plasmid in which the *MRP13* gene was disrupted with *HIS3* containing segment was used to transform YM078 to His+. **b.** The plasmid pM1-XbaI (see text) was digested with *Hpa* I and treated with Klenow fragment to make the ends blunt. A 2.96 kb *Bgl* II fragment containing the *LEU2* gene was treated with Klenow fragment and ligated with pretreated pM1-XbaI. A 4.0 kb *Xba* I fragment of the resultant plasmid in which the *YMR31* gene was disrupted was used to transform *mrp13::HIS3* mutants to Leu+. Disruption of *MRP13* and *YMR31* was confirmed by Southern hybridization after digestion with *Eco* RI (**c**) and *Xba* I (**d**) of chromosomal DNA from His+ transformant (*mrp13::HIS3*, lane 1), Leu+ transformant (*ymr31::LEU2*, lane 2), and His+ and Leu+ transformants (*mrp13::HIS3* and *ymr31::LEU2*, lanes 3 to 6). The fragments represented by lines with circular ends shown in **a** and **b** were used as probes shown in **c** and **d**, respectively. Abbreviation for restriction enzymes are: **B** for *Bam* HI, **E** for *Eco* RI, **G** for *Bgl* II, **Hp** for *Hpa* I, **Pv** for *Pvu* II, and **X** for *Xba* I. The sites in parentheses indicate those treated with Klenow fragment.

Therefore, these clones were concluded to encode the protein MRP13. One of them was termed pMRP13 and used in subsegment experiments. A 1.75 kb *Bam* HI fragment containing the *HIS3* gene was then purified from plasmid YIp1 (Scherer and Davis, 1979) and inserted into the coding region of *MRP13* at the *Bam* HI site of plasmid pMRP13. The 4.2 kb *Pvu* II fragment of resultant plasmid shown in Fig. 21 **a** was used to replace the corresponding genomic region of strain YM078 (*leu2, his3, ura3, trp1*). His+ transformants were isolated and it was confirmed that the recombination had been directed to the putative *MRP13* locus by Southern blot analysis (data not shown). These His+ transformants were able to grow on the nonfermentable medium, as expected. In the next step, a plasmid carrying *ymr31::LEU2* was constructed as shown in Fig. 21 **b**. A 2.96 kb *Bgl* II fragment containing the *LEU2* gene was purified from YEp13 and treated with Klenow fragment. The resultant fragment was inserted into the coding region of *YMR31* at the *Hpa* I site. The 7.4 kb *Xba* I fragment of a resultant plasmid was used to transform *mrp13::HIS3* strain to Leu+. The results of Southern blot analyses shown in Fig. 21 **c** and **d** clearly demonstrated that integrations occurred at the *MRP13* and at the *YMR31* locus as anticipated. All of these His+ and Leu+ transformants were observed to grow normally when their ability to grow on nonfermentable carbons was tested. The result indicates that the mitochondrial function is not affected by disrupting both *MRP13* and *YMR31* genes at the same time. The growth rate of this double-gene disruptant mutant was measured in comparison of YM078 (wild-type control), *mrp13::HIS3* mutant, *ymr31::LEU2* mutant and *mrp13::HIS3* and *ymr31::LEU2* mutant on YPD and YPG media. No difference among the mutants and wild type was observed (data not shown).

Discussion

1. Characteristics of the *YMR26* and *MRP-L33* genes

In this thesis, I described the cloning of two nuclear genes for mitochondrial ribosomal protein, YMR26 and YmL33. The screening procedure for recombinant plasmids containing these genes was to use the N-terminal amino acid sequences of the purified proteins. The gene for protein YMR26 was cloned by hybridization with the oligonucleotide mixtures corresponding to its N-terminal amino acid sequence. The nucleotide sequence of a 1.4 kb region of a resultant clone showed the presence of an open reading frame (ORF) which is capable of encoding a basic protein (net charge = +21) with 158 amino acid residues. Because its N-terminal amino acid sequence completely matched that of protein YMR26, this ORF was concluded to be the *YMR26* gene. The cloning of the *MRP-L33* gene was carried out by first generating amplified genomic DNA fragments by polymerase chain reaction using oligonucleotide mixtures as primers which were synthesized on the basis of the N-terminal amino acid sequence of YmL33 protein. Nucleotide sequence of a resultant clone containing *MRP-L33* revealed that the presence of four ORF's in the sequenced region. Of them the second one from the 5'-terminus in the cloned segment was found to be capable of encoding a protein with 99 amino acid residues and a calculated molecular weight of 11,012. Its deduced N-terminal amino acid sequence completely matched that of protein YmL33. Thus, the ORF was concluded to be the structural gene for YmL33. In addition, three additional ORF's were found to exist in the segment flanking *MRP-L33*. The proteins encoded by these open reading frames showed no significant similarity to any other proteins in the NBRF protein data base. Two of them are in

the opposite direction to the *MRP-L33* gene and are capable of encoding basic proteins. Judging from the codon usage pattern in these ORF's, if they are actually transcribed, they are expected to be only lowly expressed. As shown in Fig. 17, only a single transcript was detected when the Northern hybridization was carried out using a 1.1 kb *Hin* dIII-*Xba* I fragment which should contain not only *MRP-L33* but also part of the down stream ORF. The detected transcript was concluded to be that of *MRP-L33* because its size coincided well with the one predicted from the nucleotide sequence (Fig. 13). Thus, the down stream ORF is likely to be either a very lowly expressed gene or not an active gene.

The codon usage pattern in the *YMR26* and *MRP-L33* genes was examined as shown in Table 2. As in other mitochondrial ribosomal protein genes so far analyzed, no highly biased codon usage was observed towards a particular subset of codons which are recognized by most abundant cognate tRNAs in the cell (Sharp et al., 1986). This suggests that the expression of the genes for YMR26 and YmL33 proteins is not high.

Comparison of the amino acid sequence determined by direct protein sequencing with that deduced from the DNA sequence indicates that neither YMR26 nor YmL33 protein has a leader sequence. The N-terminal leader sequence is considered to play an important role in targeting proteins into various subcellular structures including mitochondria. However, several mitochondrial proteins such as cytochrome c and ADP/ATP translocator protein (Douglas et al., 1986) were found to lack a leader sequence. Moreover, several other mitochondrial ribosomal proteins characterized so far were found to lack a leader sequence as well (see Table 3). Of course, mitochondrial proteins lacking an N-terminal leader sequence must be also transported into mitochondria. Therefore,

Table 2. Codon usage in *YMR26* and *MRP-L33* [a]

		Y-L	Y-H	E-H	A	B			Y-L	Y-H	E-H	A	B
Phe	UUU	1.17	0.42	0.46	0.89	1.33	Ser	UCU	1.87	3.17	2.57	1.50	1.20
	UUC	0.83	1.58	1.54	1.11	0.67		UCC	0.93	2.17	1.91	1.00	1.20
Leu	UUA	1.63	0.80	0.11	1.06	1.62		UCA	1.17	0.23	0.20	1.50	0.60
	UUG	1.95	4.50	0.11	2.47	1.62		UCG	0.50	0.09	0.04	0.50	1.20
Leu	CUU	0.67	0.13	0.22	0.71	1.08	Pro	CCU	1.18	0.50	0.23	1.09	1.00
	CUC	0.33	0.02	0.20	0.71	0.00		CCC	0.62	0.03	0.04	0.72	0.00
	CUA	0.80	0.42	0.04	0.35	0.54		CCA	1.81	3.44	0.44	1.09	2.00
	CUG	0.62	0.13	5.33	0.71	1.08		CCG	0.39	0.03	3.29	1.09	1.00
Ile	AUU	1.52	1.36	0.47	1.20	0.60	Thr	ACU	1.43	1.94	1.80	1.60	0.00
	AUC	0.79	1.58	2.53	1.20	0.60		ACC	0.91	1.78	1.87	0.80	0.00
	AUA	0.70	0.06	0.01	1.00	1.00		ACA	1.17	0.22	0.14	1.60	2.40
Met	AUG	1.00	1.00	1.00	1.00	1.00		ACG	0.48	0.06	0.18	0.00	1.60
Val	GUU	1.61	2.18	2.24	0.57	0.36	Ala	GCU	1.57	2.72	1.88	1.33	2.00
	GUC	0.92	1.65	0.15	0.57	1.44		GCC	0.98	1.13	0.25	2.00	1.00
	GUA	0.75	0.04	1.11	2.29	1.08		GCA	1.10	0.12	1.10	0.67	1.00
	GUG	0.72	0.13	0.50	0.57	1.08		GCG	0.35	0.04	0.80	0.00	0.00
Tyr	UAU	1.05	0.26	0.39	1.33	0.00	Cys	UGU	1.41	1.78	0.67	0.00	0.00
	UAC	0.95	1.74	1.61	0.67	2.00		UGC	0.59	0.22	1.33	0.00	0.00
*	UAA	1.34	2.61				*	UGA	1.06	0.24		3.00	3.00
*	UAG	0.60	0.16				Trp	UGG	1.00	1.00	1.00	1.00	1.00
His	CAU	1.29	0.52	0.45	2.00	1.34	Arg	CGU	1.06	0.64	4.39	1.71	0.00
	CAC	0.71	1.48	1.55	0.00	0.66		CGC	0.30	0.01	1.56	0.00	0.00
Gln	CAA	1.42	1.89	0.22	1.50	0.66		CGA	0.34	0.00	0.02	0.86	3.00
	CAG	0.58	0.11	1.78	0.50	1.34		CGG	0.18	0.00	0.02	0.00	0.00
Asn	AAU	1.12	0.28	1.10	1.14	0.50	Ser	AGU	0.93	0.17	0.22	1.50	1.20
	AAC	0.88	1.72	1.90	0.86	1.50		AGC	0.60	0.16	1.05	0.00	0.60
Lys	AAA	1.11	0.38	1.60	1.03	1.50	Arg	AGA	3.00	5.20	0.02	3.43	1.98
	AAG	0.89	1.62	0.40	0.97	0.25		AGG	1.12	0.14	0.00	0.00	1.02
Asp	GAU	1.31	0.84	0.61	1.50	2.00	Gly	GGU	2.23	3.80	2.28	2.40	1.72
	GAC	0.69	1.16	1.39	0.50	0.00		GGC	0.72	0.15	1.65	0.80	0.56
Glu	GAA	1.43	.183	1.59	1.27	1.14		GGA	0.67	0.02	0.02	0.80	0.56
	GAG	0.57	0.17	0.41	0.73	0.86		GGG	0.38	0.03	0.04	0.00	1.16

[a] Y-L, Y-H and E-H denote data for yeast lowly expressed, yeast highly expressed and *E. coli* highly expressed genes, respectively, taken from Sharp et al. (1986) A: *YMR26* B: *MRP-L33*

a sequence or sequences other than the N-terminal leader sequence must be recognized as a mitochondrial targeting signal. Recently, the carboxyl terminal regions of ADP/ATP translocator protein were shown to be sufficient to be signaling transport into mitochondria (Pfanner et al., 1987a). More systematic analysis of mitochondrial ribosomal proteins with respect to their transport signals must be performed.

Based on its primary structure, the product of *MRP-L33* can be regarded as being homologous to protein L30 (to be abbreviated as EL30, etc. in the following discussion) of the large ribosomal subunit of *E. coli* and *B. stearothermophilus*. It thus suggests that these proteins have similar functions and evolved from a common ancestor. Three of eleven other mitochondrial ribosomal protein genes of yeast so far characterized were found to encoded proteins with a high degree of sequence similarity to *E. coli* ribosomal proteins: namely, MRP2 to S14, MRP7 to L27 and YmL8 to L17 as shown in Table 3. In addition, two nuclear genes for mitochondrial ribosomal proteins in *Neurospora crassa* showed sequence similarity to *E. coli* ribosomal proteins (Kreader et al., 1988; Kuiper et al., 1988). These data support the endosymbiont hypothesis that the mitochondrion is a descendent of an endosymbiotic prokaryote (Schwartz and Dayhoff, 1978; Gray and Doolittle, 1982). However, YMR26 protein was found to have no similarity to any ribosomal proteins of *E. coli* or other organisms. These findings suggest the existence of three classes of proteins in mitochondrial ribosomes in yeast. One is those originated from the original prokaryote, the putative mitochondrial ancestor, and still contains several conserved primary sequences due most likely to some functional constraints. Another class of proteins is those which are derived from the original prokaryote but have undergone extensive amino acid changes during the

Table 3. The characteristics of mitochondrial ribosomal protein genes

Protein	Molecular weight [a]	Leader sequence	Chromosomal location	Sequence [b] similarity	Gene disruption	Reference
YMR26	18,454	-	VII / XV	-	RD	This thesis
YMR31	12,792	8	VI	-	+	Matsushita et al., 1989
YMR44	11,476	-	XIII / XVI	-	?	Matsushita et al., 1989
YmL 8	26,822	-	X	L17, S13	RD	Kitakawa et al., 1990
YmL20	20,626	18	XI	-	RD	Kitakawa et al., 1990
YmL31	14,246	12	XI	-	RD	Grohmann et al., 1989
YmL33	10,881	-	XIII / XVI	L30	RD	This thesis
MRP 1	36,628	?	IV	-	RD	Myers et al., 1987
MRP 2	13,522	?	XVI	S14	RD	Myers et al., 1987
MRP 7	40,091	27 [c]	?	L27	RD	Fearon and Mason, 1988
MRP13	37,366	?	IV [d]	-	+	Partadelis and Mason, 1988
PET123	41,650	?	XV	-	RD	Mcmullin et al., 1990

[a] The molecular weight is calcuated for mature protein predicted from the nucleotide sequence.

[b] Sequence similarity to *E. coli* ribosomal proteins is shown.

[c] Determined as described in this thesis.

[d] Unpublished observation.

evolution of mitochondria so that their sequence similarity to *E. coli* ribosomal proteins can no longer traced. A third class of proteins is those which are derived from preexisting nuclear genes that were originally unrelated to mitochondria and were newly adopted for mitochondrial ribosomes. However, it is, at least at present, difficult to distinguish between the second and third classes of mitochondrial ribosomal proteins.

In this connection, it should be noted that the genes for mitochondrial ribosomal proteins must have been transferred from the genome of original endosymbiotic prokaryote to the nucleus during the course of mitochondrial evolution, if mitochondria are indeed described from endosymbiotic bacteria. Most of the ribosomal protein genes are clustered with each other in bacteria. In contrast, the nuclear genes for mitochondrial ribosomal proteins so far characterized are not clustered on the chromosomes as shown in Table 3. I have demonstrated that the *YMR26* gene is located on either chromosome VII or XV (Fig. 5) and the *MRP-L33* gene on either chromosome XIII or XVI (Fig. 15). The gene for protein YMR44 has been located also on chromosome XIII or XVI (Matsushita et al., 1989). However, no evidence has been found to indicate the proximity of *MRP-L33* and *YMR44* on this chromosome. Therefore, it is implied that the "movement" of the ancestral mitochondrial ribosomal protein genes occurred independently from one another. It might be that the movement was achieved via incomplete reverse transcription of polycistronic mRNAs. At any rate, loss of the corresponding genes from the mitochondrial genome must have occurred afterwards. In this connection it seems interesting to note that the C-terminal one-third of YmL33 protein have a sequence similarity to yeast cytoplasmic ribosomal protein L16. L16 protein in turn shows a high degree of similarity to *E. coli*

ribosomal protein L5 (Teem et al., 1984). The gene for EL5 is located in the same operon together with the gene for EL30 which is a homologue of YmL33. These findings suggest that some of the proteins might have arisen as the results of gene fusion during their transfer to the nucleus. There is an additional evidence in this line. One of the mitochondrial ribosomal proteins in yeast, YmL8, has a significant sequence similarity to EL17. In addition, a short stretch in the C-terminal region of this protein has a similarity to ES13 (Table 3). The genes for EL17 and ES13 belong to the same operon (Bedwell et al., 1985). It will be interesting to investigate if such fusions correspond to spacial proximity of the two proteins in the bacterial ribosomes.

As described in the Introduction section, eukaryotic cells use structurally and functionally different ribosomes for protein synthesis in the cytoplasm and in the mitochondria. The cytoplasmic ribosomal proteins are different from their mitochondrial counterparts and the two sets of proteins appear to be encoded by separate sets of genes. Many of the genes for cytoplasmic ribosomal proteins are duplicated and contain a single intron, an unusual feature in *S. cerevisiae* in which nuclear genes generally lack introns (Warner, 1989). Unlike the genes for cytoplasmic ribosomal proteins, the mitochondrial ribosomal genes exist in single copy and have no intron except for the gene for YMR44 (Table 3). These data seem to imply the evolution of two different populations of ribosomal proteins in a eukaryotic cells. It is interesting to note here that the same product of histidyl tRNA synthetase gene in yeast is utilized in the cytoplasm and in mitochondria (Natsoulis et al., 1986). The histidyl tRNA synthetase is synthesized in two ways and the one harboring a presequence is imported into mitochondria, while the other lacking it is used in the cytoplasm. Therefore, it may be that common ribosomal proteins also

exist which are present in the cytoplasm as well as in the mitochondria, though no such an evidence has so far been present. Further analyses are needed to trace evolutionary interrelation between various ribosomal proteins in mitochondria and in the cytoplasm.

2. Function of YMR26 and YmL33 proteins

The high level of sequence conservation between EL30 and YmL33 described above suggests that the functional properties of EL30 will hold true for YmL33. From the *in vitro* ribosomal reconstitution experiments by omitting a single protein in *E. coli* (Hampl et al., 1981) and *B. stearothermophilus* (Auron and Fahnestock, 1981; Cohlberg and Nomura, 1976). EL30 (BL30) was implicated to be involved in the peptidyltransferase activity. While the similarity to EL30 implicates YmL33 as a component harboring the peptidyltransferase activity, possible function of YMR26 remains obscure because it bears no obvious sequence similarity to any of the *E. coli* ribosomal proteins. It is interesting to note that, despite the role of EL30 in peptidyltransfer, an *E. coli* mutant lacking L30 was found to be viable with a growth rate similar to that of a wild type strain (Dabbs, 1979). Therefore, L30 appears to be not absolutely essential for the ribosomal function in *E. coli*. In contrast, YmL33 protein was found to be essential for mitochondria, because *MRP-L33* null mutants created by gene disruption were respiration-deficient (Fig. 16). YMR26 protein was also concluded to be essential for mitochondria from the gene disruption experiment (Fig. 7). Moreover, the inactivation of the *YMR26* or *MRP-L33* gene led to instability of the mitochondrial genome, resulting in the creation of ρ^- or ρ^o derivatives. These data support that the function of these mitochondrial ribosomal proteins, and hence the

mitochondrial protein synthesis, is necessary for the maintenance of the mitochondrial DNA as pointed out by Myers et al. (1987). The molecular basis for the linkage between mitochondrial protein synthesis and the maintenance of [ρ^+] mitochondrial DNA has not been established with respect to either how much protein synthesis is required or what the essential product(s) might be. As shown in Table 3, almost all other mitochondrial ribosomal protein genes are essential for the mitochondrial function. On the other hand, there are *E. coli* mutants which lack several ribosomal proteins and yet suffer no anomaly in growth. EL30 is one of such proteins as described above (Dabbs et al., 1981; Dabbs et al., 1983; Lotti et al., 1983). The apparent difference between the *E. coli* ribosome and the yeast mitochondrial ribosome with respect to the essentialness of protein L30 and its equivalent could be due to the structural differences between the two ribosomes. In fact, in support of this notion, many yeast mitochondrial ribosomal proteins show no sequence similarity to any *E. coli* ribosomal proteins. Furthermore, many of the yeast mitochondrial proteins which show sequence similarity to *E. coli* ribosomal proteins are much larger than their *E. coli* counterparts (Fearon and Mason, 1988; Kitakawa et al., 1990). Therefore, it is conceivable that the assembly and/or location of individual ribosomal proteins may be different in the two ribosomes.

Only two mitochondrial ribosomal protein genes so far characterized are known to be nonessential for the mitochondrial function, because the corresponding disrupted mutants were respiration-competent (Table 3). Thus, it is interesting to create a *mrp13::HIS3* and *ymr31::LEU2* double mutant, because it is conceivable that these proteins complement their function with each other so that one of them can be disrupted without affecting the mitochondrial function. When, however, both of them are disrupted simultaneously, it may be that they will cause

loss of certain function of mitochondria. When the respiration ability of a *mrp13::HIS3* and *ymr31::LEU2* doubly disrupted mutant was investigated, the growth was found normal on nonfermentable medium. In addition, the growth rate of this mutant on YPD and YPG media was the same to that of wild type. Thus, the function of mitochondria in this doubly disrupted mutant was concluded to be normal. In an evolutionary point of view, these two proteins may be considered as proteins which have been newly adopted for mitochondrial ribosomes and their function is not crucially established yet. Conversely, they are no longer needed for mitochondrial ribosomes but present there because the other proteins supplemented their functions.

3. Regulation of expression

A major objective of this thesis is to understand the regulatory mechanism that coordinate the synthesis of mitochondrial ribosomal components the genes of which exists in the mitochondrial and nuclear genomes. Northern analyses demonstrated that transcription of *YMR26* or *MRP-L33* is catabolite repressed. The level of the transcripts in derepressed cells growing on galactose or glycerol is significantly higher than in repressed cells growing in a medium with a high concentration of glucose. It appeared that the repression of transcription of *YMR26* and *MRP-L33* is similar to that of other nuclear genes for mitochondrial proteins including *MRP1*, *MRP2* (Myers et al., 1987), *MRP13* (Partaledis and Mason, 1988), and *RPO41* (Masters et al., 1987). However, the molecular basis of the regulation is not clear. There is evidence for the involvement of upstream elements in catabolite repression (Guarente and Mason, 1983; Pfeifer et al., 1987), and the *trans*-acting elements responsible for the catabolite repression of *CYC1* have been

identified (Forsburg and Guarente, 1988). However, it is not yet clear how extensively these regulatory circuits overlap with those regulating mitochondrial ribosomal proteins.

The levels of *YMR26* and *MRP-L33* mRNAs are higher in glucose-grown ρ^o strain than in ρ^+ strain. This support the findings by Parikh et al. (1987) that some nuclear genes respond to the absence (ρ^o) or small deletion (ρ^- or mit$^-$) of mitochondrial DNA. The mechanism of this response is unclear but does not seem to depend on the export of macromolecules from mitochondria. In addition, the extent of repression by glucose in ρ^o strain is smaller than in ρ^+ strain as shown in Figs. 9 and 17.

In *E. coli*, the accumulation of many ribosomal proteins is tightly linked to subunit assembly through an autogenous feedback mechanism in which excess ribosomal proteins act as translational repressor (Lindahl and Zengel, 1986). In contrast, balanced accumulation of yeast cytoplasmic ribosomal proteins appears to be the net result of similar transcription rates and transcript stabilities (Warner, 1989) coupled with the rapid degradation of excess ribosomal proteins that are not assembled into ribosomal subunits (Maicas et al., 1988). I have examined whether the transcription of *MRP-L8*, *MRP-L20*, *MRP-L33*, *YMR26*, *YMR31* and *YMR44* genes is coordinated or not by introducing extra copies of each gene. The overexpression of one gene did not affect the synthesis of other mitochondrial ribosomal proteins, in contrast to the situation in *E. coli*, in which whole operons can be controlled by overexpression of a single protein (Lindahl and Zengel, 1986). However, these experiments revealed some features of the control of expression. In a case, the copy number of a plasmid carrying the *YMR31* gene is smaller than that of the vector alone. This suggests the existence of selection against excess

copies of the mitochondrial ribosomal protein gene. In addition, in some cases (*YMR26* and *YMR44*), the amount of mRNA is less than expected from the number of copies of the gene. I have not analyzed whether the translation of these genes is controlled post-transcriptionally or not. However, it is clear that the accumulation of these mRNA does not influence the transcription level of other mitochondrial ribosomal proteins and that the regulatory mechanism is not through autogenous feedback. The expression of three nuclear encoded mitochondrial ribosomal proteins has been examined at both the transcriptional and posttranscriptional levels and each shows distinctive regulatory properties. The expression of MRP7 appears to be controlled at the level of protein synthesis, compensating for imbalances in increased gene copy number (Fearon and Mason, 1988). Whether this form of control is related to the autogenous feedback mechanism described for *E. coli* ribosomal proteins has not been elucidated. The accumulation of MRP1 and MRP13 is controlled post-transcriptionally by an increased rate of degradation, a property shared with cytoplasmic ribosomal proteins (Dang et al., 1990; Partaledis and Mason, 1988). Therefore, it seems that the regulatory properties of mitochondrial ribosomal proteins may be diverse.

In this work, I cloned and characterized two nuclear genes for mitochondrial ribosomal proteins and investigated the genetic interactions between the nucleus and mitochondria on the synthesis of mitochondrial ribosomes. The data described above provide further insights into the interactions of the nucleus and mitochondria and the evolution of mitochondrial proteins.

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