Studies on the structure and expression of mitochondrial ribosomal protein genes in Saccharomyces cerevisiae

Matsushita, Yasuhiko

(Degree)
博士（学術）

(Date of Degree)
1991-03-31

(Date of Publication)
2015-03-30

(Resource Type)
doctoral thesis

(Report Number)
甲0978

(JaLCDOI)
https://doi.org/10.11501/3057162

(URL)
https://hdl.handle.net/20.500.14094/D1000978

博 士 論 文

Studies on the Structure and Expression of Mitochondrial Ribosomal Protein Genes in *Saccharomyces cerevisiae.*

平成３年１月

神戸大学大学院自然科学研究科

松 下 保 彦

博士論文

Studies on the Structure and Expression of Mitochondrial Ribosomal Protein Genes in *Saccharomyces cerevisiae.*

＜パン酵母 (*Saccharomyces cerevisiae*) ミトコンドリアの
リボソーム蛋白質遺伝子の構造と発現に関する研究＞

平成３年１月

神戸大学大学院自然科学研究科

松 下 保 彦

Studies on the Structure and Expression of Mitochondrial Ribosomal Protein Genes in *Saccharomyces cerevisiae*

A dissertation for partial fulfilment of a Doctral Degree

at the Department of Science and Technology

of the Postgraduate School of Kobe University

January, 1991

Yasuhiko Matsushita

Contents

Contents ---------- 1

List of Figures and Tables ---------- 4

Acknowledgements ---------- 7

Abbreviations ---------- 8

Summary ---------- 9

Introduction

1. My personal interests in biology ---------- 12

2. Evolutionary view of mitochondria and chloroplasts ---------- 13

3. Gene expression on mitochondria and chloroplasts ---------- 15

4. Protein transport into mitochondria and chloroplasts ---------- 17

5. Ribosomes in yeast mitochondria ---------- 18

Materials and Methods

1. Strains and cloning vectors ---------- 21

2. Media ---------- 22

3. Preparation of ribosomal proteins from *S.cerevisiae* ---------- 23

   3-1. Preparation of mitochondria and extraction of ribosomes ------ 23

   3-2. Purification and amino acid sequencing of mitochondrial ribosomal proteins ---------- 23

4. Cloning and characterization ---------- 24

4-1. Cloning of the genes for mitochondrial ribosomal proteins ----- 24

4-2. Nucleotide sequencing ---------- 25

4-3. Miscellaneous methods ---------- 25

5. Analysis of messenger RNA ---------- 25

5-1. Isolation of total RNA ---------- 25

5-2. Electrophoresis and blotting ---------- 26

5-3. Northern hybridization ---------- 26

6. Yeast genetics ---------- 27

6-1. Transformation ---------- 27

6-2. Mating and sporulation ---------- 27

6-3. Tetrad analysis ---------- 28

Results

1. Mitochondrial ribosomal proteins in *S. cerevisiae* ---------- 29

2. Cloning and characterization of the gene for protein YMR31 -------- 29

2-1. Cloning of the *YMR31* gene ---------- 31

2-2. Nucleotide sequences of the *YMR31* gene ---------- 35

2-3. Gene disruption analysis of *YMR31* ---------- 40

3. Cloning and characterization of the gene for protein YMR44 ------- 40

3-1. Cloning of the *YMR44* gene ---------- 40

3-2. Nucleotide sequences of the *YMR44* gene ---------- 44

4. Gene organization of *YMR31* and *YMR44* ---------- 49

4-1. Copy number of the *YMR31 and YMR44* genes ---------- 49

4-2. Chromosomal locations of the *YMR31 and YMR44* genes ----- 49

5. Expression of mitochondrial ribosomal protein genes ---------- 52

5-1. Analyses of transcripts of *YMR31* ---------- 52

5-2. Transcription of other mitochondrial ribosomal protein genes ---- 55

6. Transport of mitochondrial ribosomal protein YmL8 into mitochondria -- 62

Discussion

1. Cloning methods for yeast mitochondrial ribosomal protein genes ---- 81

2. Characteristics of *YMR31* and *YMR44* genes ---------- 82

3. Evolution of mitochondrial ribosomal proteins ---------- 84

4. Transfer of mitochondrial ribosomal protein genes from mitochondria to nucleus ---------- 85

5. Expression of mitochondrial ribosomal protein genes ---------- 88

6. Transport of ribosomal proteins into mitochondria ---------- 88

References ---------- 92

List of Figures and Tables

List of Figures

Fig. 1. Two-dimensional electropherogram of mitochondrial ribosomal proteins from baker's yeast used. ---------- 30

Fig. 2. Design of oligonucleotide probes for cloning of *YMR31*. ---------- 33

Fig. 3. Genomic Southern hybridization for *YMR31*. ---------- 34

Fig. 4. Restriction map of the chromosomal segment containing the gene for YMR31. ---------- 36

Fig. 5. Strategy for sequencing *YMR31*. ---------- 37

Fig. 6. Nucleotide sequence of the gene for YMR31. ---------- 39

Fig. 7. Gene disruption analysis of *YMR31*. ---------- 41

Fig. 8. Design of oligonucleotide probes for cloning of *YMR44*. ---------- 42

Fig. 9. Genomic Southern hybridization for *YMR44*. ---------- 43

Fig. 10. Restriction map of the chromosomal segment containing the gene for YMR44. ---------- 45

Fig. 11. Strategy of sequencing *YMR44*. ---------- 46

Fig. 12. Nucleotide sequence of the gene for YMR44. ---------- 48

Fig. 13. Copy number of *YMR31* and *YMR44*. ---------- 50

Fig. 14. Chromosomal locations of the genes for YMR31 and YMR44. --- 51

Fig. 15. Estimation of relative amounts of transcripts. ---------- 53

Fig. 16. Northern hybridization with the DNA fragment containing *YMR31* gene. ---------- 54

Fig. 17. Analysis of transcript of *YMR31* by strand- and region-specific probes. ---------- 56

Fig. 18. Analysis of transcript of *YMR31* by strand-specific probes. ------ 57

Fig. 19. Analysis of transcript of *MRP-L8, MRP-L20, MRP-L31* and *MRP-L33.* ---------- 60

Fig. 20. Analysis of transcript of *YMR26* and *YMR44.* ---------- 61

Fig. 21. Changes in transcription prior to and after release from glucose repression. ---------- 63

Fig. 22. Schematic illustration of homologous region between mitochondrial ribosomal proteins from *S. cerevisiae* and *E. coli.* ---------- 66

Fig. 23. Motifs in the C-termini of mitochondrial ribosomal proteins. ----- 68

Fig. 24. Experimental scheme for analysis of the structure of YmL8. ----- 69

Fig. 25. Disruption of the *MRP-L8* gene. ---------- 70

Fig. 26. Construction of plasmids containing the *MRP-L8* gene with 3'-terminal deletions. ---------- 71

Fig. 27. Function of the *MRP-L8* deletions on YCp-type plasmid. ------- 77

Fig. 28. Analysis of the *MRP-L8* deletions on YEp-type plasmid. ------- 78

Fig. 29. Summary of structural analysis of YmL8. ---------- 79

List of Tables

Table 1. N-terminal amino acid sequences of mitochondrial ribosomal proteins in *S. cerevisiae.* ---------- 32

Table 2A. Plasmid clones for the *YMR31* gene. ---------- 38

Table 2B. M13 phage clones for the *YMR31* gene. ---------- 38

Table 3A. Plasmid clones for the *YMR44* gene. ---------- 47

Table 3B. M13 phage clones for the *YMR44* gene. ---------- 47

Table 3C. Oligonucleotides synthesized for DNA sequencing of *YMR44* gene. ---------- 47

Table 4. Probes for Northern hybridization. ---------- 59

Table 5. Classification of the mitochondrial ribosomal proteins of *S. cerevisiae.* ---------- 65

Table 6. Plasmids used for complementation analysis of *MRP-L8* gene. ---------- 72

Table 7A. Deletion constructs of *MRP-L8* gene. ---------- 74

Table 7B. Plasmids used for transformation of YMD002. ---------- 74

Table 8. Results of structural analysis of YmL8. ---------- 76

Acknowledgements

I am grateful to Dr. Katsumi Isono, Prof. of Genetics, for his general guidance and valuable advice throughout this work, to Dr. Madoka Kitakawa, for her valuable discussion and providing clones containing *MRP-L8* and *MRP-L20* genes, to Miss Wonkyung Kang for her encouragements and discussion and providing clones containing *YMR26* and *MRP-L33* genes, and to Dr. Setsuko Isono and all other members of the laboratory of Genetics for their help, discussion and encouragements. I also thank Prof. Dr. Akira Ishihama and Dr. Nobuyuki Fujita of National Institute of Genetics for their help in amino acid sequencing, Prof. Dr. Akio Toh'e and Dr. Kouichi Kawakami of Tokyo University for providing yeast strains and plasmids, Dr. Lutz Grohmann for providing a clone containing *MRP-L31* gene, and Dr. Dieter Gallwitz, Prof. of Max-Planck-Institut für Biophysical Chemistry, for providing plasmid pYA301 containing the actin gene.

January, 1991

Yasuhiko Matsushita

Abbreviations

A_{260}	Absorbance at 260 nm
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
Amp	Ampicillin
bp	Base pair
BPB	Bromophenol Blue
cpm	Count per minute
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
HPLC	High pressure liquid chromatography
kb	Kilo base pairs
min	Minute
MOPS	Morpholinopropanesulfonic acid
OD_{600}	Optical density at 600 nm
PAGE	Polyacrylamide gel electrophoresis
PEG	Polyethylene Glycol
RNA	Ribonucleic acid
rpm	Round per minute
rRNA	Ribosomal RNA
SDS	Sodium dodecyl sulfate
Tet	Tetracycline
tRNA	Transfer RNA
Tris	Tris-(hydroxymethyl)aminoethane
XC	Xylene cyanol

Summary

The mitochondrial ribosome of yeast (*Saccharomyces cerevisiae*) consists of two RNA molecules (15S and 21S) and approximately 80 proteins. Of these, the two RNAs and one protein are synthesized from the mitochondrial genes, while all other proteins are from the nuclear genes. Consequently, most of the protein components of the mitochondrial ribosome must be synthesized in the cytoplasm and transported into mitochondria. These facts suggest that the genes for mitochondrial ribosomal proteins, if not all of them, are likely to have been transferred from the mitochondrial genome to the nucleus during the course of mitochondrial evolution. Therefore, it is interesting to ask how the exression of genes in the nucleus and mitochondria is regulated coordinately and how such a gene transfer occured.

To approach these problems, I first undertook systematic cloning of individual mitochondrial ribosomal protein genes and investigated their structural characteristics and organization in the nucleus from evolutionaly points of view. For this purpose, I isolated each ribosomal protein and determined the N-terminal amino acid sequences for seven purified proteins. The sequence data were used for the synthesis of oligonucleotide probes to clone the corresponding genes. Thus, the genes for two proteins, termed YMR31 and YMR44, were cloned and their nucleotide sequences determined. From the nucleotide sequence data, the coding region of the gene for YMR31 protein was found to be composed of 369 nucleotide pairs. Comparison of the amino acid sequence of YMR31 protein and the one deduced from the nucleotide sequence of its gene suggests that it contains an

octapeptide leader sequence. The calculated molecular weight of YMR31 protein without the leader sequence is 12,792. Similarly, the gene for YMR44 protein was analyzed. It was found to contain a 147 bp intron which contains two sequences conserved among yeast introns. The length of the two exons flanking the intron totals 294 nucleotide pairs which can encode a protein with a calculated molecular weight of 11,476. Gene disruption analysis showed that the gene for YMR31 was not essential for mitochondrial function. The gene for YMR31 protein was found to be located on chromosome VI, while the gene for YMR44 protein resides on either chromosome XIII or XVI.

To investigate how the expression of mitochondrial ribosomal protein genes is regulated in the nucleus and mitochondria, I examined transcripts of some of the mitochondrial ribosomal protein genes including *YMR31* and *YMR44* in yeast cells possessing either ρ^+ or ρ^0 phenotypes under various growth conditions. The results showed that the transcription of the nuclear genes for mitochondrial ribosomal proteins occured in ρ^0 cells and that their transcription was repressed by glucose in both ρ^0 and ρ^+ cells. The degree of repression was observed to vary between ρ^+ and ρ^0 cells and the transcripts in ρ^0 cells accumulated more than those in ρ^+ cells. These suggest that the presence or absence of mitochondrial DNA influences the transcription of the mitochondrial ribosomal protein genes in the nucleus. Furthermore, the results showed that the amounts of transcripts from *MRP-L8* and *MRP-L33* the products of which do not contain a presequence for mitochondrial transport was more than that of *MRP-L20* and *MRP-L31* transcripts the products of which contain a presequence. It thus seems to indicate a possible correlation between the transcription level of a gene and the transport efficiency into mitochondria of its protein product.

Transport of proteins into mitochondria is one of the important steps for the biogenesis of mitochondrial ribosomes. Structural comparison of mitochondrial ribosomal protein genes including *YMR31* and *YMR44* suggested that the C-terminus of some of the proteins would function as signals for mitochondrial transport. To investigate this possibility, I undertook functional analyses of the C-terminus region in one of the mitochondrial ribosomal proteins, YmL8. For this purpose, I constructed deletion series of *MRP-L8* gene encoding YmL8 on a single copy or a high copy plasmid and examined their function in yeast strains in which the chromosomal *MRP-L8* gene was disrupted. When the 3'-terminus of *MRP-L8* gene was deleted so that the YmL8 protein would lack ten amino acid residues in the C-terminus, the gene on a single copy plasmid was incapable of complementing the defect. In contrast, when it was on a high copy plasmid, it was able to complement. These suggest that the C-terminus of YmL8 is important for either transport into mitochondria or assembly into mitochondrial ribosomes or both of the proteins. The data obtained in this work are discussed from the evolutionary point of view of mitochondrial ribosomal protein genes.

Introduction

1. My personal interests in biology

All necessary information required by a living organism is stored and appended in the forms of "genes". In multicellular organisms, although cells in different organs and tissues contain the same set of genes, particular genes are expressed in cells positioned at certain places at certain times. These make it possible to destine each cell during the development of a living organism and further to cause different organisms to evolve. Why each cell expresses different genes as cell division proceeds despite that they are derived from a single cell such as a fertilized egg or a germinated spore? Recently, studies in *Drosophila* (Roth et al. 1989, Hülskamp et al. 1989, reviewed in Manseau and Schüpbach 1989), *Xenopus* (reviewed in Woodland 1989, Altaba and Melton 1989) and *C. elegans*, showed that there is intra-cellular localization of some key proteins or mRNAs in the egg, which is to be inherited to cause differences in the cytoplasm of each cell after division and suggested that those are one of the reason why cell fate is determined (reviewed in Kenyon and Kamb 1989, Cooke and Smith 1990). Furthermore, cell fate is likely to be determined by factors such as communications between cells, differences in their position to environments. Thus, in multicellular organisms, particular genes in each cell seem to be selected and expressed by various factors.

In unicellular organisms, each cell is an independent organism by itself and contains the same set of genes and cytoplasm. Therefore, the same genes are selected and expressed in each cell under the same conditions and at the same

time. Among unicellular organisms, prokaryotes contain no membrane systems except for cytoplasmic membrane. Thus, they have less distinction between the place where genes exist and their information is transcribed into mRNAs and the place where proteins are synthesized from the transcribed information and play their functions. Therefore, the mechanisms by which genes to be expressed are selected and regulated seem to be comparatively simpler. On the other hand, unicellular eukaryotes contain membrane systems and subcellular organelles surrounded by membranes. This causes membranes to separate the place for primary gene expression and that for the function of their products. Therefore, the mechanisms for regulation of gene expression seem to be more complex in contrast to those in prokaryotes. Especially, it is interesting how the expression of genes for constituents in mitochondria and chloroplasts are regulated. It is because these organelles have their own DNA and some of the constituents are composed of the products from genes in both nuclear DNA and in organellar DNA.

In short, I am interested how a particular gene or genes are selected out of genes collected in each cells, how their expression is regulated and how such mechanisms were acquired in the course of evolution. Bearing these in mind, I decided to investigate on the expression of genes for mitochondrial constituents and on their evolutionary point as a subject for my doctor thesis.

2. Evolutionary view of mitochondria and chloroplast

Mitochondria and chloroplasts have their own DNA. Most of the constituents of these organelles are, however, encoded by the genes located in the nucleus. The complete nucleotide sequence of chloroplasts from *Marchiantia polymorpha* (Ohyama et al. 1986), *Nicotiana tabacum* (Shinozaki et al. 1986) and *Oriza sativa* (Hiratsuka et al. 1989) and that of mitochondria from human (Anderson et al. 1981),

mouse (Bibb et al. 1981), cow (Anderson et al. 1982), frog (Roe et al. 1985), *Drosophila* (Clary and Wolstenholme 1985), sea urchin (Jacobs et al. 1988), *Paramecium* (Pritchard et al. 1990), *Podospora* (Cummings and Domenico 1988), yeast (Zamaroczy and Bernardi 1986), *Neurospora* (Dujon 1983), *Aspergillus* (Brown et al. 1985), *Leishmania* (Simpson et al. 1987), *Trypanosome* (Simpson et al. 1987) and *Marchantia polymorpha* (Ohyama et al. 1990) have been determined. Consequently, it was shown that organellar DNA code for only genes for the respiratory system, photosynthesis system, tRNA, rRNA, and some ribosomal proteins. It has been accepted that mitochondria and chloroplasts are derived from endosymbiotic bacteria (reviewed in Gray et al. 1982, 1984, Gray 1989). In fact, many characteristics of these subcellular organelles are "prokaryotic". Therefore, it seems likely that an extensive gene transfer from such organelles to the nucleus occurred during the evolution of eukaryote, since most of the genes that encode present day organellar proteins are in the nucleus.

The *tufA* gene which encodes the protein synthesis elongation factor Tu (EF-Tu) in chloroplast should be noted as an example suggesting that gene transfer from chloroplasts to the nucleus occurred (Baldauf et al. 1990). The *tufA* gene is located in chloroplasts of green algae such as *Chlamydomonas reinhardtii* and *Codium fragile,* while it is located in the nucleus of land plants such as *Arabidopsis thaliana* and *Marchantia polymorpha.* Interestingly, the *tufA* gene is located in both chloroplast and the nucleus in *Coleochaete*, one of the green algae proposed to belong to a sister group of land plants.

The genes for some ribosomal proteins in mitochondria and chloroplasts are known to be located in organellar DNA in some organisms but not in other organisms. The genes for mitochondrial ribosomal proteins, L2, L14, S12 and S14,

are located in the mitochondrial genome of *Paramecium* but not in animal or fungal (Pritchard et al. 1990). Of these four genes, the gene for L14 does not exist in the mitochondrial genome in *Marchiantia polymorpha* (Ohyama et al. 1990). Little is known about whether a certain gene which does not exist in the organellar genome of a certain organism is located in the nucleus or not. One of such examples is the gene for mitochondrial ribosomal protein S14. This protein is encoded in the mitochondrial genome in *Paramecium* (Pritchard et al. 1990) and *Marchiantia polymorpha* (Ohyama et al. 1990) while the gene for a mitochondrial ribosomal protein termed MRP2, which have sequence similarity to S14, is located in the nucleus (Myers et al. 1987).

Examples described above are important to understand the gene transfer from organelles to the nucleus from an evolutionary point of view. In addition, it is reported that a single gene product makes a common soil bacterium to grow in the cytoplasm of a mammalian cell like a parasite (Bielecki et al. 1990). This is also interesting in view of origin of mitochondria and chloroplasts.

3. Gene expression on Mitochondria and chloroplasts

As mentioned above, most of the constituents in mitochondria and chloroplasts are encoded by nuclear DNA, synthesized on cytoplasmic ribosomes, and then transported into the organelles (Tzagoloff et al. 1979, 1986). Thus, expression of these genes are likely to be regulated in various ways at transcription, translation and transport. Furthermore, it is known that constituents such as F1-ATPase, a complex in electron transport pathway, and ribosomes, are constructed from products synthesized from nuclear as well as organellar genes. In these cases, communication of the two separate genetic systems are required. It is interesting how the gene expression of nuclear and organellar genes which are

located at different places within the cell is regulated coordinately and how the information passes from one place to another through membranes.

Of the nuclear gene-encoded products in organella, there are several examples in which a single gene encodes both mitochondrial and nuclear/cytoplasmic forms of an enzyme; they include *HTS1* (Natsoulis et al. 1986), *MOD5* (Najarian et al. 1987, Dihanich et al. 1987), *LEU4* (Beltzer et al. 1986, 1988), *VAS1* (Chatton et al. 1988), *FUM1* (Wu et al. 1987), and *TRM1* (Ellis et al. 1987) genes of *S. cerevisiae,* gene of leucyl-tRNA synthetase of *Neurospora crassa* (Beauchamp et al. 1977), gene of mouse and human fumarase (Doonan et al. 1984, Zinn et al. 1986) and goose malonyl-CoA decarboxylase (Flurkey et al. 1982). The mechanisms by which single genes code for isozymes destined to different intracellular compartments are diverse. For the *HTS1*, *LEU4*, *VAS1* and *FUM1* genes of *S. cerevisiae*, a single open reading frame encodes isozymes that differ from each other by amino-terminal extension. Two in-frame AUGs are used to initiate the translation of proteins that differ in size according to the length of the amino-terminal extension. The extension determines the final location of the longer protein and which AUG is used to initiate translation is determined by the 5' ends of the mRNA. Although, for the *TRM1* genes of *S. cerevisiae*, a single open reading frame codes for isomers like the *HTS1* and *LEU4* genes, functional mitochondrial targeting information is not located within the amino-terminal extension region, but within a region of *TRM1* that is present in both forms of isomers (Ellis et al. 1987). There are two in-frame ATGs in the *MOD5* gene of *S. cerevisiae*, and a model is proposed to suggest that in this case the choice between the AUGs may be made at the translational level (Dihanich et.al., 1987, Najarian et.al., 1987). This is interesting with respect to endosymbiotic origin of mitochondria and chloroplasts.

4. Protein transport into mitochondria and chloroplasts

Sorting of a protein into a target organelle requires that the protein in question has information for its destination, and that each organelle has a system to recognize the information. Comparison of protein transport into mitochondria and chloroplasts revealed several common features: most of the proteins have presequences in their N-terminus which are removed during or after import. That the presequences contain targeting information was proven by deletion analyses and gene fusion experiments. For example, the presequence of yeast cytochrome c oxidase subunit IV (Cox IV) directs the cytosolic protein, mouse dihydrofolate reductase (DHFR), into the mitochondrial matrix in vitro (Hurt et al.1984). Similar observations have been reported for the presequence of other matrix proteins including human ornithin transcarbamylase (OTC; Horwich et al. 1986), yeast alcohol dehydrogenase III (van Loon et al. 1986) and a chloroplast stromatic protein such as pea ribulose bisphosphate carboxylase small subunit (SSU; van den Broeck et al. 1985). Even in those proteins synthesized without presequences, the precise targeting information has been localized to N-terminal regions; for example, the first 12 amino acids of a yeast 70 kDa outer membrane protein were shown to be essential for proper targeting (Hurt et al. 1985).

On the contrary, examples that targeting information does not exist in the N-terminal region have also been reported. Smagula and Douglas (1988) reported that the region between amino acid 72 and 111 of ADP/ATP carrier protein (309 a.a.) in *S. cerevisiae* constitutes at least part of mitochondrial transport signal. In this connection, it should be noted that some mitochondrial ribosomal proteins in *S. cerevisiae* have no leader sequences in their N-terminus and some of them have sequence similarity in their N-terminal region to bacterial ribosomal proteins (Myers

et al. 1987, Fearon and Mason 1988, Partaledis and Mason 1988, Grohmann et al. 1989, Kitakawa et al. 1990, Kang 1991, Kang et al. 1991, this work).

In contrast to the amount of our knowledge concerning the signals for transported proteins, little is unknown about the mechanism by which organelles recognize the signals (reviewed in Pfanner and Neupert 1990). Recently, Söller et al. (1989, 1990) reported the existence of distinct specific receptor sites, MOM19 and MOM72, on the mitochondrial surface in *N. crassa* by using monospecific antibodies against mitochondrial outer membrane proteins. They also reported that MOM19 functions as an import receptor for mitochondrial precursor proteins with presequences in their N-terminus while MOM72 functions towards an ADP/ATP carrier protein. It should be noted that the ADP/ATP carrier protein contains s transport signal in its central region (Smagula and Douglas 1988). It thus appears that there are two major mechanisms in transport systems into mitochondria.

5. Ribosomes in yeast mitochondria

The yeast has many advantages for the studies of mitochondrial biogenesis. First, when grown on glucose, yeast has an ability to live through glycolysis alone and therefore without mitochondrial functions. This makes it possible to study mutations in both mitochondrial and nuclear genes that crucially interfere with mitochondrial biogenesis; such mutations are lethal in nearly all other organisms. Second, yeast is a simple unicellular eukaryote, easy to culture and to characterize genetically and biochemically. This makes it easy to introduce in vitro manipulated genes to investigate their functions.

There are many nuclear genes that code for organellar proteins suitable for the studies described in previous sections. Mitochondrial ribosomes seem to be best suited, because they are composed of many constitutes encoded in both the

nuclear and mitochondrial genes. For these reasons, I selected the yeast *Saccharomyces cerevisiae,* focused my doctorate work on their mitochondrial ribosomes and investigated the structural and functional characteristics of their genes from an evolutionary point of view to establish how their expression are regulated in the nucleus and mitochondria.

Ribosomes in yeast mitochondria are composed of two RNAs and about 80 ribosomal proteins. The rRNAs are encoded in the mitochondrial DNA while all of the proteins except for one termed VAR1 (Hudspeth et.al., 1982) are encoded in the nuclear genes. Little were studied about these genes when I started my doctorate work presented here. Four nuclear-coded genes for ribosomal proteins, MRP1 (Myers et al. 1987), MRP2 (Myers et al. 1987), MRP7 (Fearon and Mason 1988) and MRP13 (Partaledis and Mason 1988), were cloned and characterized by other people. Therefore, I first undertook systematic cloning of individual ribosomal protein genes and succeeded in cloning of the genes for two proteins, termed YMR31 and YMR44, which characterized in detail. I performed systematic cloning of the genes for mitochondrial ribosomal proteins including these two and collaborated with other people in this laboratory and in the Max-Plank-Institute für Molekulare Genetik in Berlin in further cloning. Thus, we are able to clone a total of eleven mitochondrial ribosomal protein genes (Grohmann 1989, Grohmann et al. 1989, Kitakawa et al. 1990, Graack 1990, Kang 1991, Kang et al. 1991, this work). Therefore, using the cloned genes, I examined in the next step how their expression was regulated. Furthermore, comparison of their gene structures brought to my attention a possibility that some of the proteins contain signals for targeting into mitochondria in their C-terminus. Therefore, I examined this possibility with protein YmL8. These results raised several features with respect to

the points described above.

Materials and Methods

1. Strains and cloning vectors

E. coli strains

HB101 F$^-$, *leu, thi, lacY, str^r, hsdR, hsdM, endI$^-$, recA*

XL-1 *endA1, hsdR17, supE44, thi-1, recA, gyrA, relA, Δ(lac)* F' (*proAB, lacI^{q}ZΔM15,* Tn10)

S. cerevisiae strains

ORI1087	**a**/α, commercial baker's yeast	Oriental Yeast Co.Ltd.
07173	**a**/α	M. Kitakawa
DC-5	**a**, *leu2-3, leu2-112, his3, can1*	A. Toh'e
DC-5 ρ^o	**a**, *leu2-3, leu2-112, his3, can1* (derived from DC-5)	A. Toh'e
KWK002	**a**, *ade1, leu2, his3, trp1* (derived from D13-1A x DC04)	W. K. Kang
KWK002ΔL8	**a**, *ade1, leu2, his3, trp1, MRP-L8/MRP-L8::HIS3* (*MRP-L8* disruptant derived from KWK002)	this work
JSS122-14B	α, *his3-Δ200, leu2-Δ1, trp1-Δ1, tyr7-1, ura3-52*	K. Kawakami
YMD002	**a**/α, *leu2, trp1, his3, MRP-L8/MRP-L8::HIS3* (derived from KWK002ΔL8 x JSS122-14B)	this work

Vectors

E. coli plasmids

pBR322 Amp^r, Tet^r

pUC118 Amp^r

pUC119	Amp^r	

Shuttle vectors in *E. coli* and *S. cerevisiae*

YCp19	Amp^r, Tet^r, *URA3, TRP1*	A.Toh'e
YEp13	Amp^r, Tet^r, *LEU2*	A.Toh'e
YEpH	a derivative of YEp13, Amp^r, *HIS3*	W.K.Kang

M13 phage vectors

mp18

mp19

2. Media

Media for *E. coli*

LT* 1 % tryptone, 0.5 % yeast extract, 0.5 % NaCl, thymidine

*When necessary, 50 μg/ml of ampicillin (final concentration) was added.

Media for yeast# (Sherman et al., 1983)

YPD	2 % peptone, 1 % yeast extract, 2 % glucose or 6 % glucose
YPG	2 % peptone, 1 % yeast extract, 2 % 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; 30 μg/ml, trp; 20 μg/ml, ura; 20 μg/ml, tyr; 30 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. Preparation of ribosomal proteins from *S. cerevisiae*

3-1. Preparation of mitochondria and extraction of ribosomes

Frozen cells of commercial baker's yeast were obtained from Oriental Yeast, Osaka, Japan. Cells were thawed in 10 mM Tris-HCl, pH7.0, containing 0.65 M sorbitol, 0.1 mM EDTA and 0.025 % bovine serum albumin, washed and disrupted mechanically with glass beads (0.5 mm in diameter) in a Dyno Mill according to the procedure described by Schatz (1967) and by Guérin et al. (1979). The resultant lysate was cleared by differential centrifugations for 10 min each: first at 4,700 and 10,000 rpm in a JA-10 rotor then twice at 3,500 and 10,000 rpm in a JA-17 rotor of the Beckman J2-21 centrifuge. Mitochondria thus obtained were solubilized by incubating with 2 % Triton X-100 (Lambowitz 1979) with or without prior treatment with puromycin (50 mg/ml) (Faye and Sor 1977) and then cleared by a low speed centrifugation. The supernatant was either placed on a 30 % sucrose cushion prepared in 10 ml tubes and centrifuged at 4°C and 40,000 rpm for 2.5 hours in a Ti50 rotor of the Spinco L8 centrifuge, or on a 10-30 % sucrose gradient prepared in 35 ml tubes and centrifuged at 2 °C and 19,000 rpm for 16 hours in a SRP-28SA rotor of the Hitachi 80P centrifuge. Fractions containing ribosomes were identified by measuring absorbance at 260 nm and combined. Ribosomes were then precipitated with 10 % PEG-6000 (Faye and Sor 1977). From 150 to 200 g cells, about 10 A_{260} units of ribosomes were usually obtained.

3-2. Purification and amino acid sequencing of mitochondrial ribosomal proteins

Proteins were extracted from ribosomes prepared as described above with 68 % acetic acid containing 68 mM $MgCl_2$, dialyzed against 2 % acetic acid and lyophilized. They were then separated by two-dimensional gel electrophoresis

(Geyl et al. 1981). Each protein spot was cut out of the gel, electro-eluted with 0.15 % cetyl-trimethyl-ammonium bromide (Wu et al. 1982) and purified by reverse phase HPLC using a ODS-120A column (Toyo Soda) equilibrated with 0.1 % trifluoroacetic acid (TFA) and 10 % acetonitrile. Proteins were eluted with a 10-70 % acetonitrile gradient in 0.1 % TFA. Purified proteins were then applied to a model 477A amino acid sequencer (Applied Biosystems, Inc.).

4. Cloning and characterization

The standard manipulation of DNA was carried out as described by Maniatis et al. (1982), and Sambrook et al. (1989).

4-1. Cloning of the genes for mitochondrial ribosomal proteins

Oligonucleotide mixtures (17mer) of appropriate sequences were synthesized using a model 381A DNA synthesizer (Applied Biosystems, Inc.) by choosing the regions of amino acid sequences containing least degenerate codons. They were end-labelled with γ-[^{32}P]ATP and used as probes in Southern hybridization with the genomic DNA of strain DC-5 ρ^0 digested with *Bam* HI, *Hin*d III or *Pst* I. Hybridization was carried out overnight at 37°C and the filer was washed at 52°C for 20 min twice in a solution containing 3 M tetramethylammoniumchloride after washing in ice cold 6x SSC (0.9 M NaCl, 0.09 M sodium citrate) five times as described by Wood et al. (1985). The portion of agarose gel containing the chromosomal DNA fragments hybridizing with the probe was cut out and the DNA fragments electroeluted. They were then ligated with pBR322 DNA cleaved with either *Bam* HI or *Pst* I and transformed in *E. coli* HB101. Clones containing the gene in question were screened by colony hybridization (Grustein and Wallis, 1979).

4-2. Nucleotide sequencing

Nucleotide sequencing was performed according to Sanger et al. (1977) and the data were stored and similarities were compared using the computer programs (Isono 1984). For rapid identification of clones, double-strand plasmid DNA was used directly as the template in the sequencing reaction without prior cloning into M13 phages and chemically synthesized oligonucleotide mixtures were used as such to prime the sequence reaction.

4-3. Miscellaneous methods

SDS-PAGE (Laemmli 1970) was performed on 12.5 % polyacrylamide gel using monomeric and oligomeric cytochrome c as the molecular weight standards. Pulse-field and field inversion gel electrophoresis of the chromosomal DNA was performed as described by Carle et al. (1986). Large scale preparation of the yeast genomic DNA for Southern hybridization was carried out as described by Niederacher and Entian (1987). Small scale of chromosomal DNA or plasmid DNA in yeast were rapidly isolated as described by Hoffman and Winston (1987).

5. Analysis of messenger RNA

5-1. Isolation of total RNA

Yeast total RNA was isolated as described bellow according to Sprague et al. (1983). Cells harvested from 40 ml of yeast culture (ρ^+; OD_{600}=1~3, ρ^0; OD_{600}=0.7~2) were resuspended in 0.2 ml of 0.2 M Tris-HCl (pH 7.6), 10 mM EDTA, 0.5 M NaCl, 1 % SDS in 2 ml sampling tube, and then 0.2 ml of phenol/chloroform and 0.3 g of glass beads were (0.5 mm ϕ) added. The mixture was vigorously vortexed for 60 sec and immediately cooled in ice bath for 30 sec. After repeating this step three times, the aqueous layer obtained by centrifugation

was further extracted twice with phenol/chloroform and once with chloroform. The total DNA and RNA were precipitated with ethanol, dissolved in 0.6 ml of 10 mM Tris-HCl (pH 7.6), 10 mM EDTA, 0.1 M LiCl, 0.2 % SDS. Then, total RNA was precipitated by adding of 0.4 ml of 5 M LiCl and storing on ice for more than 1 hour. The resultant RNA was dissolved in 200 μl of TE (10 mM Tris-HCl (pH 8.0), 1 mM EDTA).

5-2. Electrophoresis and blotting

The gel tray and electrophoretic chamber were pretreated with 0.2 N NaOH for 30 min and washed with H_2O. Agarose-formaldehyde gel was prepared from 100 ml of the solution containing 10 ml of 10 x MOPS buffer (0.2 M MOPS (pH 7.0), 50 mM sodium acetate, 10 mM EDTA), 1.2 g of agarose and 5.4 ml of 37 % formaldehyde. Loading-samples were prepared by adding of 2 μl of loading buffer (50 % glycerol, 1 mM EDTA, 0.4 % BPB, 0.4 % XC, 0.5 mg/ml EtBr) after boiling at 90°C for 2 min of mixtures composed of 4.5 μl of total RNA (10 ~40 μg), 2.0 μl of 10 x MOPS buffer, 3.5 μl of 37 % formaldehyde, and 10μl of deionized formamide. Electrophoresis was performed in 1x MOPS buffer at 4 V/cm for 1 hour. The separated RNA was transferred to nitrocellulose or nylon membrane using a vacuum blotter (Sanplatec) for 90 min with 1 M ammonium acetate.

5-3. Northern hybridization

Probes for Northern hybridization were prepared by the random priming method (Feinberg and Vogelstain 1983). Strand specific probes were prepared using single strand DNA as templates by the random priming method or by DNA sequencing reaction with universal primer M3 of pUC plasmids. Hybridization and washing were performed according to the standard protocols (Maniatis et al. 1982, Sambrook et al. 1989).

6. Yeast Genetics

Standard procedures for yeast genetics were carried out according to Sherman et al. (1983) and Ausubel et al. (1987).

6-1. Transformation

Transformation of yeast cells was carried out with Li-acetate according to Ito et al. (1983) as follows. An overnight culture was diluted 1/10 with 20 ml YPD and cells were grown to log-phase (OD_{600}=4~8) at 30°C. Cells were harvested, washed once with TE, and suspended in 1 ml of a solution containing 0.1 M lithium acetate, 15 % glycerol , 10 mM Tris-HCl (pH 8.0), and 1mM EDTA, and stored at 30°C for 1 hour. Then, DNA (no more than 15 μl) was added to 0.3 ml of the suspension and stored for 30 min with occasional mixing. 0.3 ml of 70 % PEG-4000 was added and the mixture further incubated at 30°C for 1 hr and then at 42°C for 5 min. The cells were washed with H_2O two times, resuspended in 0.2 ml H_2O and spread onto selecting agar plates.

6-2. Mating and sporulation

Mating and sporulation were performed according to Ausubel et al. (1987) as described bellow. The cells of each haploid parent were mixed on YPD plates and incubated overnight at 30°C. The mating mixture was streaked onto appropriate agar plates to select for the diploid phenotype. The resultant diploid cells were purified to single colonies on the same selecting plates. For sporulation, the diploid cells were grown to log-phase (OD_{600} = 2.5~3.0) in YPD medium. Cells from 3 ml culture were washed twice with H_2O, resuspended in 3 ml sporulation medium and incubated for 2~3 days at 30°C.

6-3. Tetrad analysis

Tetrad analysis was performed with a micromanipulator system (Leitz) according to Sherman et al. (1983).

Results

1. Mitochondrial ribosomal proteins in *S. cerevisiae*

There are three possible approaches to the cloning of the genes for mitochondrial ribosomal proteins in *S. cerevisiae*. One is to raise antibodies against purified mitochondrial ribosomal proteins and use them in screening of the clones. A second is to isolate *PET* mutants and to screen clones by complementation analysis of the mutants. A third is to synthesize oligonucleotide mixtures based on the N-terminal amino acid sequence data of mitochondrial ribosomal proteins and to use them as probes on hybridization. Of these, the last method is best suited for systematic cloning of individual ribosomal protein genes.

During the course of my work for a Master's degree, I established the procedures for isolation of mitochondrial ribosomes with negligible contamination of cytoplasmic ribosomes. Two-dimensional electrophoretic analysis of the mitochondrial ribosomal proteins prepared on that way from a commercial bakers yeast cells was very similar to those from laboratory strains of *S. cerevisiae* including strain DC-5. Therefore, I decided to use the amino acid sequence data obtained for commercial baker's yeast cells for the synthesis of oligonucleotides probes to clone the corresponding genes from a ρ^0 derivative of strain DC-5.

The mitochondrial ribosomes were accordingly isolated from commercial yeast cells, and their proteins were separated by two-dimensional gel electrophoresis to purify individual proteins. A typical electropherogram is shown in Fig.1. About seventy proteins were recognized on such an electropherogram. Several ribosomal proteins were well separated from others on two-dimensional gels.

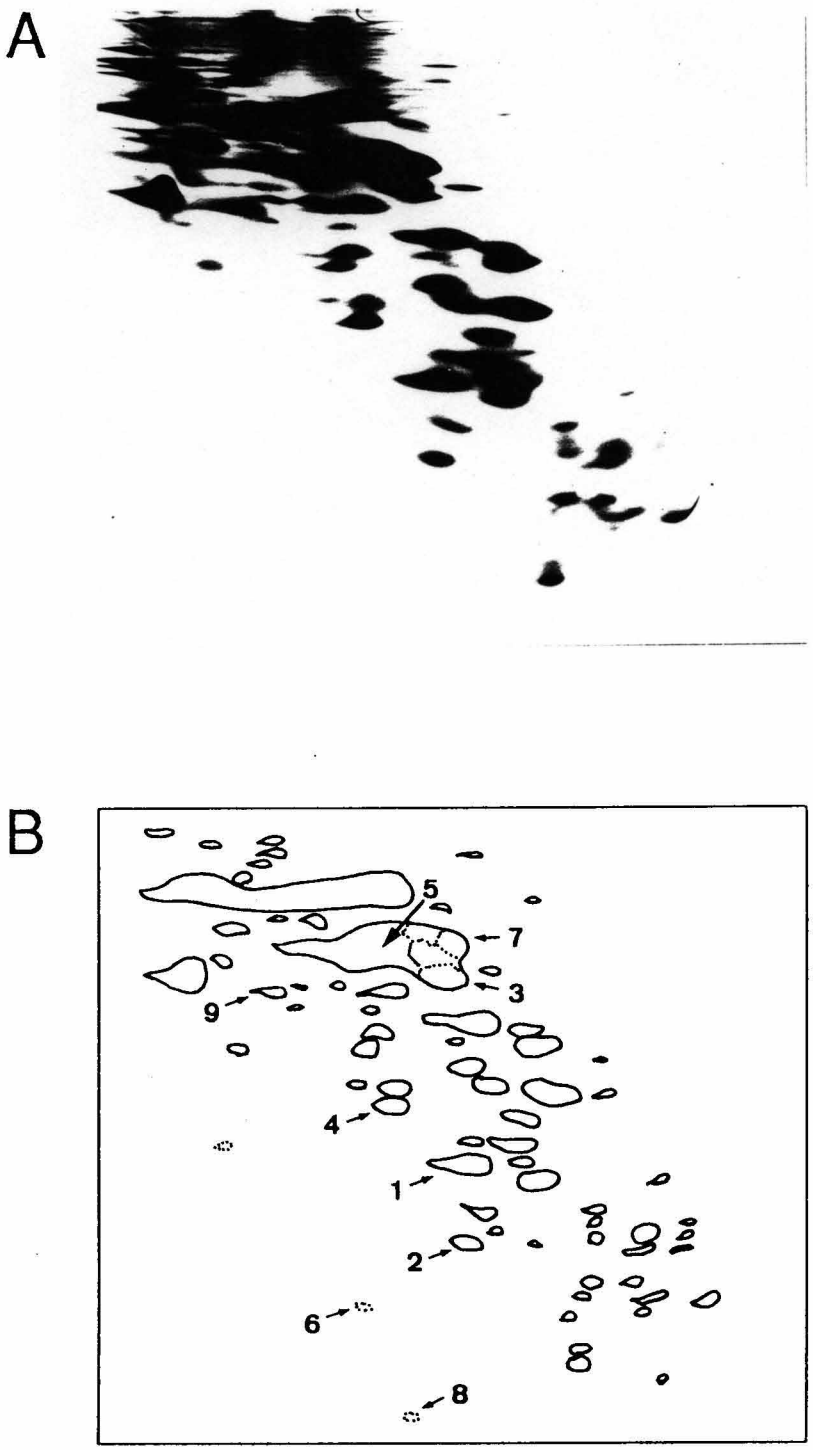


Fig.1. (A) Two-dimensional electropherogram of mitochondrial ribosomal proteins from baker's yeast used in this work. Electrophoresis was performed in the first dimension (horizontal) and the second dimension (vertical) as described by Geyl et al. (1981). (B) Schematic drawing of the electropherogram shown in (A). Numbered arrows indicate proteins whose N-terminal amino acid sequences were determined:1.YMR31, 2.YMR44, 3.Ka-1, 4.L-4, 5.S-2-3, 6. D-3, 7.Ki-2, 8.C-4, 9.P-4.

Therefore, I recovered them by electroelution and further purified them by reversed phase HPLC. Then, their N-terminal amino acid sequences of several proteins were determined. The N-termini of some of them were found to be blocked (Table 1). Two proteins, termed YMR31 and YMR44, gave rise to amino acid sequence data for longer than 20 residues and they contained stretches with least degenerate codons. Therefore, I decided to clone the genes for the two proteins by synthesizing oligonucleotides probes based on their amino acid sequence data.

2. Cloning and characterization of the gene for protein YMR31

2-1. Cloning of the *YMR31* gene

The amino acid sequence of protein YMR31 was determined for the N-terminal 24 residues (Table 1 and Fig. 2). It was found that there were two overlapping regions which contained amino acid sequences with least degenerate codons (Fig.2). They were termed regions **a** and **b**, respectively. Oligonucleotide mixtures (probes **a** and **b**) were accordingly synthesized and were used to hybridize the genomic DNA prepared from strain DC-5 ρ^o. Thus, *Pst* I fragments of approximately 4.4 kb and 9.4 kb were found to hybridize with the probe **a** (Fig. 3A). Since the probe was a mixture of 48 different oligonucleotides, it was not surprising to observe several bands hybridizing with the probe. To distinguish between true and false signals, additional hybridization experiments with a different probe was necessary. Of the two fragments hybridizing with probe **a**, the 4.4-kb fragment showed positive hybridization with probe **b** as well, whereas the 9.4-kb fragment did not (Fig. 3B). Therefore, the 4.4 kb *Pst* I fragment appeared to be a good candidate of a chromosomal fragment containing the gene for protein YMR31. DNA fragments of this size were accordingly eluted out from the agarose gel, phenol extracted, ligated with pBR322 and used to transform *E. coli* strain HB101.

Table 1. N-terminal amino acid sequences of mitochondrial ribosomal proteins in *S. cerevisiae*

protein	N-terminal amino acid sequence #
YMR31	A K S A Y E P M I K F V G T (R) H P L V K (H) A T E - -
YMR44	M I T K Y F S K V I V R F N P F G K E A K V A R L V L A - -
Ka-1	? Q (F) (G) L I E T H (V) I A (P) Q P N Y Q V - - (N)
L-4	(M) L Y E L I G L V R I T N ? N A P ? L - - (A)
S-2-3	? V G I V (E) F L (P) ? Q (P) A ? Q D N L L - - (K)
D-3	(S) A ? ? K A G I S Y A A Y L N V A A Q ? I (R) (S) (S) L - -
Ki-2	P S ? D A F A R F L D Q ? A ? - -
C-4	blocked ?
P-4	blocked ?

Amino acid sequences are shown by single letter codes. Residues in parentheses are slightly ambiguous. Unidentified residues are indicated by ?.

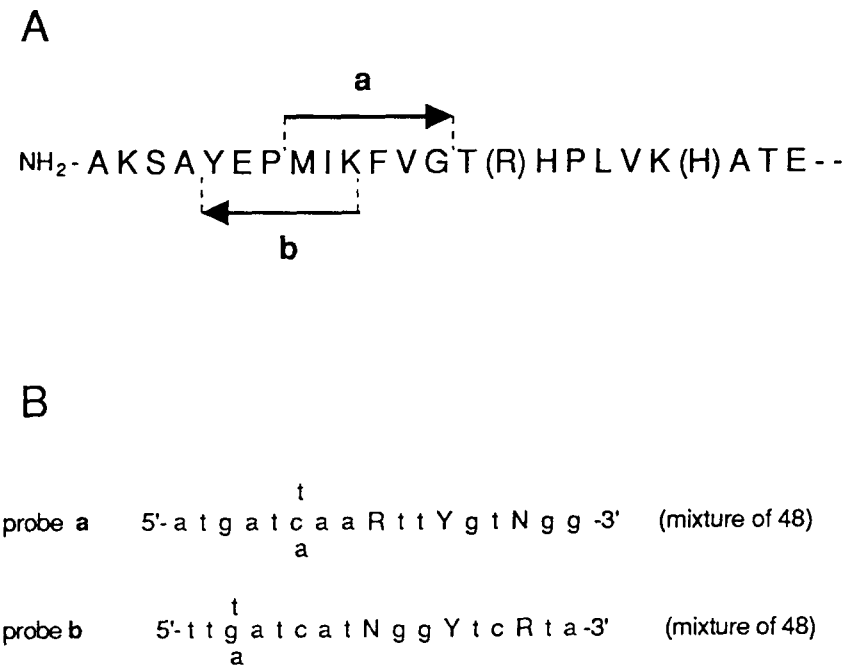


Fig.2. Design of oligonucleotide probes for cloning of *YMR31*. (A) The N-terminal amino acid sequence of ribosomal protein YMR31 was determined for twenty-four residues as shown. Residues in parentheses are slightly ambiguous. Oligonucleotide probes **a** and **b** were synthesized based on the sequence indicated by arrows **a** and **b**, respectively. The direction indicates from the 5' to 3' end of the synthesized nucleotides. (B) Oligonucleotide sequence of probes **a** and **b** used for cloning of the *YMR31* gene. N indicates a, g, c, and t mixture; R, a and g mixture; and Y, c and t mixture.

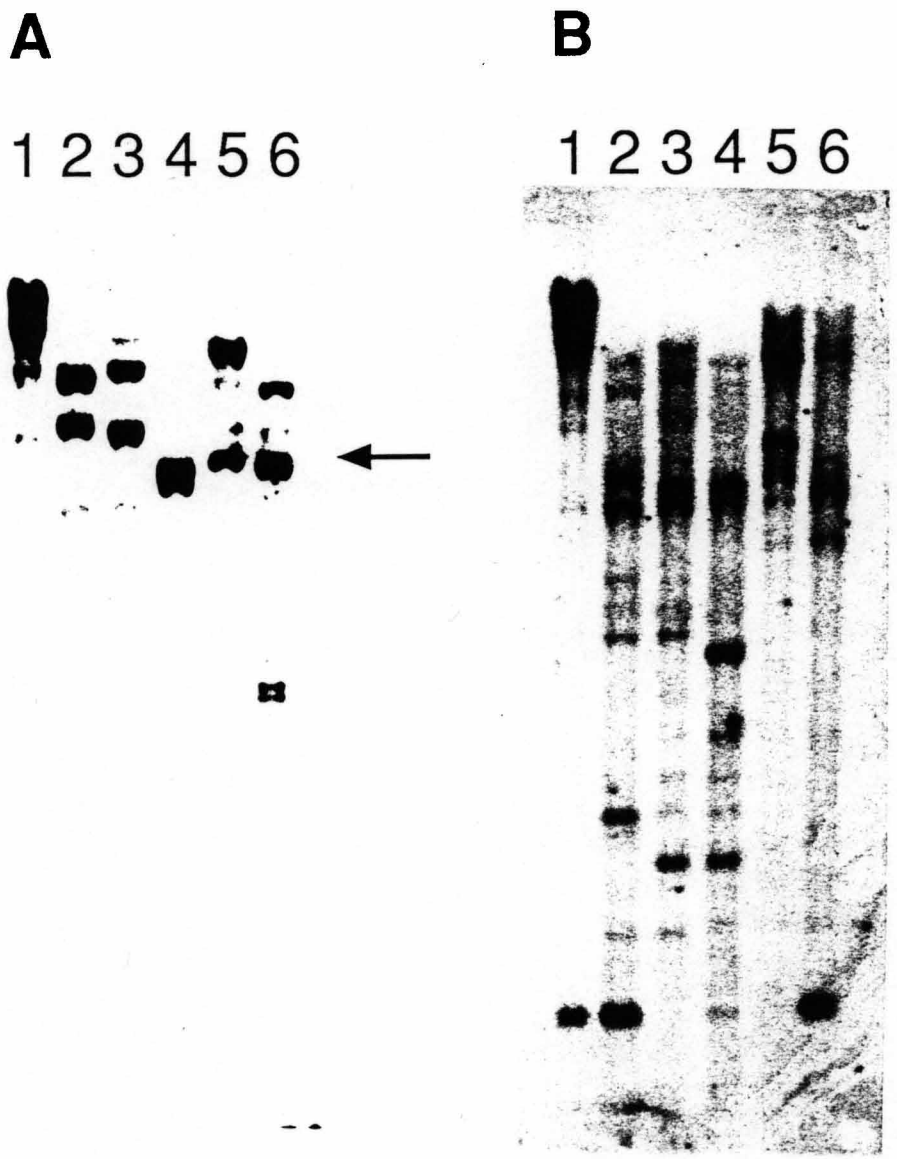


Fig.3. Genomic Southern hybridization of *YMR31*. (A) Ten micrograms of the genomic DNA from DC-5 ρ^0 cells were digested with restriction enzymes *Bam* HI (lane 1), *Bam* HI and *Hin* dIII (lane 2), *Hin* dIII (lane 3), *Hin* dIII and *Pst* I (lane 4), *Pst* I (lane 5), or *Bam* HI and *Pst* I (lane 6), electrophoresed in 1 % agarose gel, and transferred to nitrocellulose membrane (Schleicher and Schüll) for Southern hybridization (Southern 1975) with probe **a** shown in Fig.3. (B) The same membrane used in (A) was reused in hybridization with probe **b** after washing in 6x SSC for 30 min at 65°C. Arrow points to a 4.4 kb *Pst* I fragment in lane 5 which showed hybridization with both probes **a** and **b**.

The resultant transformants were first screened for hybridization with oligonucleotide probes **a** and **b**, and then subjected to double-strand nucleotide sequencing using these oligonucleotide mixtures to prime the sequence reaction. Several clones were obtained which showed positive hybridization with the two probes and gave rise to short nucleotide sequence data the amino acid translation of which agreed completely with that shown in Fig. 2. One of such clones was termed pYM-31 and used in subsequent experiments.

2-2. Nucleotide sequences of the *YMR31* gene

A detailed restriction map analysis was performed with pYM-31 as shown in Fig. 4. Both oligonucleotide probes, **a** and **b**, were found to hybridize with a 755 bp *Xba*I-*Eco*RI fragment of this clone as indicated. Accordingly, this fragment and its neighboring segments were cloned into M13 phages (Fig.5, Table2B) and their nucleotide sequences were determined. Thus, complete nucleotide sequence of the gene for YMR31 and its flanking regions was established as shown in Fig. 6. The amino acid sequence of YMR31 protein (Fig. 2) was found to match the 9th Ala onward of that predicted from the nucleotide sequence of its gene. It is likely, therefore, that the first eight amino acid residues constitute a short leader sequence. The predicted open reading frame for YMR31 encodes a protein with a calculated molecular weight of 12,792.14 when the molecular weight for the putative leader sequence is subtracted. This is in good agreement with the value, 12.5 kDa, determined for protein YMR31 by SDS-PAGE (data not shown). Presence of many basic amino acids in the predicted amino acid sequence also supports its identity as the gene for a basic ribosomal protein, YMR31. No distinct sequence similarity to other proteins including *E. coli* and yeast cytoplasmic ribosomal proteins was observed.

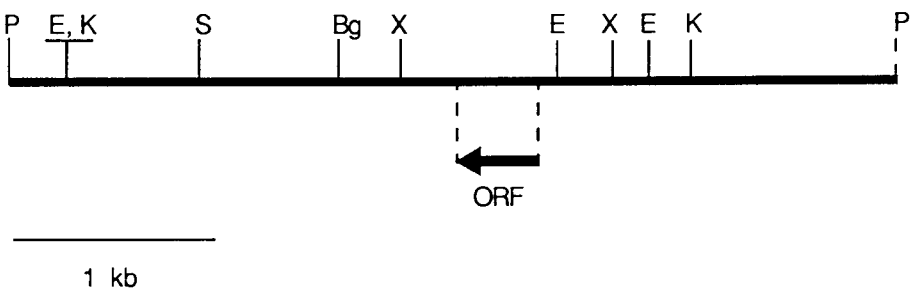


Fig.4. Restriction map of the chromosomal segment containing the gene for YMR31. Plasmid pYM-31 was digested with *Eco* RI (**E**), *Kpn* I (**K**), *Sac* I (**S**), *Bgl* II (**Bg**), or *Xba* I (**X**), and their cleavage sites mapped as shown. Thick arrow labelled as ORF indicates the size and the 5' to 3' direction of the gene for YMR31. No site was found for restriction enzymes *Bam* HI, *Cla* I, *Hin* dIII, *Mlu* I, *Pvu* II, *Sal* I, and *Sma* I.

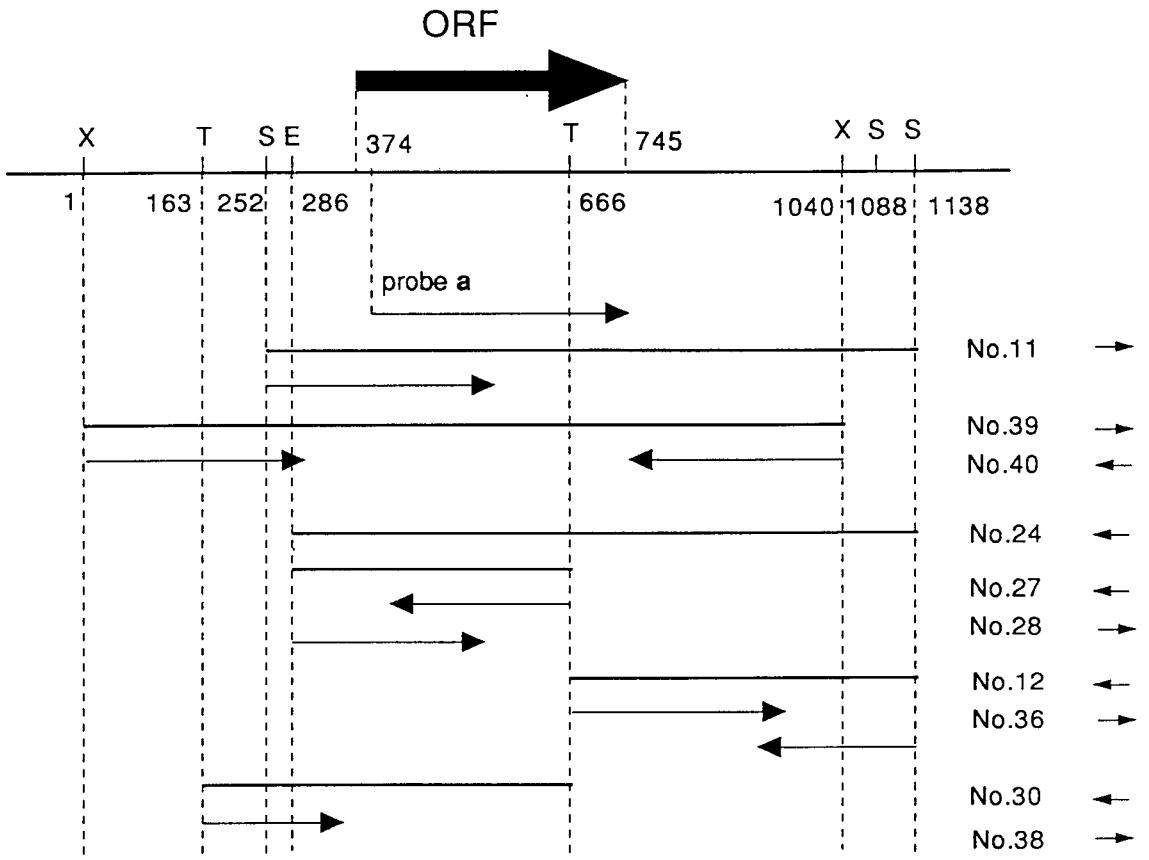


Fig.5. Strategy for sequencing *YMR31*. The region analyzed by DNA sequencing are shown. Relative positions are numbered as in Fig.6. Thick arrow indicates the open reading frame of *YMR31* gene in the 5' to 3' direction. Subfragments cloned into M13 phage are indicated by horizontal lines. M13 phage subclones (Table 2B) and their direction of sequencing are shown at right. Thin arrows indicate the direction and approximate extent of the sequence data obtained. Abbreviations for restriction enzyme cleavage sites are : E, *Eco* RI ; S, *Sau* 3A ; T, *Taq* I ; X, *Xba* I.

Table 2A. Plasmid clones for the *YMR31* gene

Plasmid	Insert	Vector (sites used)
pYM-31	DC-5ρ°*Pst* I 4.4kb	pBR322 (*Pst* I)
pM1-118A	pYM-31 *Pst* I 4.4kb insert	pUC118 (*Pst* I)
pM1-118B	pYM-31 *Pst* I 4.4kb insert	pUC118 (*Pst* I)
pM1-XbaI-1	M13 No.40 RF *Bam* HI *Pst* I 1kb insert	pUC118 (*Bam* HI *Pst* I)
pM1-XbaI-1 Hpa I LEU2	YEp13 *Bgl* II 3kb (*LEU2*)	pM1-XbaI-1 (*Hpa* I)

(host strain used was HB101)

Table 2B. M13 phage clones for the *YMR31* gene

Plasmid	Insert	Vector (sites used)
M13 No.11	pYM-31 *Pst* I 4.4kb fragment *Sau* 3A mixtures	mp19 (*Bam* HI)
M13 No.12	M13 No.11 RF *Eco* RI *Pst* I 1kb fragment *Taq* I mixtures	mp18 (*Acc* I)
M13 No.24	M13 No.11 RF *Eco* RI *Pst* I 1kb fragment	mp18 (*Eco* RI *Pst* I)
M13 No.27	M13 No.11 RF *Eco* RI *Pst* I 1kb fragment *Taq* I mixtures	mp18 (*Eco* RI *Acc* I)
M13 No.28	M13 No.11 RF *Eco* RI *Pst* I 1kb fragment *Taq* I mixtures	mp19 (*Eco* RI *Acc* I)
M13 No.30	pYM-31 *Xba* I 1kb fragment *Taq* I mixtures	mp18 (*Acc* I)
M13 No.36	M13 No.12 RF *Bam* HI *Pst* I 500bp fragment	mp19 (*Bam* HI *Pst* I)
M13 No.38	M13 No.30 RF *Bam* HI *Pst* I 520bp fragment	mp19 (*Bam* HI *Pst* I)
M13 No.39	pYM-31 *Xba* I 1kb fragment	mp19 (*Xba* I)
M13 No.40	pYM-31 *Xba* I 1kb fragment	mp19 (*Xba* I)

(host strain used was XL1)

Fig.6. Nucleotide sequence of the gene for YMR31. Nucleotide sequence was determined for the region flanked by two *Xba* I cleavage sites indicated in Figs. 4 and 5. The amino acid translation of the gene is given in single letter codes.

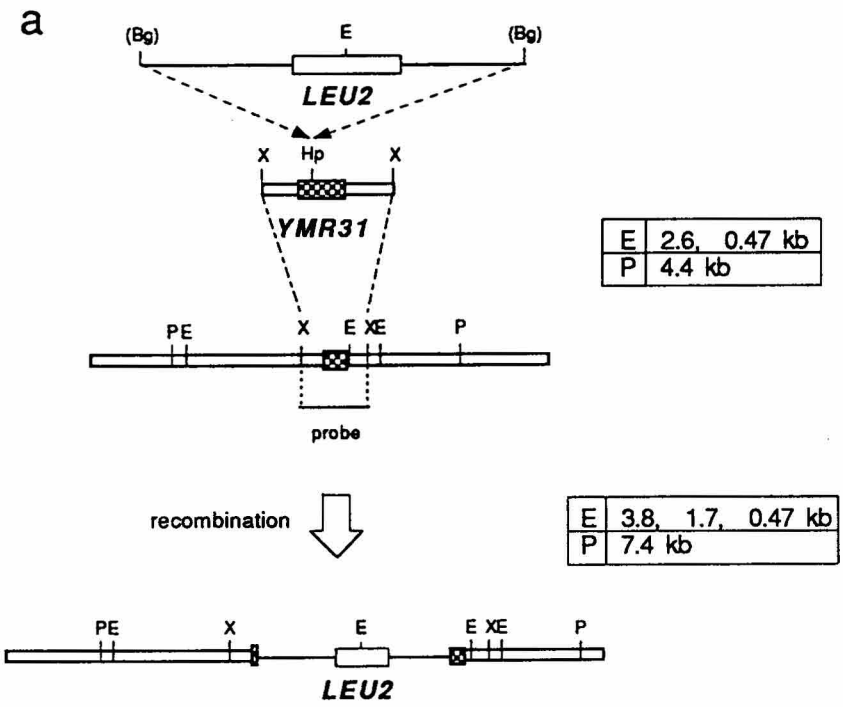
2-3. Gene disruption analysis of *YMR31*

To determine whether the product of *YMR31* is essential for the mitochondrial function in yeast cells, a gene disruption experiment was performed. For this purpose, a disrupted allele was constructed as outlined in Fig.7. The 2.9 kb *Bgl* II fragment containing *LEU2* gene was filled in and inserted into the coding region of *YMR31* at the *Hpa* I site of pM1-XbaI-1 (Table 2A). The resultant plasmid (pM1-XbaI-1-HpaI-Leu2-1, Table2A) was digested with *Eco* RI and used to replace the corresponding genomic region by transformational integration. LEU+ transformants were selected for in strain DC-5. They were then examined for their ability to grow on non-fermentable carbon sources. It was found that the LEU+ transformants were all respiration proficient and that their growth rate was indistinguishable from DC-5. Southern blot analysis (Fig. 7) of the *YMR31* gene in the genomic DNAs prepared from these transformants confirmed that recombination had occurred at the expected site and the disrupted allele had been incorporated into the chromosome. These results indicate, therefore, that the *YMR31* gene is not essential for the mitochondrial function in yeast.

3. Cloning and characterization of the gene for protein YMR44

3-1.Cloning of the *YMR44* gene

The amino acid sequence of protein YMR44 was determined for N-terminal 28 residues as shown in Table 1 and Fig.8. For the cloning of its gene, I selected two underlined regions (regions **c**, **d-1** and **d-2**) for the synthesis of 48 and 64 mixtures of 17mer oligonucleotides (probes **c**, **d-1** and **d-2**). Southern hybridization was then performed using the resultant oligonucleotide mixtures as in the case of YMR31. Consequently, a chromosomal *Bam*HI fragment of 4.4 kb was found to hybridize with probe **c** as shown in Fig.9 (lane 1). However, no



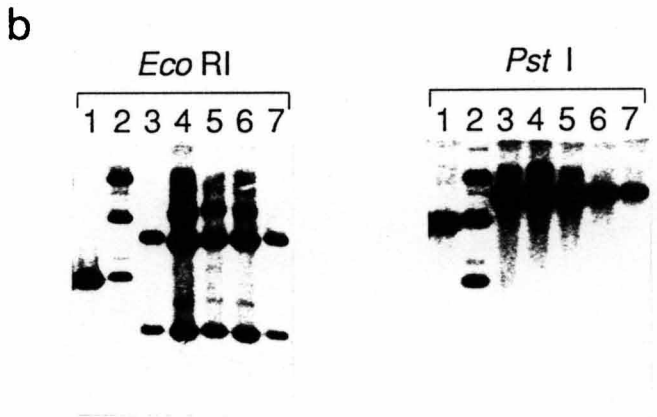


Fig.7. Gene disruption analysis of *YMR31*. **a.** A 1.0 kb *Bam* HI-*Pst* I fragment of RF form of M13 No.24 (Table 2B and Fig.5) which contained the *YMR31* gene (hatched box) and its upstream and down stream regions was subcloned into the *Bam* HI-*Pst* I sites of pUC118 and named pM1-Xbal-1 (only the relevant portion is shown). A 2.9 kb *Bgl* II fragment of YEp13 containing the *LEU2* gene (open box) was ligated into the *Hpa* I site of pM1-Xbal-1 after filling the cohesive ends. A 3.9 kb *Xba* I fragment of the resultant plasmid (pM1-Xbal-1-Hpal-Leu2-1, Table2A) in which the *YMR31* gene was disrupted was then used to transform DC-5 cells. The chromosomal *YMR31* gene was expected to be disrupted as indicated. **b.** The disruption of *YMR31* was confirmed by Southern hybridization analysis. The DNAs from five LEU+ transformants (lanes 3-7) and DC-5 (undisrupted control : lane 1) digested with *Eco* RI and *Pst* I were analyzed with a 1.0 kb *Eco* RI-*Pst* I fragment of RF form of M13 No.24 (Table 2B and Fig.5) containing *YMR31* gene as a probe. As size markers, a mixture of the DNA of plasmid pYM-31 digested with *Eco* RI or *Pst* I was used (lane 2). Their sizes are, from top to bottom, 8.7, 4.3 and 2.4 kb, respectively. Abbreviations for restriction enzymes are **Bg** for *Bgl* II, **E** for *Eco* RI, **Hp** for *Hpa* I, **P** for *Pst* I, and **X** for *Xba* I.

A

B

probe **c**	5'-a t g a t c a c N a a R t a Y t t -3' t a	(mixture of 48)
probe **d-1**	5'-g c N a c Y t t Y g c Y t c Y t t -3'	(mixture of 64)
probe **d-2**	5'-g c N a c Y t t R g c Y t c Y t t -3'	(mixture of 64)

Fig.8. Design of oligonucleotide probes for cloning of *YMR44*. (A) The N-terminal amino acid sequence of ribosomal protein YMR44 was determined for twenty-seven residues as shown. Oligonucleotide probes **c** and **d** were synthesized based on the sequence indicated by arrows **c**, **d-1** and **d-2** whose direction is indicated in the 5' to 3' end of the synthesized oligonucleotides. (B) Oligonucleotide sequence of probes **c**, **d-1** and **d-2** used for cloning of the *YMR44* gene. For N, R, and Y, see the legend to Fig.2.

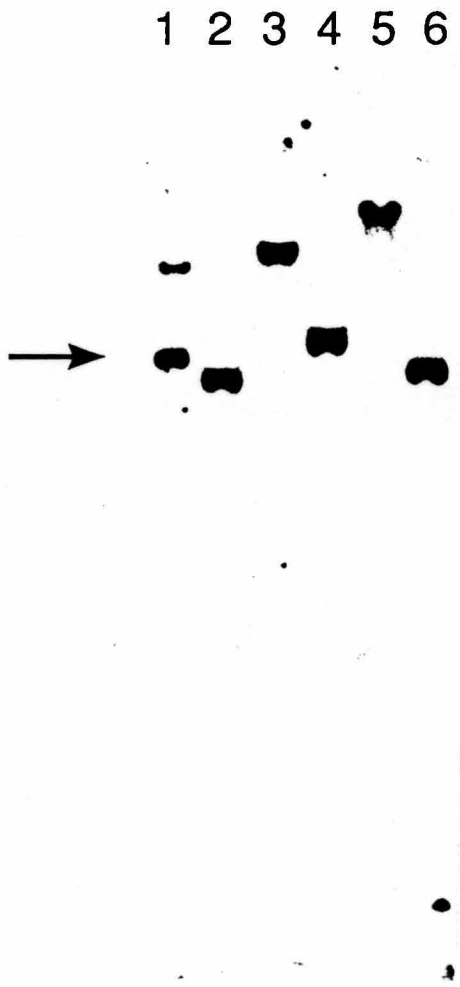


Fig.9. Genomic Southern hybridization of *YMR44*. Experiments were performed as in Fig. 3 with probe **c** shown in Fig. 8. Arrow points to a 4.4 kb *Bam* HI fragment (lane 1) which was cloned into pBR322 for further analyses.

hybridization was observed with probe **d-1** and **d-2** (data not shown). The reason why probe **d-1** and **d-2** did not show any hybridization will be described later. The chromosomal *Bam* HI fragments of approximately 4.4 kb were recovered from agarose gel and ligated into plasmid pBR322. Transformants were screened for hybridization with probe **c** and their plasmids were analyzed by the double-strand sequencing method. One of the clones was found to contain a nucleotide sequence whose amino acid translation matched well with a stretch of several amino acid residues of the amino acid sequence shown in Fig.8. This clone was termed pYM-44 and used in subsequent experiments.

3-2. Nucleotide sequences of the *YMR44* gene

A detailed restriction map was established with pYM-44 as shown in Fig10. By Southern hybridization analysis, the oligonucleotide probe **c** was found to hybridize with a 470 bp *Bam*HI-*Pvu*II fragment of pYM-44 as indicated. Therefore, I cloned this region into M13 phage and pUC plasmid and determined their nucleotide sequences by using universal M3 primer and chemically synthesizing primers as indicated in Fig.11 and Table 3.

As presented in Fig.12, the amino acid sequence deduced from the nucleotide sequence data agreed perfectly with the first 19 amino acid residues of protein YMR44. However, match was lost at the Ala at position 20 and afterwards. By further examination of the nucleotide sequence data, I found that there was an intron-like sequence starting at the 119th and ending at the 265th nucleotide in this gene as indicated. The predicted amino acid sequence after the putative intron again matched completely with the amino acid sequence presented in Fig.12. The intron-like sequence is only 147 nucleotides long. However, it has typical donor and acceptor sites of yeast introns. Furthermore, it has two sequences very similar

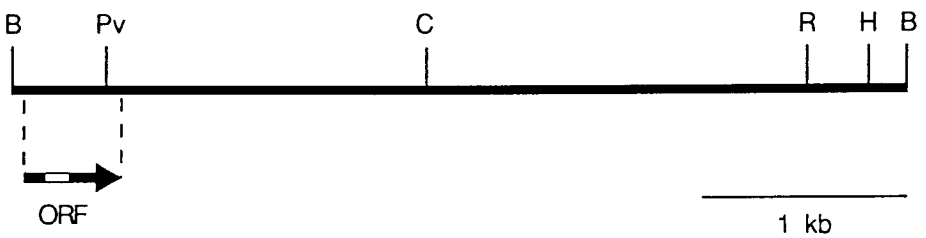


Fig.10. Restriction map of the chromosomal segment containing the gene for YMR44. The 4.4 kb *Bam* HI fragment shown in Fig. 9 (lane 1) was cloned into pBR322, digested with *Pvu* II (**Pv**), *Cla* I (**C**), *Eco* RV (**R**), or *Hin* dIII (**H**), and their cleavage sites mapped as shown. Thick arrow labelled as ORF indicates the size and the 5' to 3' direction of the gene for YMR44 which contains an intron (blank). No site was found for restriction enzymes *Bgl* II, *Eco* RI, *Kpn* I, *Pst* I, *Sac* I, *Sal* I, *Sma* I, and *Xba* I.

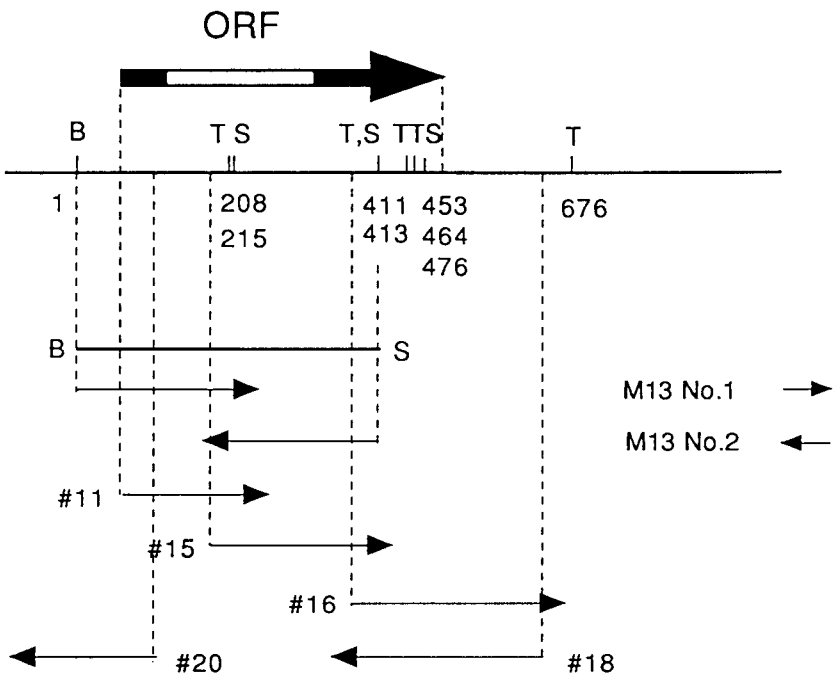


Fig.11. Strategy of sequencing *YMR44.* The regions analyzed by DNA sequencing are shown. Relative positions are numbered as in Fig.12. Thick arrow indicates the open reading frame of *YMR44* gene in the 5' to 3' direction. The intron of *YMR44* is indicated by an open box. Subfragments cloned into M13 phage are indicated by horizontal lines. The clone numbers (see Table 3B) and their direction of sequencing are shown at right. Thin arrows indicate the direction and approximate extent of the sequence data obtained. DNA sequencing with #11, #15 and #16 primers was performed with the single strand DNA derived from pN4-119- 1 as templates while sequencing with #18 and #20 was performed with the DNA from pN4-118-1 (Table 3A and 3C). Abbreviations for restriction enzyme cleavage sites are : B, *Bam*HI ; S, *Sau*3A ; T, *Taq*I.

Table 3A. Plasmid clones for the *YMR44* gene

Plasmid	Insert	Vector (sites used)
pYM-44	DC-5ρ°*Bam* HI 4.4kb fragment	pBR322 (*Bam* HI)
pN4-119-1	pYM-44 *Bam* HI *Hind* III 4.0kb insert	pUC119 (*Bam* HI *Hind* III)
pN4-118-1	pYM-44 *Bam* HI *Hind* III 4.0kb insert	pUC119 (*Bam* HI *Hind* III)
pN4HP4.5-1	DC-5ρ°*Hind* III *Pst* I 4.5kb fragment	pUC119 (*Hind* III *Pst* I)
pN4HcII-1	pN4HP4.5-1 *Hinc* II 2kb insert	pBR322 (*Eco* RV *Pvu* II)
pN4HcII-1-ΔE	deletion of 620bp *Eco* RI fragment from pN4HcII-1	
pN4HcII-1-ΔE-BPvuII-His3-1	YEpH *Bam* HI 1.75kb (*HIS3*)	pN4HcII-1-ΔE (*Bam* HI *Pvu* II)

(host strain used was HB101)

Table 3B. M13 phage clones for the *YMR44* gene

Phage No.	Insert	Vector (sites used)
M13 No.1	pYM-44 *Bam* HI 4.4kb fragment Sau 3A mixtures	mp19 (*Bam* HI)
M13 No.2	M13 No.1 RF *Eco* RI *Pst* I 450bp fragment	mp18 (*Eco* RI *Pst* I)

(host strain used was XL1)

Table 3C. Oligonucleotides synthesized for DNA sequencing of *YMR44* gene

Name	Length	Sequence*	Position#
#11 (=probe c)	17 mer	5'-atgatBacNaaRtaYtt-3'	61 - 77
#15	17 mer	5'-ttggctaaactaggctg-3'	182 - 196
#16	17 mer	5'-aagtaacctacaaggac-3'	377 - 393
#18	19 mer	5'-gggtatatctatctatacg-3'	639 - 621
#20	17 mer	5'-gccaaagggattaaacc-3'	111 - 95

* Abbrebiations of nucleotide are: N; a, g, c, and t, R; a and g,Y; c and t, B; a, c, and t .

\# Relative positions are numbered as shown in Fig.12 .

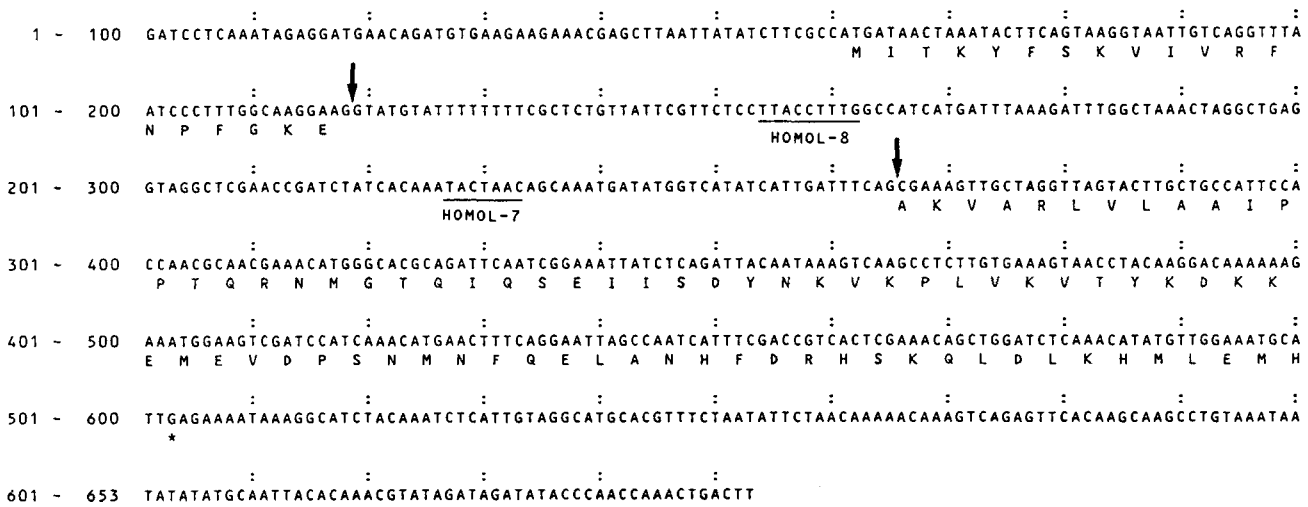


Fig.12. Nucleotide sequence of the gene for YMR44. Nucleotide sequence was determined for the region from the left-hand side *Bam* HI site through the *Pvu* II site shown in Fig. 10. Arrows indicate the boundaries of an intron in this gene. Two sequences similar to the conserved sequences in yeast introns (Teem et al. 1984) are marked as HOMOL-7 and HOMOL-8.

to those conserved among yeast introns, HOMOL7 and HOMOL8 (Teem et al. 1984), as indicated. For these reasons I consider it very likely that this is indeed an intron. Thus, the gene for YMR44 protein is the first example of an intron-containing gene for a mitochondrial ribosomal protein in yeast. The open reading frame in the first and second exons totals 297 base pairs which can encode a basic protein with a calculated molecular weight of 11,475.88. The value was in good agreement with that estimated for YMR44 protein in SDS-PAGE (data not shown). Therefore, the open reading frame was concluded to be the gene for protein YMR44. As in the case of protein YMR31, I searched for similarity of the amino acid sequence predicted for YMR44 protein to other known ribosomal proteins. However, none was found to be significantly similar to this protein.

4. Gene organization of *YMR31* and *YMR44*

4-1. Copy number of the *YMR31* and *YMR44* genes

To determine the copy number of the two cloned genes described above, the segments containing the gene for *YMR31* and *YMR44* were used for Southern hybridization to the yeast chromosomal DNA digested with various restriction enzymes as shown in Fig.13. Only one band was detected in each lane. Therefore, it was concluded that the genes for YMR31 and YMR44 exist in a single copy.

4-2. Chromosomal locations of the *YMR31* and *YMR44* genes

To establish the chromosomal locations of the two genes described above, the cloned segments containing the genes for YMR31 and YMR44 were used to hybridize the yeast chromosomes separated by pulse-field or field inversion electrophoresis on agarose gels. As shown in Fig.14, the gene for YMR31 was found to hybridize with chromosome VI (Fig.14A) and that for YMR44 with chromosome XIII or XVI (Fig.14B). The location of the gene for YMR31 on

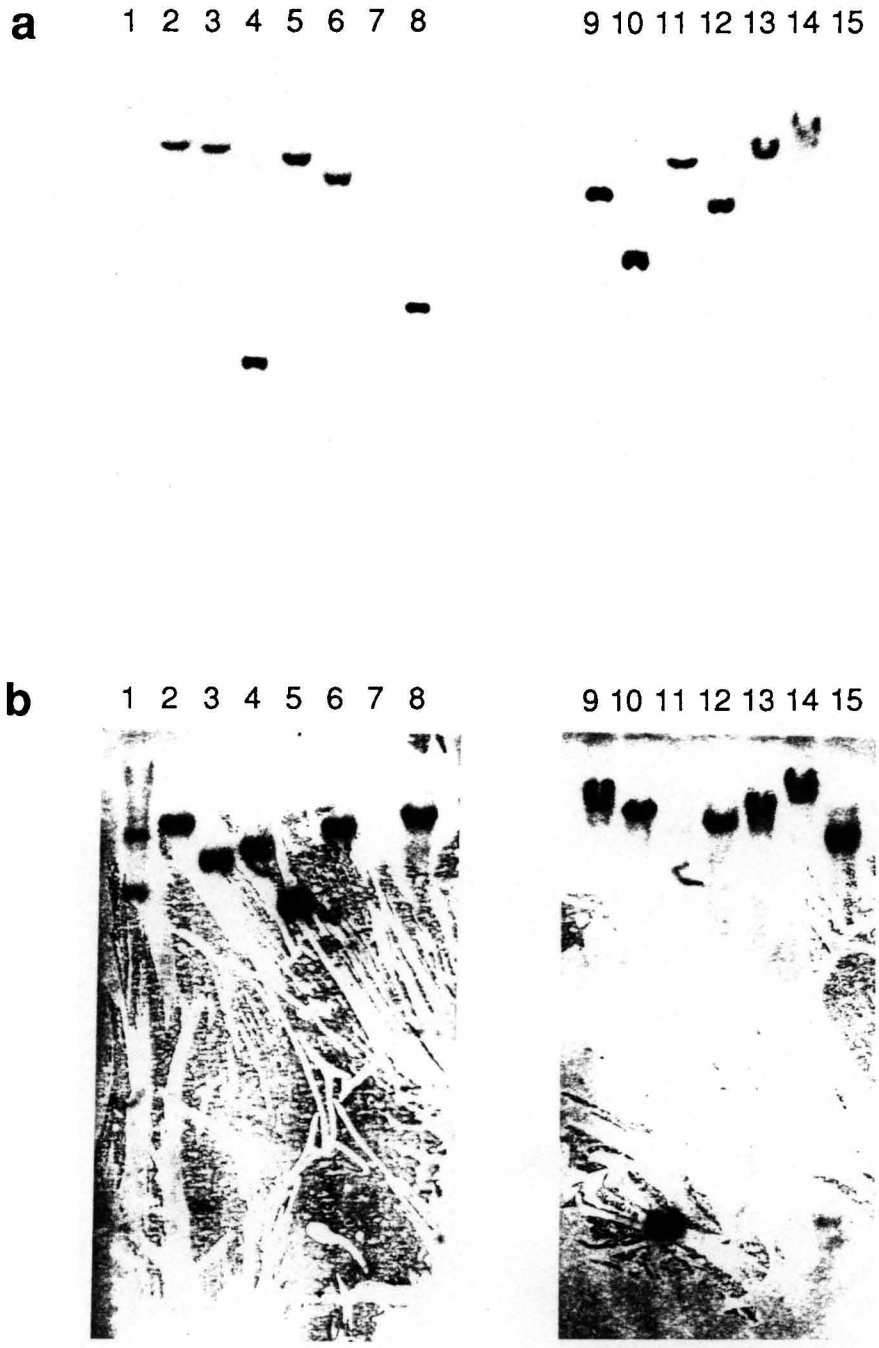


Fig.13. Copy number of *YMR31* and *YMR44*. The genomic DNA from DC-5 ρ^0 cells were digested with restriction enzymes *Bam* HI (lane 1), *Bgl* II (lane 2), *Cla* I (lane 3), *Eco* RI (lane 4), *Eco* RV (lane 5), *Hin* dIII (lane 6), *Hin* fI (lane 7), *Kpn* I (lane 8), *Mlu* I (lane 9), *Pst* I (lane 10), *Pvu* II (lane 11), *Sac* I (lane 12), *Sal* I (lane 13), *Sma* I (lane 14), or *Xba* I (lane 15), electrophoresed in 1% agarose gel, and transferred to nitrocellulose membrane for Southern hybridization with the 380 bp *Eco* RI-*Pst* I fragment of RF form of M13 No.27 (Table 2B) containing *YMR31* gene (**a**) or the 450 bp *Eco* RI-*Pst* I fragment of RF form of M13 No.1 (Table 3B) containing a portion of *YMR44* gene (**b**).

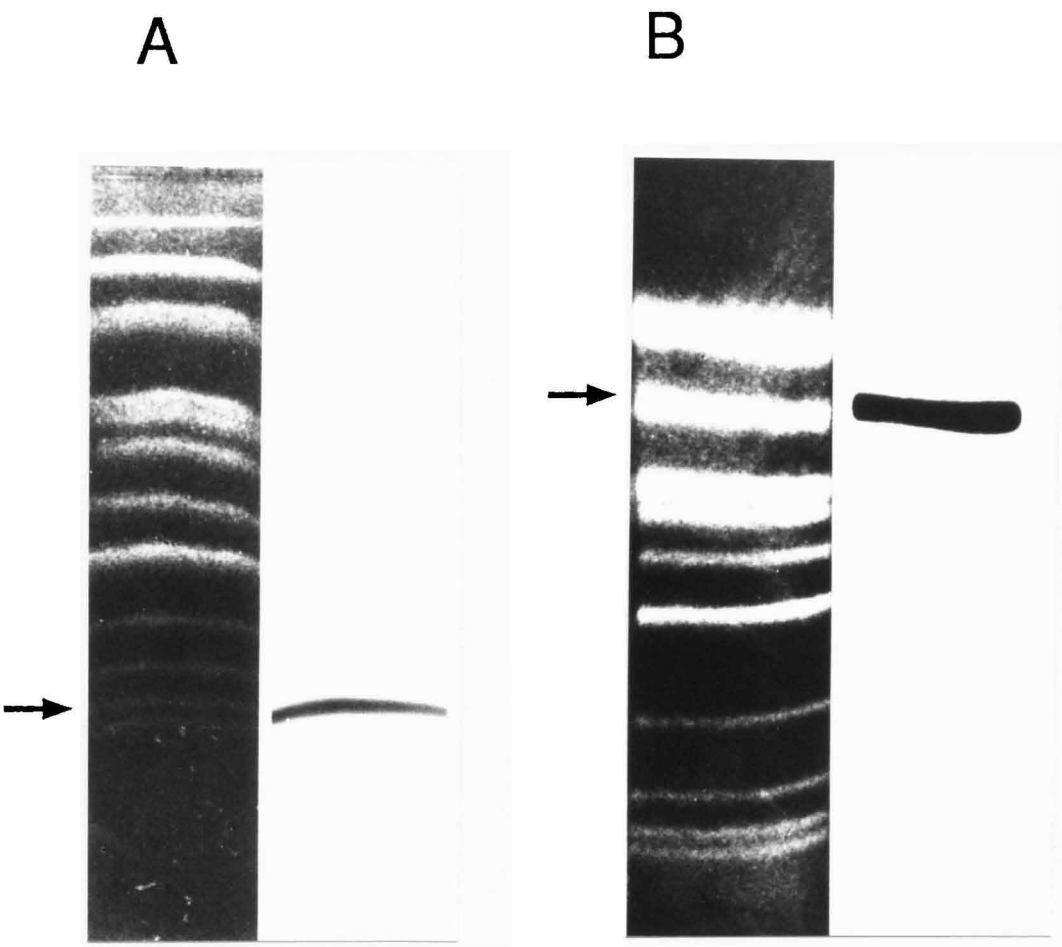


Fig.14. Chromosomal locations of the genes for YMR31 and YMR44. The chromosomes of strain DC-5 ρ^0 were separated either by pulse field electrophoresis on 1.5 % agarose gel (A) or by field inversion electrophoresis on 1 % agarose gel (B). Each gel was stained with ethidium bromide (1 µg/ml) to visualize DNA bands (left-hand side panels) and then the DNA bands were transferred to nitrocellulose membrane and hybridized with (A) the 4.4 kb *Pst* I fragment shown in Fig. 4, or (B) a 412 bp *Bam* HI-*Sau* 3AI fragment corresponding to nucleotides 1 through 412 of *YMR44* in Fig. 12 (right-hand side panels).

chromosome VI was further confirmed by an additional hybridization experiment with a λ phage clone bank for this chromosome (Yoshikawa A and Isono K, manuscript in preparation).

5. Expression of mitochondrial ribosomal protein genes

Mitochondrial ribosomes in *S. cerevisiae* are said to be composed of about eighty different proteins (Graack et al. 1989, Graack 1990) synthesized from the genes in the nucleus and two RNA molecules and one protein synthesized from the genes in mitochondria. Therefore, it is interesting how the expression of these genes are regulated to achieve cooperation with other genes in the nucleus, or between the genes in the nucleus and mitochondria. To acquire information for such presumed cooperative gene expression, I examined the transcripts from the cloned mitochondrial ribosomal protein genes by Northern hybridization to total RNAs purified from cells with various genetic backgrounds and growing under various conditions.

For this purpose, it was examined whether differences in the quantity of transcripts were amenable to detection by Northern hybridization using the DNA fragments containing the *YMR31* and *YMR44* genes as probes. Fig.15A and Fig.15B show that the intensity of a band showing hybridization with the probes increased as the total RNA loaded in the electrophoresis was increased from 2.5 μg to 40 μg. The results indicate, therefore, that differences could be detected easily when the quantity of a particular transcript varies two fold or more.

5-1. Analyses of transcripts of *YMR31*

By overexposing the autoradiogram shown in Fig.15C and the data from Northern hybridization experiment (Fig.16), it was found that two bands (0.83 kb and 1.19 kb) were detected when the 1 kb *Xba* I fragment or 0.85 kb *Eco* RI-*Pst* I

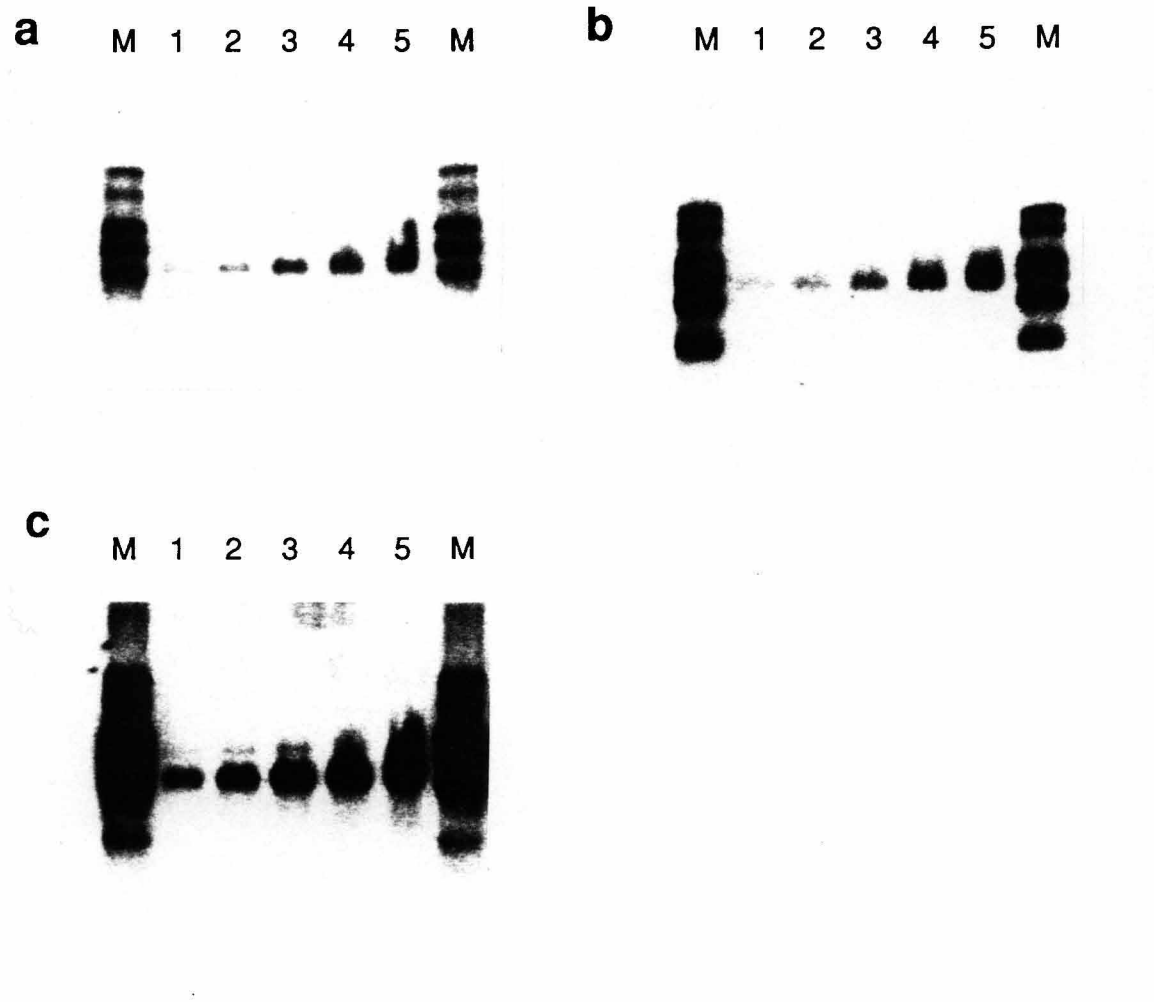


Fig.15. Estimation of relative amounts of transcripts. Total RNA was prepared from early log-phase cultures of DC-5 cells growing in YPG medium. 2.5 μg (lane 1), 5.0 μg (lane 2), 10 μg (lane 3), 20 μg (lane 4), or 40 μg (lane 5) of the resultant RNA per lane were electrophoresed in formaldehyde-agarose gel, transferred to nitrocellulose membrane, and hybridized with a 1.0 kb *Bam* HI-*Pst* I fragment of pM1-XbaI-1 (Table 2A) containing *YMR31* gene (**a**) or a 450 bp *Eco* RI-*Pst* I fragment of RF form of M13 No.1 (Table 3B) containing a portion of *YMR44* gene (**b**). As size markers, a mixture of the DNA of plasmid pM1-XbaI-1 digested with *Bam* HI-*Pst* I, *Bam* HI- *Eco* RI-*Pst* I or *Pvu* II and the DNA of plasmid pYM-44 digested with *Sau* 3A, *Bam* HI- *Pvu* II, *Eco* RV-*Pvu* II or *Hin* dIII- *Pvu* II was used after denaturation (lane **M**, in **a** and **b**). Their sizes are, from the third band in top to bottom, 1.39, 1.04 and 0.76 kb in (**a**) and 0.82, 0.66, 0.47 and 0.21 kb in (**b**). Two larger bands detected in lane M of (**a**) and (**b**) are likely due to the partial digestion. Longer exposure of autoradiogram in (**a**) is shown in (**c**).

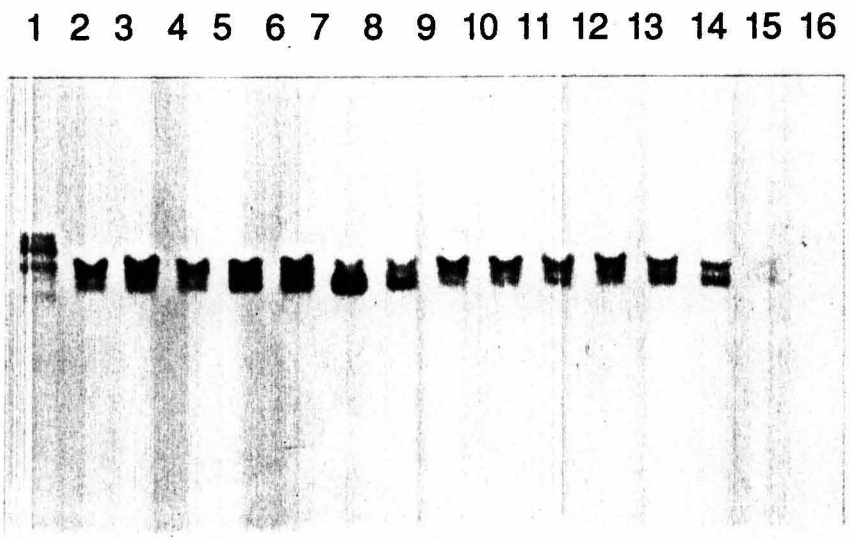


Fig.16. Northern hybridization with the DNA fragment containing *YMR31* gene. DC-5 (lanes 2-8) or DC-5ρ^0 (lanes 9-15) cells grown to early log phase in YPD medium containing 6% glucose were harvested by centrifugation and transferred to one-fourth the starting volume of fresh YPD medium containing 2% glucose. Total RNA was prepared from samples of the culture taken before transfer (lanes 2 and 9), and 15 min (lanes 3 and 10), 30 min (lanes 4 and 11), 1 hour (lanes 5 and 12), 3 hour (lanes 6 and 13), 6 hour (lanes 7 and 14), and 12 hour (lanes 8 and 15) after transfer. Thirty micrograms of total RNA from each sample were separated in formaldehyde-agarose gel, transferred to nitrocellulose filter, and hybridized with a 0.85 kb *Eco* RI-*Pst* I fragment of RF form of M13 No.24 (Fig.5, Table 2B) containing the *YMR31* gene. Size markers loaded in lane 1 and 16 were the same as in Fig. 15.

fragment from M13 No.24 (Table 2B) containing *YMR31* gene was used as a probe. To determine which band is derived from the transcript of *YMR31* gene, several region- and strand-specific probes were synthesized and used for Northern analyses. The results (Fig.17 and Fig.18) showed that the upper band was detected by the strand specific probes which should hybridize to the transcript of *YMR31* gene, and that the lower band was detected by the probes of the opposite direction. Therefore, it was determined that the upper band of 1.19 kb in size was the transcript of *YMR31* gene. When 4.4 kb *Pst* I fragment containing the *YMR31* gene was used for hybridization probes, an additional band of 1.78 kb in size was detected (Fig.18). This band was not detected when 1 kb *Xba* I fragment containing *YMR31* gene was used as a probe and when strand specific probes which should not hybridize to the transcript of *YMR31* gene were used (Fig.18). Therefore, it was interpreted to indicate that this transcript was derived from the gene in the same direction as *YMR31* gene which located in the 4.4 kb *Pst* I fragment but not in the 1.0 kb *Xba* I fragment. Recently, Yoshikawa and Isono established the entire relational clone bank and transcript mapping of yeast chromosome VI (Yoshikawa and Isono, manuscript in preparation) as well as those of chromosome III (Yoshikawa and Isono, 1990). The three transcripts of 0.83 kb, 1.19 kb (*YMR31*), 1.78 kb in size which I detected here were found to correspond to the transcripts #89 (0.70 kb), #88 (1.10 kb), and #90 (2.43kb), respectively. Thus, the physical location of the *YMR31* gene was precisely established on chromosome VI and the relative abundance of its expression during the log-phase growth of DC-5 cells could be correlated with other genes on this chromosome.

5-2. Transcription of other mitochondrial ribosomal protein genes

As a first approach to understanding of the regulation of expression of the

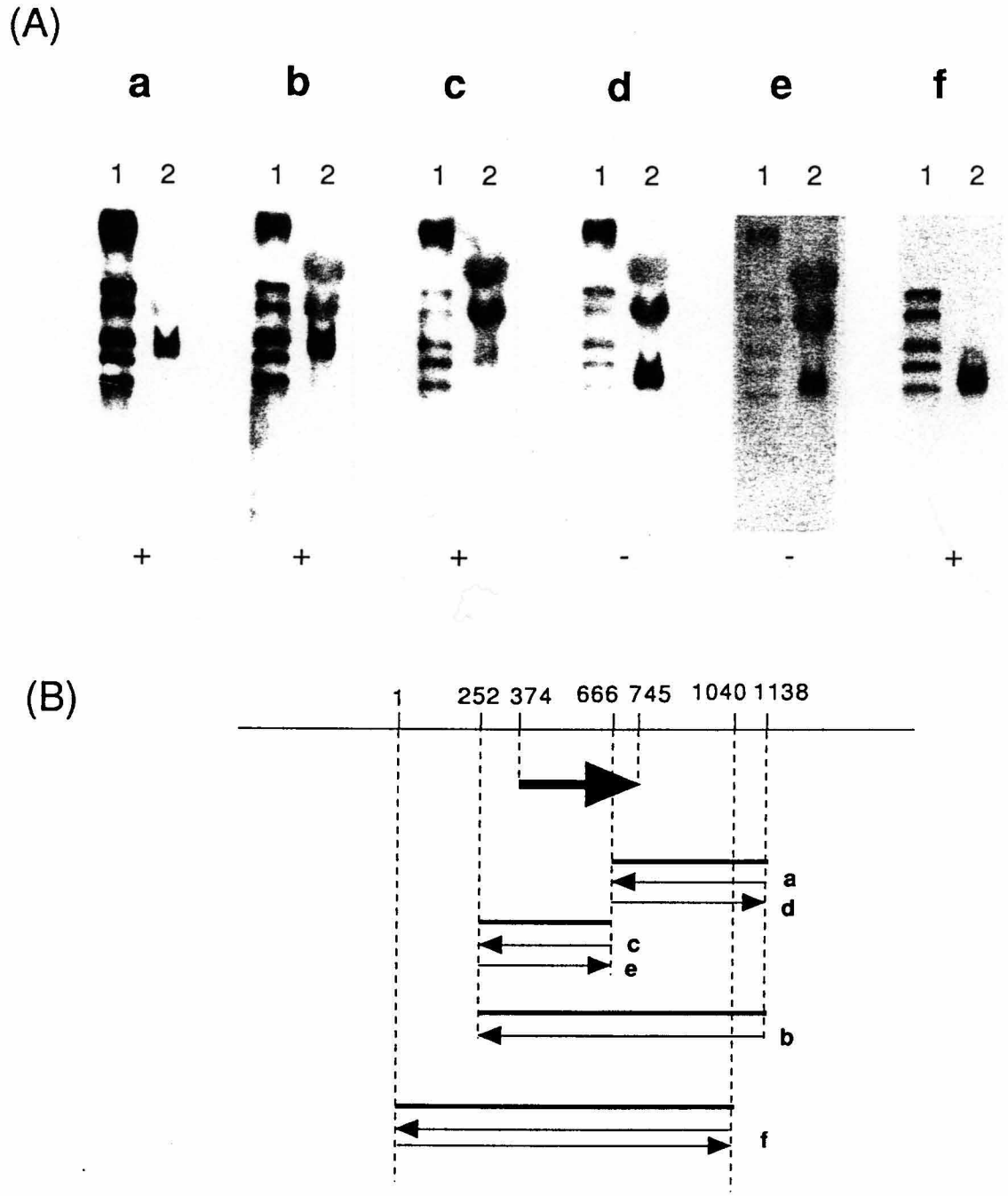


Fig.17. Analysis of transcript of *YMR31* by strand- and region-specific probes. (A) Thirty micrograms of total RNA per lane were used for Northern hybridization as described in Fig.15 (lane 2 in a-f). Probes in **a-e** were prepared by primer extension labeling using as templates single strand DNA of M13 No.12 (**a**), M13 No.24 (**b**), M13 No.27 (**c**), M13 No.38 (**d**), and M13 No.28 (**e**) (Table 2B) with a universal M3 primer for M13 phage mp18 and mp19. Probes in **f** were prepared by random primer labelling using a 1.0 kb *Xba* I fragment of pYM-31 as template. Size markers were loaded in lane 1 of **a-f** as in Fig.15. Whether probes used in each experiments are expected to hybridize with mRNA of *YMR31* or not are predicted as marked by + or -, respectively. (B) Schematic drawing of probes used in **a-f**. The region containing the *YMR31* gene is shown at the top. Relative positions are numbered as in Fig.6. Thick arrow indicates the open reading frame of *YMR31* in the 5' to 3' direction. Horizontal lines indicate the regions contained in the template DNAs used in probe preparation. Arrows indicate the direction of probes synthesized.The probes in **f** were prepared with both direction.

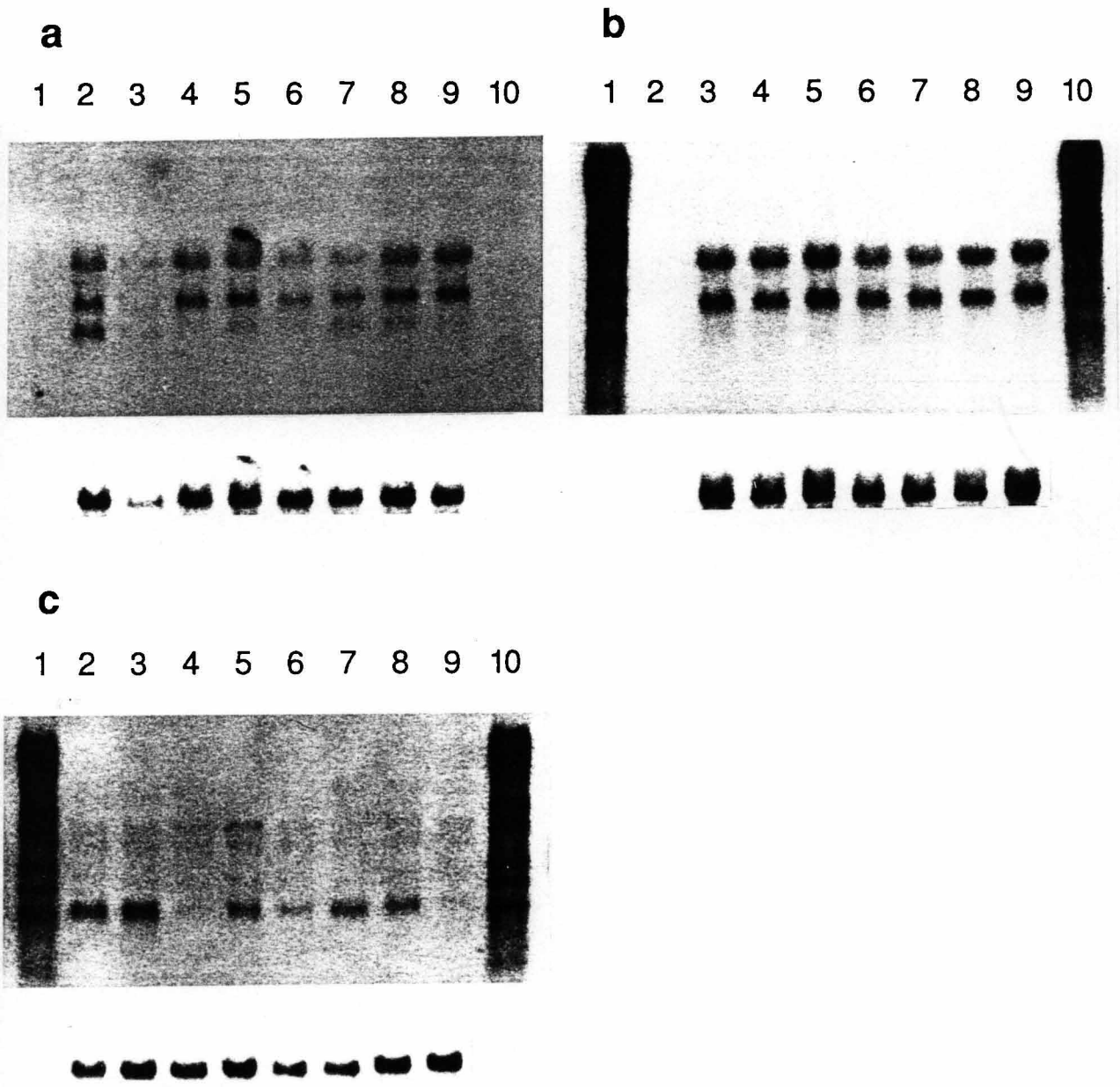


Fig.18. Analysis of transcript of *YMR31* by strand-specific probes. Total RNA was prepared from early log-phase cultures of DC-5 (lanes 2, 3 and 4) and DC-5ρ^0 (lanes 5 and 6) and strain 07173 (lanes 7, 8 and 9) growing in YP medium containing either 2% glycerol (lanes 2 and 7), 2% galactose (lanes 3, 5 and 8), or 6% glucose (lanes 4, 6 and 9). Ten micrograms of the resultant RNA per lane were used for Northern hybridization with the probes prepared by random primer labelling using as templates double strand DNA of pM1-118A after denaturation (a) or single strand DNA of pM1-118B (b) or pM1-118A (c) (Table2A). Strand specific probes in **b** were expected to hybridize with mRNA of *YMR31* while those in **c** were not. Size markers were loaded in lane 1 and 10 in **b** and **c** as in Fig.15. To calibrate the amounts of loaded RNA, the same filters were re-hybridized with a 1.0 kb *Xho* I-*Hin* dIII fragment of pYA301 containing the *ACT1* gene as shown below each hybridization profile.

mitochondrial ribosomal protein genes in the synthesis of ribosomes, the quantities of transcripts from the genes listed in Table 4 were determined in ρ^+ and ρ^0 derivatives of strain DC-5 and ρ^+ derivative of strain 07173 growing in 6% glucose or 2% galactose. In addition, the growth in 2% glycerol was monitored with ρ^+ cells. The results of the Northern blot analyses are shown in Fig.18, Fig.19 and Fig.20. In each case, except for *YMR31* gene, only one band was detected. In all cases, the following was observed: firstly, in ρ^+ derivatives of strain DC-5 and 07173, the amount of transcripts in cells growing in glucose medium was less than that in cells growing in galactose and glycerol medium. Secondly, in ρ^0 derivatives of strain DC-5, transcription was repressed in cells growing in glucose medium as compared to that in cells growing in glycerol medium, but the degree of repression in ρ^0 cells was less severe than that in ρ^+ cells. Thirdly, the amount of transcripts in ρ^0 cells were larger than that in ρ^+ cells derived from the same parental strain, DC-5. It is noteworthy in understanding of the communication in gene expression between the nucleus and mitochondria that the genes mentioned above were transcribed in ρ^0 cells without mitochondrial DNA, in which no rRNA was synthesized, more than in ρ^+ cells and that the degree of repression of transcription by glucose was different in ρ^+ and ρ^0 cells.

In investigating the transcription of some mitochondrial ribosomal protein genes, a possibility was conceived that the different responses in transcription of the genes analyzed under various conditions could be classified into several groups. However, the results shown in Fig.18, Fig.19, and Fig.20 did not suggest such a possibility. For further investigation, I examined transcription at various times after the change of glucose concentration in medium from 6% to 2%. Under these conditions, no difference was observed in the transcription of the

Table 4. Probes for Northern hybridization

gene	DNA source	Fragment		Approximate radioactivity (cpm)
MRP-L8	pL8EP	*Eco* RI *Hpa* I	1.13 kb	9.0×10^6
MRP-L20	pL20EP	*Pst* I *Kpn* I	0.69 kb	8.4×10^6
MRP-L31	pL31H	*Hind* III	1.9 kb	9.2×10^6
MRP-L33	pL33	*Hind* III *Xba* I	1.2 kb	1.1×10^7
YMR26	pEHc65-1	*Pvu* II	1.52 kb	1.0×10^7
YMR31	pM1-XbaI-1	*Bam* HI *Pst* I	1.0 kb	8.4×10^6
YMR44	M13 No.1 RF	*Eco* RI *Pst* I	0.45 kb	9.6×10^6

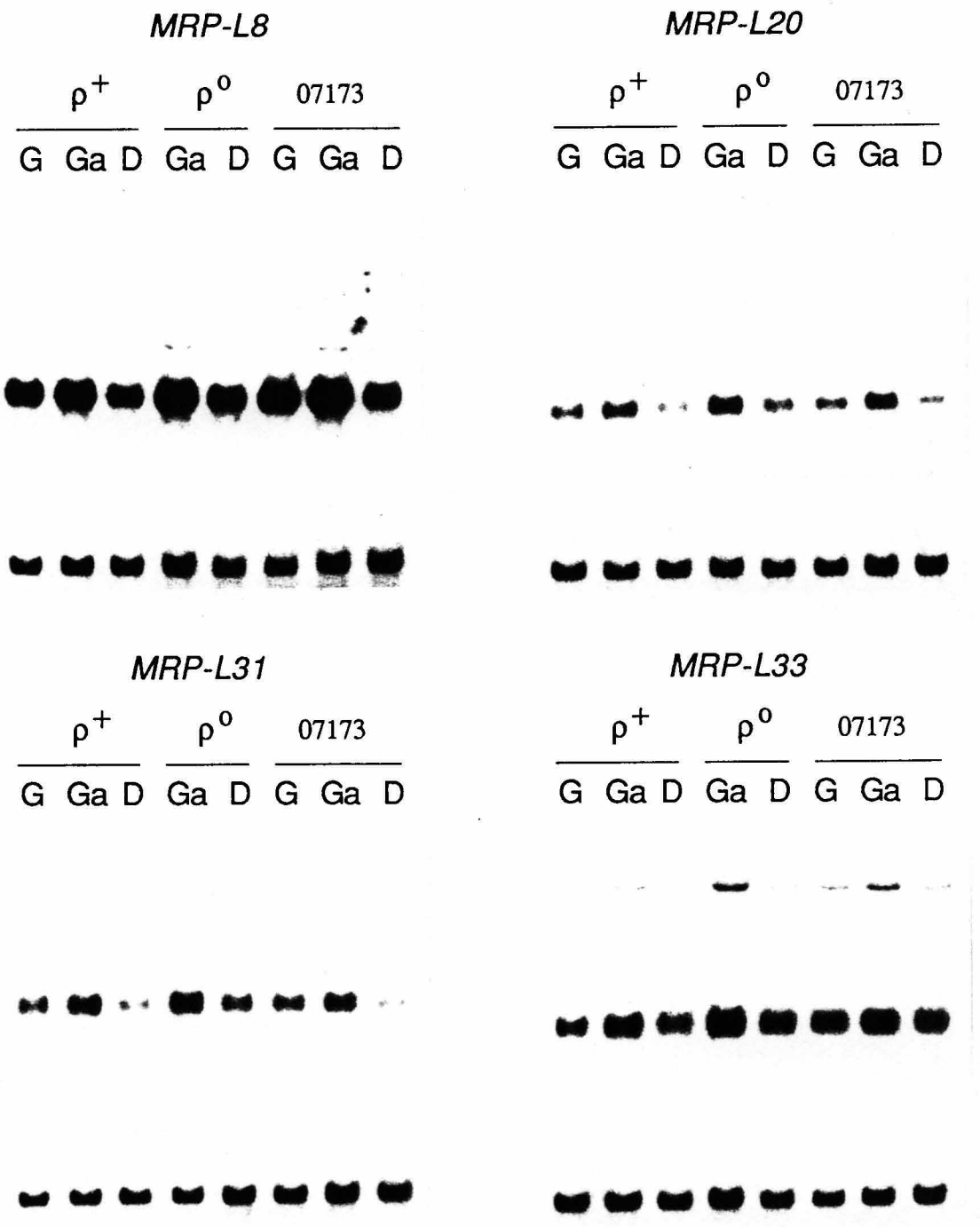


Fig.19. Analysis of transcript of *MRP-L8, MRP-L20, MRP-L31* and *MRP-L33.* Total RNA was prepared from early log-phase cultures of DC-5 (ρ+) and DC-5ρ⁰ (ρ⁰) and strain 07173 (**07173**) growing in YP medium containing either 2% glycerol (**G**), 2% galactose (**Ga**), or 6% glucose (**D**). Twenty μg of the resultant RNA per lane were electrophoresed in formaldehyde-agarose gel, transferred to nitrocellulose membrane, and hybridized with the probes for *MRP-L8, MRP-L20, MRP-L31,* and *MRP-L33* as shown in Table.4 . All the nitrocellulose filters were processed simultaneously for washing and autoradiography and photographed. To calibrate the amounts of loaded RNA, the same filters were rehybridized with a 1.0 kb *Xho* I-*Hin* dIII fragment of pYA301 containing the *ACT1* gene as shown under each panel.

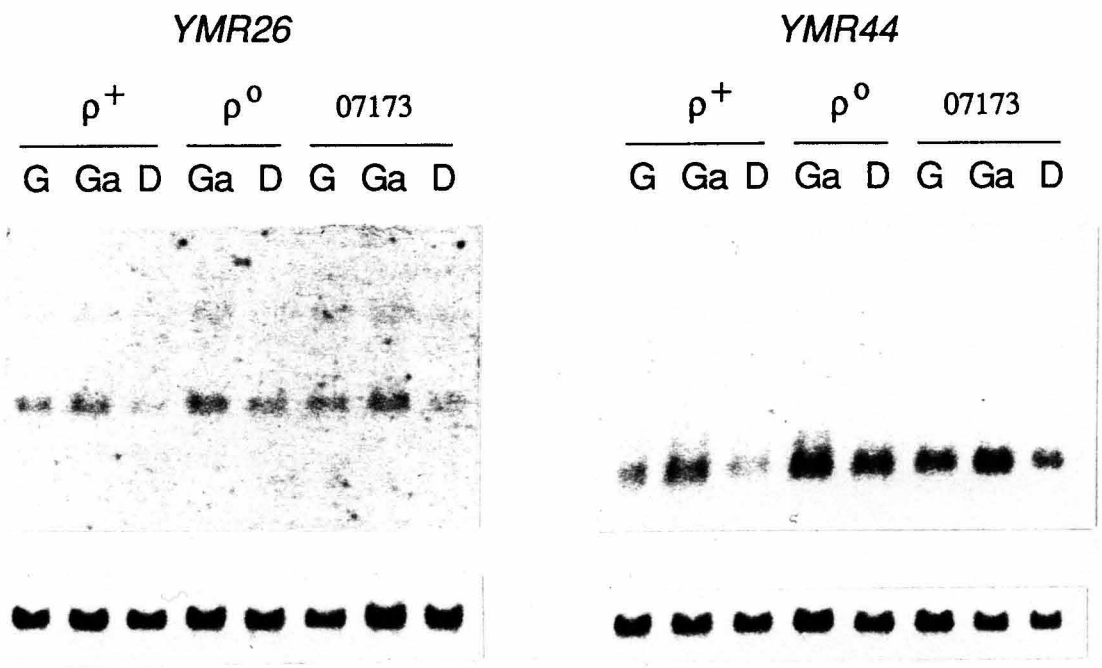


Fig.20. Analyses of transcript of *YMR26* and *YMR44*. Total RNA was prepared from early log-phase cultures of DC-5 (ρ+) and DC-5ρ^0 (ρ^0) and strain 07173 (**07173**) growing in YP medium containing either 2% glycerol (**G**), 2% galactose (**Ga**), or 6% glucose (**D**). Twenty micrograms of the resultant RNA per lane were electrophoresed in formaldehyde-agarose gel, transferred to nitrocellulose membrane, and hybridized with the probes for *YMR26* and *YMR44* as shown in Table.4 . To calibrate the amounts of loaded RNA, the same filters were re-hybridized with a 1.0 kb *Xho* I-*Hin* dIII fragment of pYA301 containing the *ACT1* gene as shown under each panel.

mitochondrial ribosomal protein genes (Fig.21).

Judging from the size and specific radioactivity of the probes as shown in Table 4 and from the size of transcripts, it appeared that the transcripts of *MRP-L8* and *MRP-L33* were more abundant than those of *MRP-L20* and *MRP-L31* since all the procedures following the hybridization in Fig.19 had been performed at the same time. In this connection, it should be noted that L8 and L33 have no signal sequence for transport into mitochondria in their N-terminus while YmL20 and YmL31 have. It might be that the transport efficiency of proteins without a pre-sequence is lower than that of proteins with a pre-sequence. Consequently, the amount of transcripts of proteins without a pre-sequence is larger than that of proteins with a pre-sequence. It might be to compensate the less efficient transport.

6. Transport of mitochondrial ribosomal protein YmL8 into mitochondria

All mitochondrial ribosomal proteins except for VAR1 in *S. cerevisiae* are encoded in nuclear genes. Therefore, they must be transported into mitochondria after synthesis in the cytoplasm. Till now, fourteen genes for mitochondrial ribosomal proteins including YMR31 and YMR44 were cloned and characterized. On the basis of their N-terminal amino acid sequences and the 5'-terminal nucleotide sequences of their genes, some proteins contain a leader sequence in their N-terminus, while others possess no leader sequence (Table 5). YmL8 and YmL33 do not have a leader sequence and show sequence similarity to the ribosomal proteins L17 and L30 of *E. coli*, respectively. Furthermore, the predicted amino acid sequence of the genes for YmL8 and YmL33 indicates that they are larger than the bacterial counterparts and that their C-terminus shows no sequence similarity to any ribosomal proteins (Fig.22). Therefore, it is conceivable that

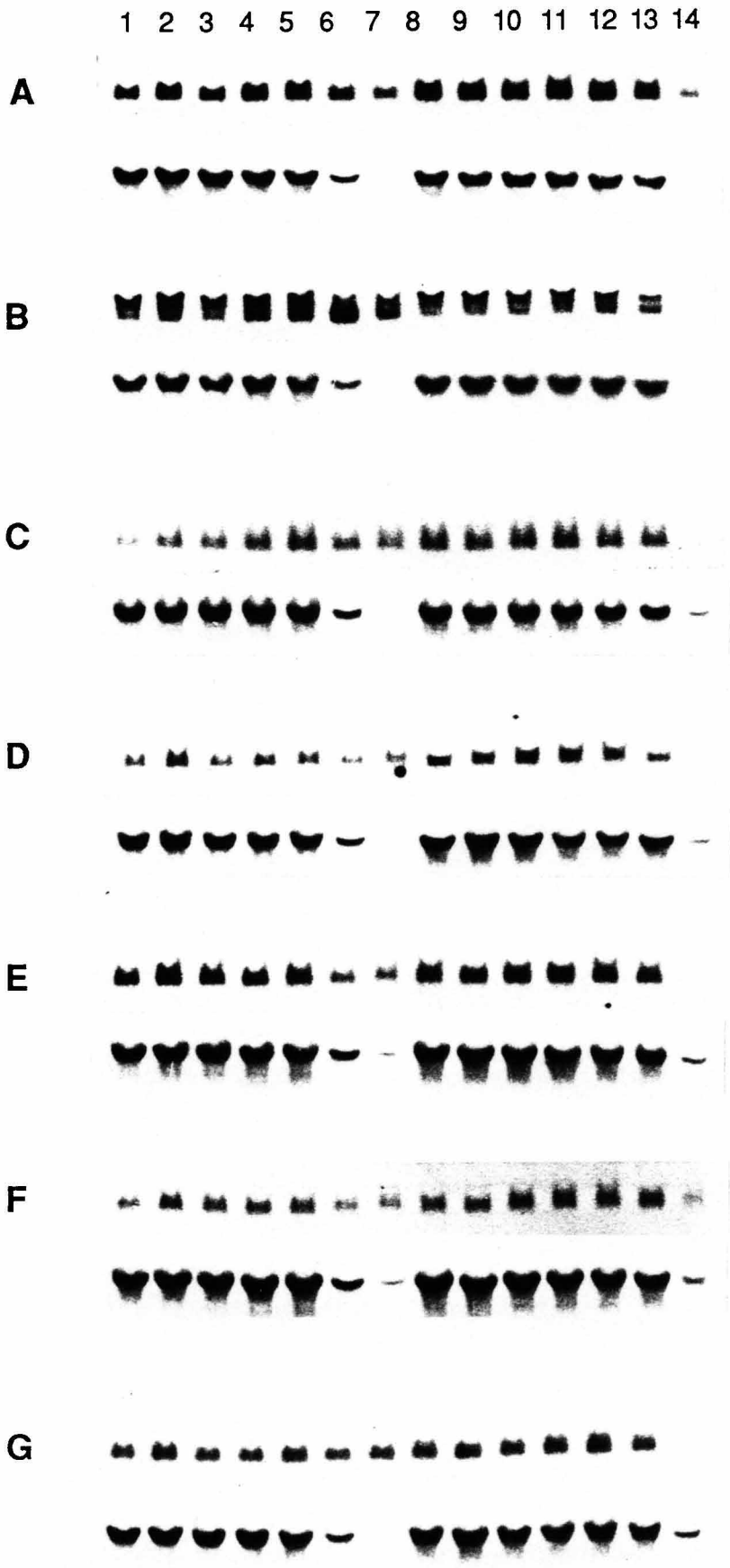


Fig. 21

Fig.21. Changes in transcription level prior to and after release from glucose repression. (A-G) DC-5 (lanes 2-8) or DC-5ρ^0 (lanes 9-15) cells grown to early log-phase in YPD medium containing 6% glucose were harvested by centrifugation and transferred to one-fourth of the starting volume of fresh YPD medium containing 2% glucose. Total RNA was prepared from aliquots of each culture taken before transfer to the YPD medium containing 2% glucose (lanes 2 and 9), and at15 min (lanes 3 and 10), 30 min (lanes 4 and 11), 1 hour (lanes 5 and 12), 3 hour (lanes 6 and 13), 6 hour (lanes 7 and 14), and 12 hour (lanes 8 and 15) after transfer to the 2% glucose medium. Thirty micrograms of total RNA from each sample was separated in formaldehyde-agarose gel, transferred to nitrocellulose membrane, and hybridized with a 1.13 kb *Eco* RI-*Hpa* I fragment of pL8EP containing *MRP-L8* gene (A), a 0.69 kb *Pst* I-*Kpn* I fragment of pL20EP containing a portion of *MRP-L20* gene (B), a 1.9 kb *Hin* dIII fragment of pL31H containing *MRP-L31* gene (C), a 1.2 kb *Hin*dIII-*Xba* I fragment of pL33 containing *MRP-L33* gene (D), a 1.5 kb *Pvu* II fragment of pEHc65-1 containing *YMR26* gene (E), a 1.0 kb *Bam* HI-*Pst* I fragment of pM1-XbaI-1 containing *YMR31* gene (F), and a 0.45 kb *Eco* RI-*Pst* I fragment of RF form of M13 No.1 (Table 3B) containing a portion of the *YMR44* gene (G). To calibrate the amounts of loaded RNA, the same filters were re-hybridized with a 1.0 kb *Xho* I-*Hin* dIII fragment of pYA301 containing the *ACT1* gene as shown under each panel.

Table 5. Classification of the mitochondrial ribosomal proteins of *Saccharomyces cerevisiae*

With presequence

Protein	Molecular weight	Leader sequence	Gene	Gene Disruption	Sequence similarity	reference
YMR31	12,792	8	372 bp	+	-	this work
YmL4	35,293	14	960	?	-	Graack 1990
YmL9	27,565	19	810	?	L3	Graack 1990
YmL13	?	36	?	?	-	Grohmann 1989
YmL20	20,626	18	588	RD	-	Kitakawa et al. 1990
YmL27	14,813	16	441	RD	-	Graack 1990
YmL31	14,246	12	396	RD	-	Grohmann et al. 1989
MRP2	11,852	15	348	RD	S14	Myers et al. 1987
MRP7	40,091	27	1116	RD	L27	Fearon and Mason 1989
MRP13	37,366	10	975	+	-	Partaledis and Mason 1989

Without presequence

Protein	Molecular weight	Leader sequence	Gene	Gene Disruption	Sequence similarity	reference
YMR26	18,586	-	474 bp	RD	-	Kang 1991
YMR44	11,476	-	297	?	-	this work
YmL8	26,822	-	717	RD	L17,S13	Kitakawa et al. 1990
YmL33	11,012	-	300	RD	L30	Kang 1991

pre-sequence ?

Protein	Molecular weight	Leader sequence	Gene	Gene Disruption	Sequence similarity	reference
MRP1	36,628	?	966 bp	RD	-	Myers et al. 1987
PET123	41,650	?	957	RD	-	Haffter et al. 1990

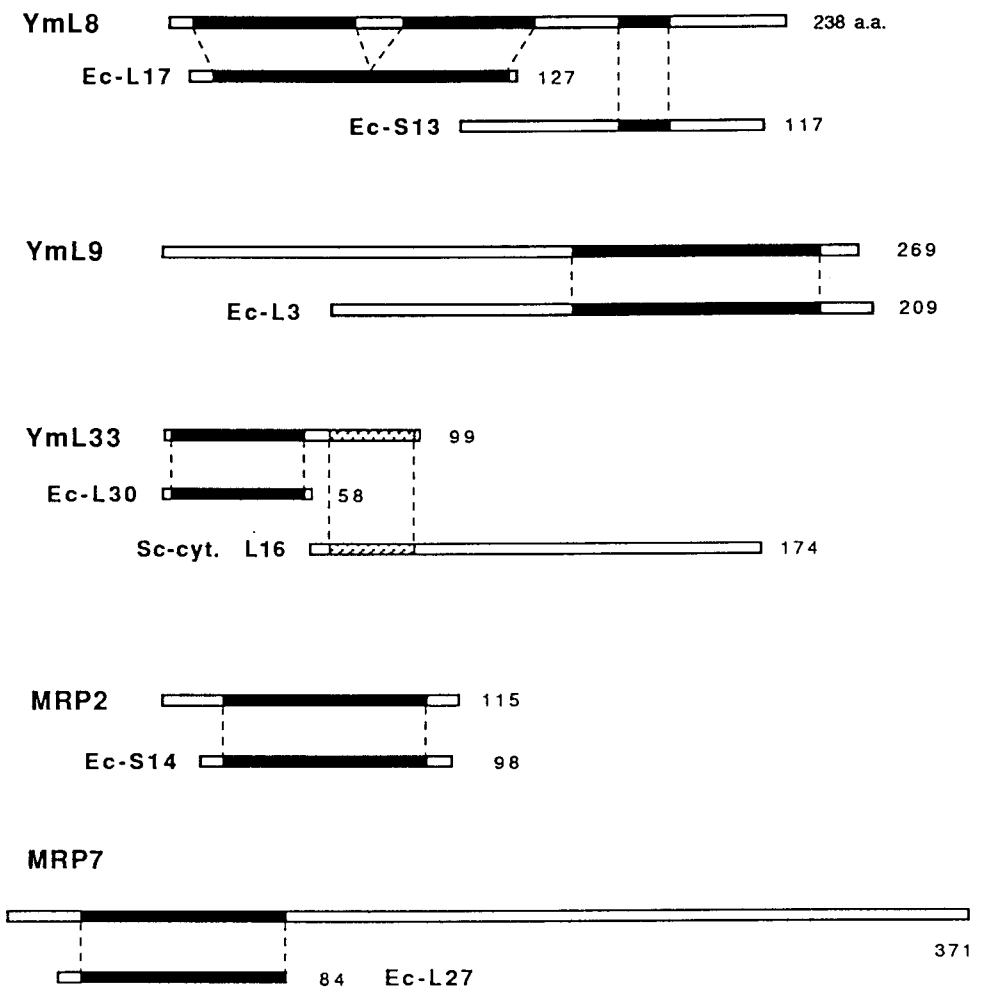


Fig.22. Schematic illustration of homologous regions between mitochondrial ribosomal proteins from *S. cerevisiae* and *E. coli*. Filled boxes indicate regions showing sequence similarity between the paired proteins. Hatched box indicates a region showing sequence similarity between a mitochondrial and a cytoplasmic ribosomal protein. Numbers indicate total lengths expressed in amino acid residues.

mitochondrial ribosomal proteins, especially those without a leader sequence, possess signals for transport into mitochondria in their C-terminus. The C-terminal amino acid sequences of them were accordingly compared. Short stretches showing similarity were found to be present on these proteins as shown in Fig.23. It is composed of two basic amino acid residues and two aliphatic amino acids and, in some cases, tyrosine or phenylalanine. To investigate the possibility that the C-terminus of the proteins containing this motif plays indeed a role in transport into mitochondria, I selected YmL8 which has no leader sequence and examined whether the *MRP-L8* gene with a small deletion on its 3'-region could function in cells in which the chromosomal *MRP-L8* gene had been disrupted. Fig.24 shows an outline of the experimental scheme.

For this purpose, the chromosomal *MRP-L8* gene in strain KWK002 was disrupted with *HIS3* gene as a marker as shown in Fig.25. One of the resultant disruptant strain, KWK002ΔL8, was then mated with strain JSS122-15B to supply functional mitochondria, since it was known that strains with disrupted *MRP-L8* gene became ρ^- or ρ^0 (Kitakawa et al. 1990). In this way, the diploid strain YMD002 was obtained. The 3'-terminus of the plasmid-borne *MRP-L8* was then deleted as shown in Fig.26. To investigate the effect of gene dosage on these experiments, two different vectors were used: a low copy number plasmid, YCp19, and a high copy number plasmid, YEp13. In the first step a DNA fragment containing the *MRP-L8* gene was isolated from pL8EP and inserted into pUC119 or pUC119link, in which a linker of 14 mer containing stop codons (TAA) in three phases had been inserted, as shown in Fig.26. The resultant plasmids pYML8A and pYML8link (Table 6) were used for the deletion of the 3'-terminus of the *MRP-L8* gene. In such

With pre-sequence

YMR31	NRPLQKDELASIFELPARFRYKPNEHELESINSGGW*
YmL4	KVPKLDELPTVRKYLKQLIHASSVEQATA*
YmL9	DENNVIWVKGSVAGPKNSFVKIQDAIKKT*
YmL20	EIQSRWKEKRRIAREDRKHRKLLWYQA*
YmL27	PRLQLLKIKEYIVNGRVQSEGATDTSCYKERG*
YmL31	AVGYRKGIHKVPKWTKISIRKAFKFF*
MRP2	RLCRYQFRENALKGNLPGVKKGIW*
MRP7	IVSRRFDYTKNKVEVIARSRRAFLSKL*
MRP13	VGNTLSGSGKRGKIVQNTHRKYINNIL*

Without pre-sequence

YmL8	ERTRTQALLKEVYSSSLPKKTKKPSSYVMVPRP*
YmL33	LRKSNPGFIVGEENHRLKQRNKALDFLSS*
YMR26	KLKKREEALKKVDELIASKKGSKYAKRVEKMKKNQSIGWF*
YMR44	KDKKEMEVDPSNMNFQELANHFDRHSKQLDLKHMLEMH*

pre-sequence ?

MRP1	LERVWDSIEWKIVESRLPQRTKIQAFHTL*
PET123	DLKNISNKIDSKLNPTSNGAGNNGNNNNTTNL*

Fig.23. Motifs in the C-termini of mitochondrial ribosomal proteins. Amino acid sequences in the C-terminus of mitochondrial ribosomal proteins are shown and putative mitochondrial transport signals are enclosed.

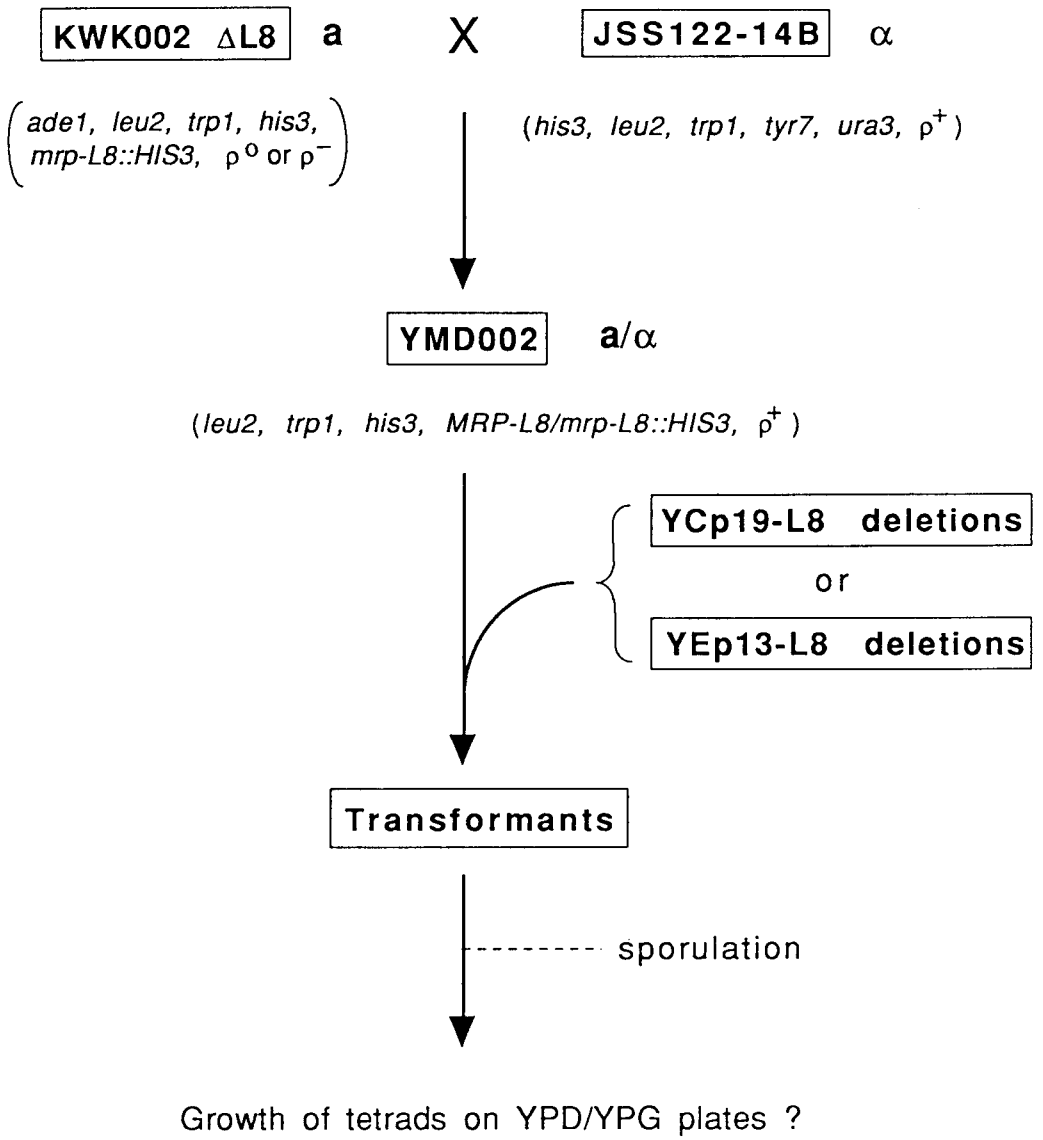


Fig.24. Experimental scheme for analysis of the structure of YmL8. KWK002 cells in which the chromosomal *MRP-L8* gene is disrupted as shown in Fig. 25 are used for mating with JSS122-14B cells to supply intact mitochondria. The resultant diploid strain YMD002 was used as recipient for transformation of a single copy or a high copy plasmid containing an intact or a 3'-terminal deleted *MRP-L8* gene constructed as shown in Fig. 26. The tetrad spores derived from the resultant transformant cells were separated with a micromanipulator system (Leitz) on YPD plates and then their ability to grow on YPG plates was examined.

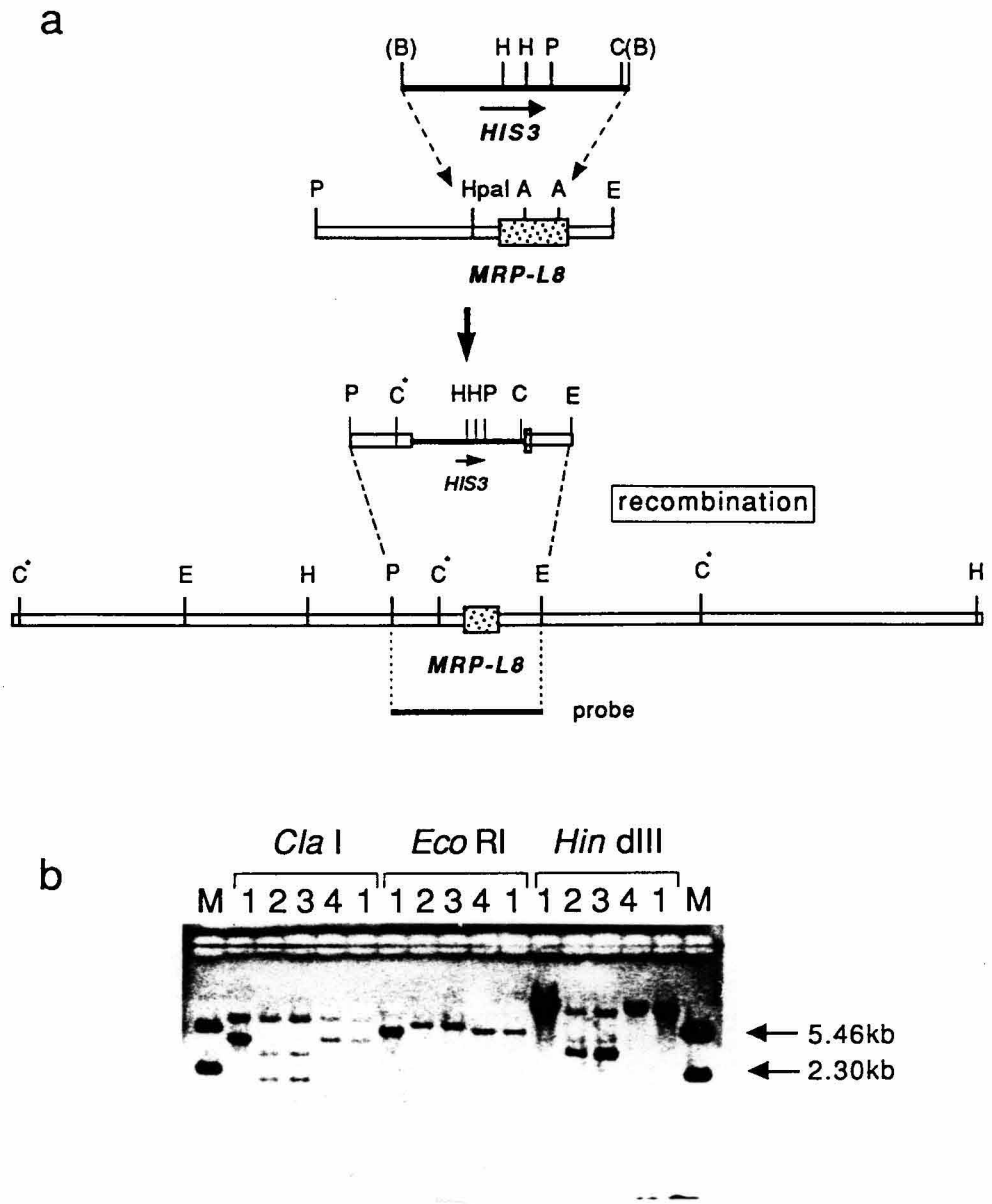


Fig.25. Disruption of the *MRP-L8* gene. **a.** A 1.75 kb *Bam* HI fragment of YEpH (Table 6) containing the *HIS3* gene (indicated by an arrow) was ligated into the *Acc* I-*Hpa* I site of pL8EP after filling of their cohesive ends. The resultant plasmid (pL8His3A, Table 6) in which the *MRP-L8* gene became disrupted was cleaved with *Eco* RI and then used to transform KWK002 cells. The chromosomal *MRP-L8* gene was expected to be replaced by the disrupted allele as indicated. Bar drawn under the gene indicates a probe for Southern hybridization. **b.** The disruption of *MRP-L8* gene was confirmed by Southern hybridization analysis of the DNA from three HIS+ transformants (lanes 2, 3, and 4) and KWK002 (undisrupted control : lane 1). Their DNA was digested with *Cla* I, *Eco* RI or *Hin* dIII and hybridized with a 2.3 kb *Eco* RI-*Pst* I fragment of pL8EP. As size markers, a mixture of the DNA of pL8EP digested with *Eco* RI or *Eco* RI-*Pst* I was used (lane M). Their size are, from top to bottom, 5.46 and 2.30 kb, respectively. Cells in lanes 2 and 3 are not able to grow on YPG plates in contrast to those in lanes 1 and 4. Cells in lane 2 were used for further experiments described in the text. Abbreviations for restriction enzymes are **A** for *Acc* I, **B** for *Bam* HI, **C** for *Cla* I, **E** for *Eco* RI, **H** for *Hin* dIII, and **P** for *Pst* I. The restriction enzyme sites in **a** whose positions are ambiguous are indicated by asterisks.

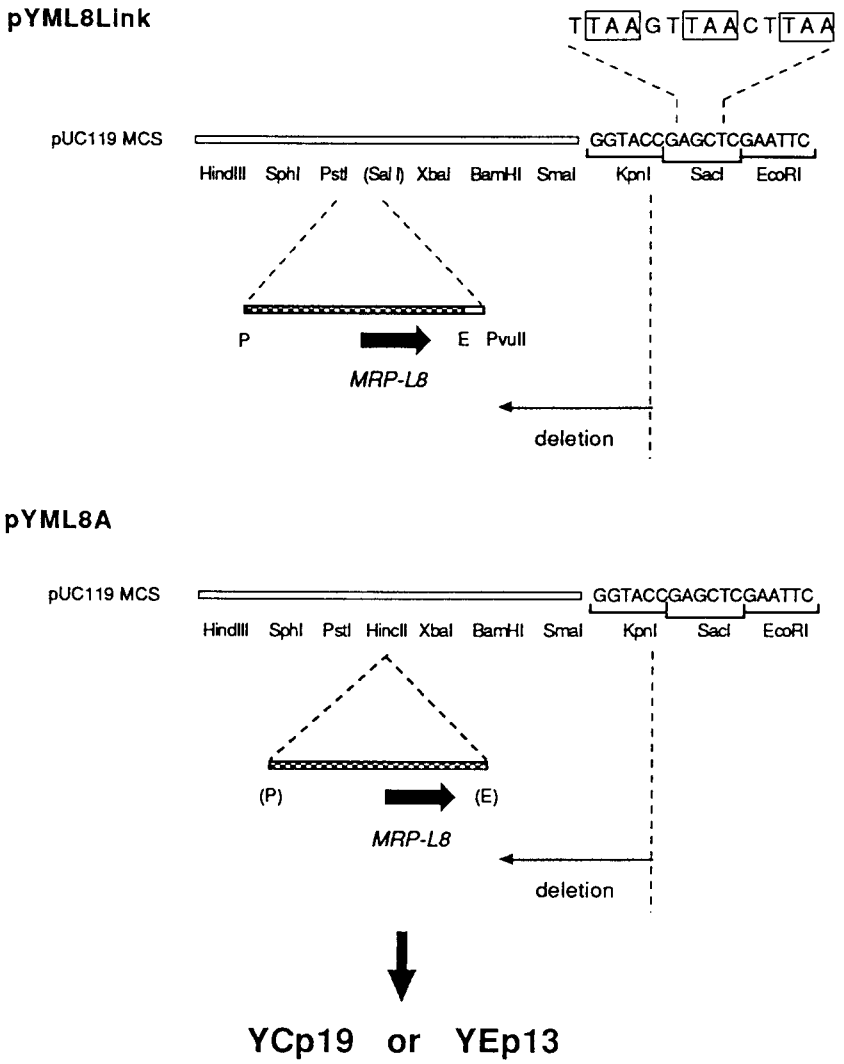


Fig.26. Construction of plasmids containing the *MRP-L8* gene with 3'-terminal deletions. The 2.3 kb *Eco* RI-*Pst* I fragment of pL8EP containing the *MRP-L8* gene was filled in and ligated into the *Hin* cII site of pUC119. The resultant plasmid which contained the *MRP-L8* gene in the direction as indicated was termed as pYML8A. The oligonucleotide linker (5'-TTAAGTTAACTTAA-3') containing stop codons (TAA) in three phases was inserted at the *Sac* I site of pUC119 after making the cohesive ends blunt with T4 DNA polymerase. The resultant vector (pUC119link) was digested with *Sal* I, filled in and then digested with *Hin* dIII. A 2.5 kb *Hin* dIII-*Pvu* II fragment of pL8EP containing the *MRP-L8* gene was then ligated in. The resultant plasmid was termed as pYML8link. The pYML8A and pYML8link were digested with *Bam* HI and *Kpn* I and used for deletion with exonuclease III as indicated by thin arrows. The deletion end points of the resultant plasmids (pYML8d23, pYML8d37, pYML8d45, pYML8Δ1, pYML8Δ2, pYML8Δ3, pYML8Δ4, see Table 7A) were examined by DNA sequencing of these plasmids. The *Pvu* II fragment containing the *MRP-L8* gene deleted in the 3'-terminus was excised and then ligated into the *Sma* I site of YCp19 or the *Pvu* II site of YEp13 (Table 7B). As control plasmids, YCp19-L8intactB and YEp13-L8B containing the intact *MRP-L8* gene were also constructed with YCp19 and YEp13, respectively (Table 7B).

Table 6. Plasmids used for complementation analyses of *MRP-L8* gene

Plasmid	Insert	Vector (sites used)	Reference
pL8EP	strain 07173 *Eco* RI *Pst* I 2.3 kb	pUC118 (*Eco* RI *Pst* I)	Kitakawa et al. 1990
pYML8A	pL8EP *Eco* RI *Pst* I 2.3 kb fill in	pUC119 (*Hinc* II)	
pUC119 link	stop linker (5'-t t a a g t t a a c t t a a -3')	pUC119 (*Sac* I, T4 Pol.)	
pYML8 link	pL8EP *Hind* III *Pvu* II 2.5 kb	pUC119 link (*Sal* I fill in, *Hind* III)	
YEpH	YIp1 *Bam* HI 1.75 kb (*HIS3*)	*Bgl* II fragment deleted-YEp13 (*Bam* HI)	Kang 1991
pL8His3A	YEpH *Bam* HI 1.75 kb (*HIS3*) fill in	pL8EP (*Hpa* I *Acc* I fill in)	

deletions of pYML8A, it was expected that translational products would be synthesized as fusion proteins. One of such constructs should have shortest extra amino acid residues of the13 residues expected, i.e. -X-ELEFTGRRFTTS. It should be noted that a fusion protein in this phase has the motif (RRFT) in its extended region. In the case of deletions derived from pYML8link, extra amino acid sequence in the C-terminus was expected to be -R, -X-LS or -X-VKLT. Accordingly, *Pvu* II segments isolated from the resultant plasmids containing 3'-deleted *MRP-L8* genes (Table 6 and Table 7A) were ligated into the *Sma* I site of YCp19 and *Pvu* II site of YEp13 (Table 7B).

Plasmids constructed in this way were then used for transformation of YMD002 described above. Colonies growing under the selection conditions were obtained in control experiments without plasmid DNA although their numbers were about two powers of less than those with plasmid DNA. It was thus necessary to confirm that each transformant indeed contained plasmids used in transformation. Therefore, the DNA isolated from each transformant was used to transform *E. coli* strain HB101, since YCp19 and YEp13 were shuttle vectors between yeast and *E. coli*. The plasmid DNA isolated from resultant transformants was digested with appropriate restriction enzymes and their restriction pattern was analyzed with agarose gel electrophoresis (data not shown). Consequently, it was found that all yeast transformants examined contained the same plasmids used in the first transformation. YMD002 cells with various plasmids thus obtained were used for tetrad analyses. After growth of tetrad spores on YPD medium, it was examined whether they could grow on YPG plate or not. If cells with both disrupted *MRP-L8* gene on the chromosome and the plasmid-borne *MRP-L8* gene with a deletion in the 3'-terminus could grow on YPG plates, then it can be concluded that the

Table 7A. Deletion constructs of *MRP-L8* gene

Plasmid	Construction
pYML8 link Δ1	deletion of pYML8 link (*Bam* HI *Kpn* I) up to position 786
pYML8 link Δ2	deletion of pYML8 link (*Bam* HI *Kpn* I) up to position 877
pYML8 link Δ3	deletion of pYML8 link (*Bam* HI *Kpn* I) up to position 912
pYML8 link Δ4	deletion of pYML8 link (*Bam* HI *Kpn* I) up to position 839
pYML8A d23	deletion of pYML8A (*Bam* HI *Kpn* I) up to position 853
pYML8A d37	deletion of pYML8A (*Bam* HI *Kpn* I) up to position 1035
pYML8A d45	deletion of pYML8A (*Bam* HI *Kpn* I) up to position 784

Table 7B. Plasmids used for transformation of YMD002

Plasmid	Insert	Vector (sites used)
YCp19		
YCp19-L8 Δ1B	pYML8 link Δ1 *Pvu* II insert	YCp19 (*Sma* I)
YCp19-L8 Δ2B	pYML8 link Δ2 *Pvu* II insert	YCp19 (*Sma* I)
YCp19-L8 Δ3B	pYML8 link Δ3 *Pvu* II insert	YCp19 (*Sma* I)
YCp19-L8 Δ4B	pYML8 link Δ4 *Pvu* II insert	YCp19 (*Sma* I)
YCp19-L8 d23B	pYML8A d23 *Pvu* II insert	YCp19 (*Sma* I)
YCp19-L8 d37B	pYML8A d37 *Pvu* II insert	YCp19 (*Sma* I)
YCp19-L8 d45B	pYML8A d45 *Pvu* II insert	YCp19 (*Sma* I)
YCp19-L8 intact B	pL8EP *Pvu* II insert	YCp19 (*Sma* I)
YEp13		
YEp13-L8 Δ3B	pYML8 link Δ3 *Pvu* II insert	YEp13 (*Pvu* II)
YEp13-L8 Δ4B	pYML8 link Δ4 *Pvu* II insert	YEp13 (*Pvu* II)
YEp13-L8 d23B	pYML8A d23 *Pvu* II insert	YEp13 (*Pvu* II)
YEp13-L8 d45B	pYML8A d45 *Pvu* II insert	YEp13 (*Pvu* II)
YEp13-L8 B	pL8EP *Eco* RI *Pst* I 2.3 kb fill in	YEp13 (*Pvu* II)

deleted gene could function in spite of the deletion. The results are shown in Table 8.

In tetrad analysis, it is expected that wild-type and disrupted alleles of chromosomal *MRP-L8* segregate in a 2 : 2 ratio and that plasmids containing *MRP-L8* with deleted 3'-terminus transferred only into two spores of the tetrads in the case of YCp19 and into all four spores in the case of YEp13. Therefore, it was required to confirm whether *MRP-L8* gene on the chromosome was disrupted or not and whether plasmids existed or not. Therefore, the DNAs isolated from several sets of tetrad spores were digested with *Eco* RI and *Hind* III and examined by Southern hybridization using *Eco* RI-*Pst* I fragment of 2.3 kb in size from pL8EP (see Fig.25) as a probe. The results are shown in Fig.27 and Fig.28 together with the results of the growth of cells derived from tetrads on YPG plates. It was expected that the DNA fragments derived from the disrupted *MRP-L8* gene on the chromosome could be detected as 3.2 kb and 1.2 kb bands, while the wild-type *MRP-L8* should be detected as a 3.6 kb band. Similarly, the *MRP-L8* deletions present on plasmids should be detected as a 1.9 to 2.3 kb band except that the band derived from YEp13-L8B containing intact *MRP-L8* gene should be 4.4 kb in size. Furthermore, it was necessary to examine whether the transcripts from *MRP-L8* with deletions in the 3'-terminus accumulated or not. By a series of Northern hybridization experiments, it was found that the transcripts from *MRP-L8* gene on YCp19 encoding proteins lacking ten residues in the C-terminus (YCp19-L8Δ3B, Table 5B) accumulated at the same level as those from wild-type *MRP-L8* gene (data not shown). The results from Table 8 and Fig.27 and Fig.28 are schematically summarized in Fig.29 which shows that, when YmL8 had lost ten amino acid residues from its C-terminus, it was no longer able to function when present on a

Table 8. Results of structural analysis of YmL8[#]

Plasmid	4/4	3/4	2/4	1/4	0/4
YCp19-L8Δ1B	0	0	8	0	0
YCp19-L8Δ2B	0	0	4	0	0
YCp19-L8Δ3B	0	1	4	0	0
YCp19-L8Δ4B	0	0	4	0	0
YCp19-L8d23B	0	0	4	0	0
YCp19-L8d37B	1	0	7	0	0
YCp19-L8d45B	0	0	5	0	0
YCp19-L8intactB	1	0	4	0	0
YCp19	0	0	6	0	0
YEp13-L8Δ3B	3	0	3	0	0
YEp13-L8Δ3B	0	0	6	0	0
YEp13-L8d23B	0	0	3	0	0
YEp13-L8d45B	0	0	4	0	0
YEp13-L8B	4	2	1	0	0
YEp13	0	0	3	0	0
no plasmid	0	0	4	0	0

Four spores contained in a single ascus were separated on YPD plate with a micromanipulator. Their growth on YPG plates was examined. The number of spores counted are shown in columns of 4/4, 3/4, 2/4, 1/4, and 0/4.

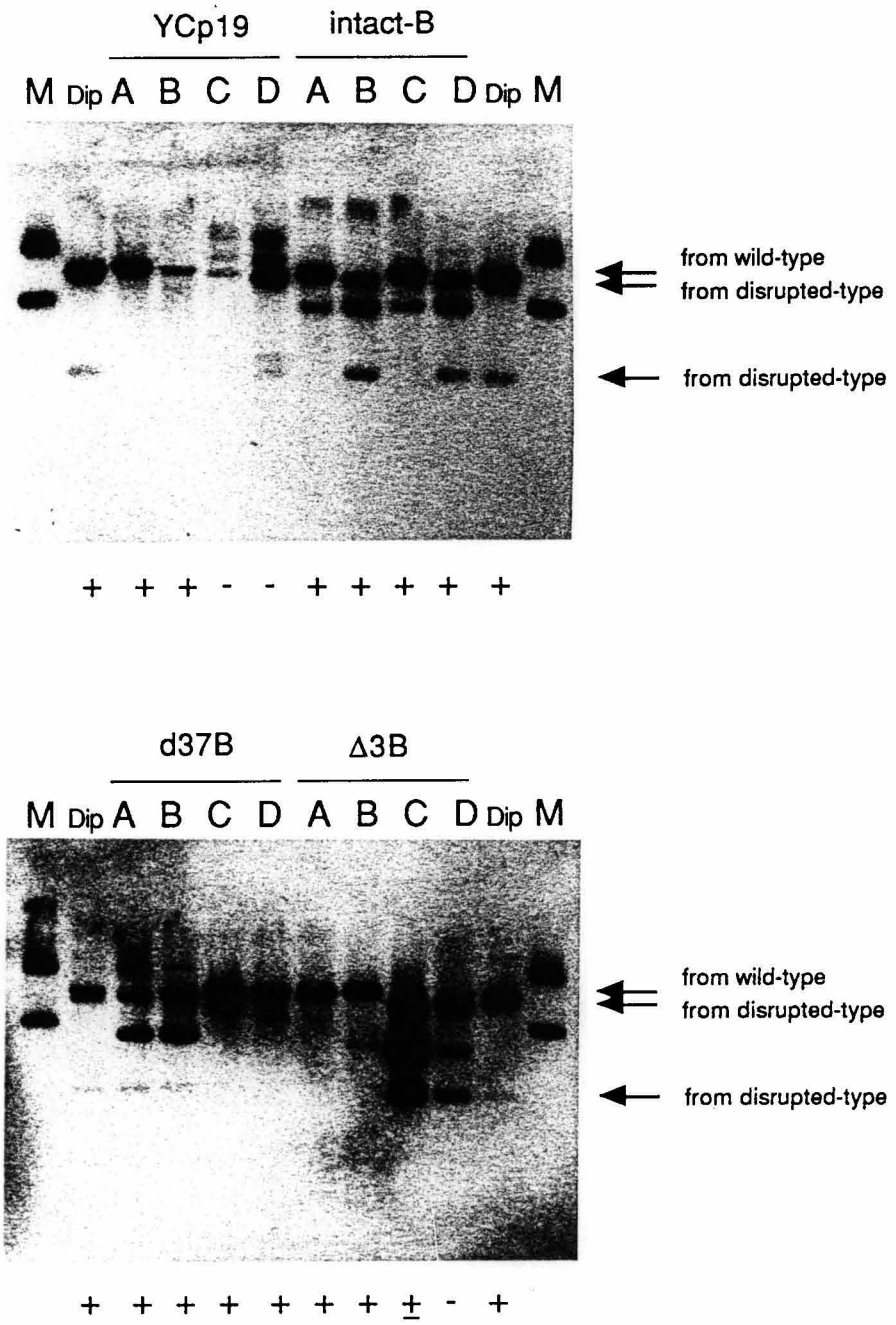


Fig.27. Function of the *MRP-L8* deletions on YCp-type plasmid. Each spore (A-D) was separated with a micromanipulator from the ascus containing tetrad spores derived from YMD002 cells containing YCp19 (**YCp19**), YCp19-L8intact-B (**intact-B**), YCp19-L8d37B (**d37B**), or YCp19-L8Δ3B (**Δ3B**) and grown on YPD plates. Total DNA was prepared from cultures of the resultant cells growing in YPD medium, digested with *Eco* RI and *Hin* dIII, electrophoresed in 1% agarose gel, and transferred to nitrocellulose membrane for Southern hybridization with the 2.3 kb *Eco* RI-*Pst* I fragment of pL8EP. As undisrupted and disrupted controls of chromosomal *MRP-L8* gene, total DNA from YMD002 cells was loaded in lane **Dip**. Size markers were loaded in lane M as in Fig.25. Disrupted and undisrupted *MRP-L8* genes on the chromosome and recombinant *MRP-L8* gene on plasmid are expected to be detected as indicated. Whether each cell is able to grow on YPG plates or not is indicated by + (growth) or - (no growth) under the results of Southern hybridization. ± shows they grew as segregants on YPG plate.

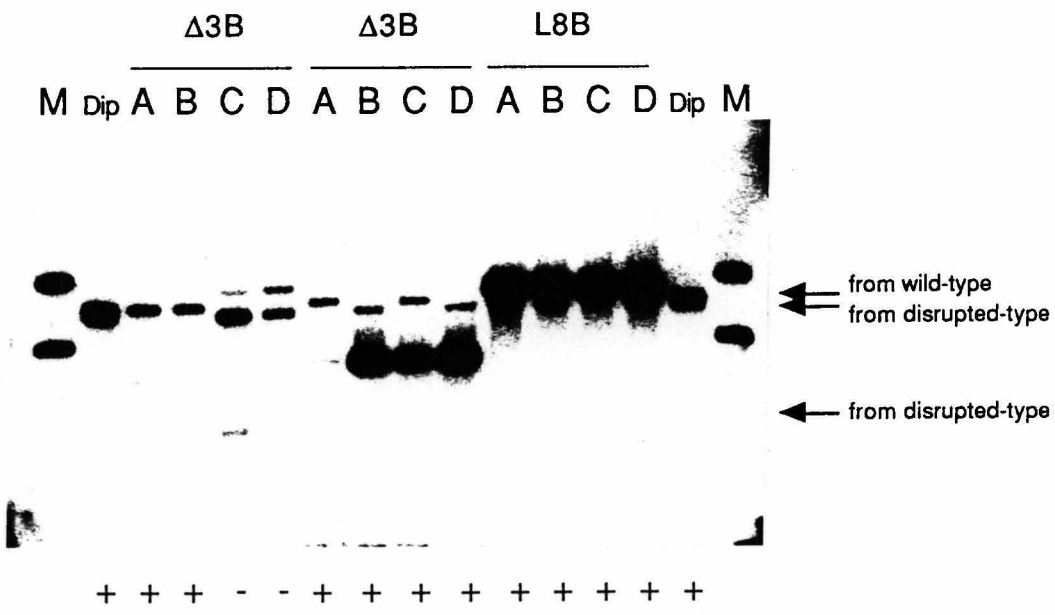


Fig.28. Analysis of the *MRP-L8* deletions on YEp-type plasmid. Each spore (A-D) was separated with a micromanipulator from the ascus containing tetrad spores derived from YMD002 cells containing YEp13-L8Δ3B (**Δ3B**) or YEpo13-L8B (**L8B**) and grown on YPD plates. Total DNA prepared from cultures of the resultant cells growing in YPD medium was used for Southern hybridization as described in Fig.27. As undisrupted and disrupted controls of the chromosomal *MRP-L8* gene, total DNA from YMD002 cells was loaded in lane **Dip**. Size markers were loaded in lane M as in Fig.25. Disrupted and undisrupted *MRP-L8* genes on the chromosome and recombinant *MRP-L8* gene on plasmid are expected to be detected as indicated. Whether each cell is able to grow on YPG plates or not is indicated by + (growth) or - (no growth) under the results of Southern hybridization.

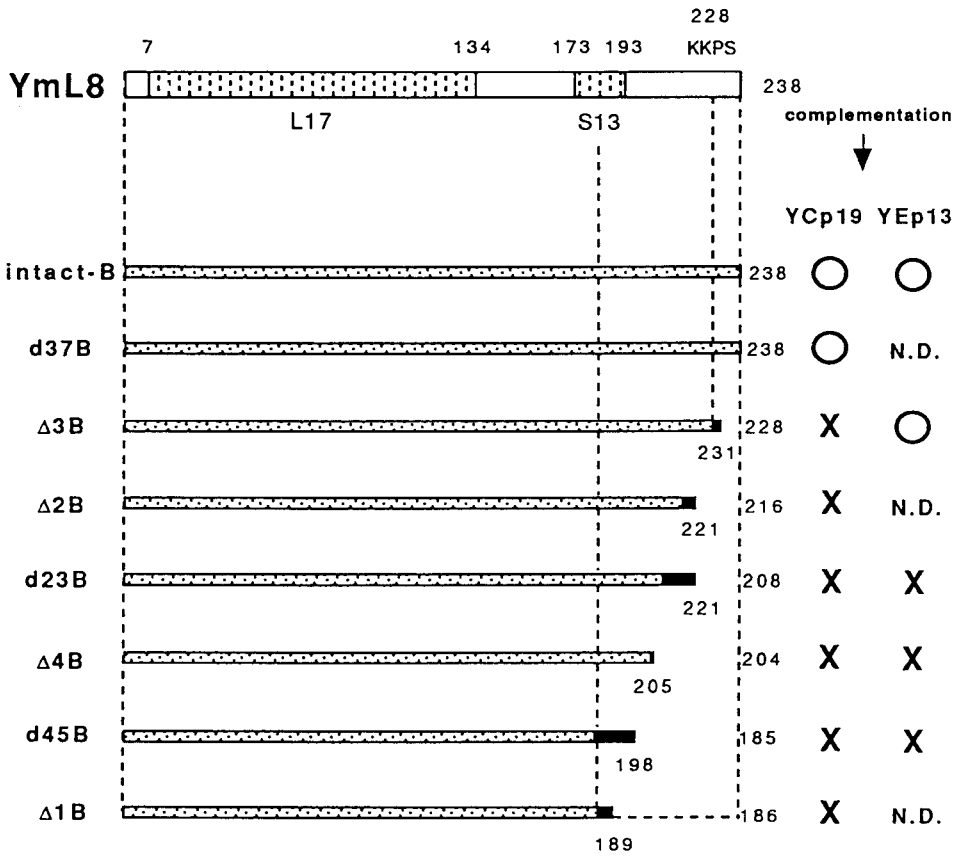


Fig.29. Summary of structural analysis of YmL8. Results of the complementation experiments in Table 8, Fig.27 and Fig.28 are summarized. The YmL8 proteins are expected to be synthesized as indicated at the top. Filled stretches indicate the regions of the protein derived from the vector sequence. Numbers at right indicate expected length and the junction positions of the deletions. Whether each construct on a YCp19 or a YEp13 could complement the disrupted *MRP-L8* gene on the chromosome or not is indicated by O and X, respectively.

low copy-number plasmid YCp19. However, it was still capable of functioning when placed on a high copy-number plasmid YEp13.

Discussion

1. Cloning methods for yeast mitochondrial ribosomal protein genes

To clone the genes for mitochondrial ribosomal proteins, I used oligonucleotides mixtures designed from the N-terminal amino acid sequences of the proteins as hybridization probes. This method is certainly a more generally applicable method for the cloning of mitochondrial ribosomal protein genes than other methods such as the isolation of nuclear *PET* mutants to use them in screening by complementation. To date, the genes for eleven mitochondrial ribosomal proteins have been cloned in yeast by this method (Grohmann et al. 1989, Grohmann 1989, Kitakawa et al. 1990, Kang 1991, Kang et al. 1991, Graack 1990, this work). Advantages of this method are as follows. First of all the proteins whose genes are planned to clone are already identified on two-dimensional gel electropherogram. Therefore, biochemical analysis for their identification is not necessary. A second advantage is that, once a candidate clone is obtained, it can quickly and easily be confirmed whether the clone indeed contains the gene in question by comparing the N-terminal amino acid sequence data of the protein with that predicted from the nucleotide sequence determined directly by using the oligonucleotide mixtures used for screening probes as sequencing primers. A third advantage is that it is possible to deduce the presence of a presequence and an intron in the protein and its gene by comparing the nucleotide sequence of a gene and its translation with the N-terminal amino acid sequence data. Thus, it was found that protein YMR31 has a presequence of eight amino acid residues and that the *YMR44* gene contains an intron of 147 bp in length.

A disadvantage of this procedure is that cloning is difficult when the available amino acid sequence data are only for a few residues and when the data do not contain amino acid sequences with least degenerate codons, because, as the number of oligonucleotides in the mixture increases, the relative probing activity of one kind of oligonucleotide in the mixture decreases and the band intensity detected in the hybridization to genomic DNA will become very weak. As a result, much more false bands are likely to emerge. In these cases, application of polymerase chain reaction (PCR) seems to be useful. Indeed it had been reported that oligonucleotide mixtures including about a thousand different oligonucleotide sequences could be used as PCR primers in order to clone the gene encoding SRP14, one of the six proteins contained in the signal recognition particle (SRP) (Strub and Walter 1989). If amino acid sequences in the internal or C-terminal regions of the protein in question can be determined in addition to that of the N-terminal region, cloning can be performed as in the case of the *SRP14* gene. In fact, some of the mitochondrial ribosomal protein genes in *S. cerevisiae* were cloned in this way using PCR (Kang 1991, Kang et al. 1991, Kitakawa et al. unpublished results).

2. Characteristics of *YMR31* and *YMR44* genes

In this work, I cloned and characterized two nuclear genes for mitochondrial ribosomal proteins termed YMR31 and YMR44. The gene for the latter, YMR44, was found to contain an intron of 147 bp in length. No other mitochondrial ribosomal protein genes cloned and characterized to date are known to contain an intron. In *Neurospora crassa*, *cyt-21* and *mrp3* genes, both of which encode mitochondrial ribosomal proteins, are known to contain one intron and two introns, respectively (Kuiper et al. 1988, Kreader et al. 1989). Various lines of evidence

seem to support the notion that the mitochondrion is a descendant of parasitic procaryote in the eukaryotic cell (reviewed in Gray 1989). Thus, it will be interesting to investigate whether the introns found in these genes are also present in the corresponding genes in related organisms or not. It will then be possible to obtain clues as to whether the introns in these genes were introduced into the genes after their transfer from the genome of the parasitic procaryote, the hypothetical ancestor of mitochondria, into the nucleus of the two *Ascomycetes* fungi during the course of mitochondrial evolution. Alternatively, the *cyt21*, *mrp3* and *YMR44* genes could have been of nuclear origin and the proteins encoded by them became adopted to the mitochondrial ribosome. I am currently investigating these possibilities.

By comparing the nucleotide sequence data with the N-terminal amino acid sequence data, YMR31 was found to contain a leader sequence of eight amino acid residues. This is the shortest of all leader sequences known to date among the proteins transported to mitochondria. The leader sequences in other mitochondrial proteins are much longer: for example, the mitochondrial F_1-ATPase β-subunit has a leader sequence of 19 amino acid residues (Takeda et al. 1985) and the leader sequence in the mitochondrial L(+)-lactate cytochrome c oxidoreductase is 81 residues long (Guiard 1985). Recently, it was found that, in addition to YMR44 presented in this work, some of the mitochondrial ribosomal proteins in *S. cerevisiae* contained no leader sequence. Also, at least two other mitochondrial ribosomal proteins in *S. cerevisiae* were found to contain relatively short leader sequences: for examples, the leader sequences of protein YmL4 and YmL31 are 14 residues and 12 residues long, respectively (see Table 5). The roles of these sequences for transport into mitochondria will be discussed later with the results of transport experiments of YmL8 in this work.

3. Evolution of mitochondrial ribosomal proteins

Among eighteen mitochondrial ribosomal proteins in *S. cerevisiae* including YMR31 and YMR44 cloned and characterized to date (Table5), seven proteins have high similarity to the ribosomal proteins in *E. coli*. These seem to support the notion that mitochondrion is a descendant of a parasitic procaryote in the eucaryotic cell. The reasons are as follows. If that is not the case, then the mitochondrial ribosome must have evolved from a common ancestor from which the cytoplasmic ribosome has evolved. In this case, it is expected that some of the cytoplasmic ribosomal proteins have similarity to bacterial ribosomal proteins since seven of the mitochondrial ribosomal proteins among eighteen have high similarity to bacterial ribosomal proteins. However, only three such examples are known among about forty cytoplasmic ribosomal proteins in *S. cerevisiae* characterized to date (Warner 1989). It thus seems that the mitochondrial ribosome is far more closely related to the bacterial ribosome, which in turn seems to support the "endosymbiont origin of mitochondria".

Eleven genes for mitochondrial ribosomal proteins in *S. cerevisiae* including *YMR31* and *YMR44* have no similarity to any bacterial ribosomal proteins. In addition, while bacterial ribosomes are composed of about fifty to sixty proteins, mitochondrial ribosomes in *S. cerevisiae* are composed of more than eighty proteins (Graack et al. 1988, Graack 1990). Therefore, it may be that there are three classes of mitochondrial ribosomal proteins in *S. cerevisiae*. One class is those proteins originated from the original prokaryote which still contain several conserved primary sequences due most likely to some functional constraints. Second class of proteins is those which have undergone extensive amino acid changes during the evolution of mitochondria so that their sequence similarity to

bacterial ribosomal proteins can no longer be traced. Third class is those which are derived from preexisting nuclear genes that were originally unrelated to mitochondria. In view of the third class of proteins, it is interesting to note that single gene products are utilized in both cytoplasm and mitochondria in the cases of *HTS1* (Natsoulis et al. 1986), *MOD5* (Najarian et al. 1987, Dihanich et al. 1987), *LEU4* (Beltzer et al. 1986, 1988), *VAS1* (Chatton et al. 1988), *FUM1* (Wu et al. 1987), and *TRM1* gene (Ellis et al. 1987) of *S. cerevisiae*. So far no evidence has been presented to indicate such is also the case with the mitochondrial ribosomal proteins in yeast.

4. Transfer of mitochondrial ribosomal protein genes from mitochondria to nucleus

If the mitochondrion is indeed a descendant of a parasitic prokaryote in the eucaryotic cell, most of the mitochondrial ribosomal protein genes located in the nucleus at present day must have transferred from mitochondria to the nucleus during the course of mitochondrial evolution. Recently, Thorsness and Fox (1990) reported that transfer of DNA from mitochondria to the nucleus occurred much more efficiently than in the opposite direction. In addition, it was reported that *tufA* gene encoding chloroplast translation elongation factor Tu (EF-Tu) exists only in chloroplasts in some green algae while it exists only in the nucleus in land plants. Furthermore, the *tufA* gene exists in both chloroplasts and nucleus in *Coleochaete*, a proposed sister group to land plants (Baldauf et al. 1990). These will give important information as to how gene transfer from subcellular organelles such as mitochondria and chloroplasts to the nucleus occurred.

According to the view of endosymbiotic origin of mitochondria, most of the mitochondrial ribosomal protein genes would also have transferred from

mitochondria to nucleus except for the case in which the products of nuclear genes which were originally unrelated to mitochondria were newly adopted as mitochondrial ribosomal proteins. Most of the genes for mitochondrial ribosomal proteins including YMR31 and YMR44 characterized in this work are located on different chromosome in *S. cerevisiae* as shown in Table 5. Of these, five genes, *MRP#A*, *MRP#B*, *MRP-L13*, *MRP-L20*, and *MRP-L31*, are located on chromosome XI. Whether these five genes are located close to one another is not known. At least it does not seem the case that these genes exist in an operon as in the case of ribosomal protein genes in bacteria, since the analysis of transcripts of *MRP-L20* and *MRP-L31* presented in this work and nucleotide sequence of these genes (Kitakawa et al. 1990, Grohmann et al. 1989) showed that each transcripts could encode only one product, respectively.

The *MRP2* and *MRP-L33* genes encode proteins which have similarity to S14 and L30 in *E. coli*, respectively. The genes for S14 and L30 belong to the same operon in *E. coli* (Ceretti et al. 1983). However, the products of *MRP2* and *MRP-L33* seem not to be derived from a single transcripts judging from the size of their transcripts and their nucleotide sequence (Myers et al. 1987, Kang 1991, Kang et al. 1991) although *MRP2* and *MRP-L33* may exist in the same chromosome XVI (Table 5). Thus, it appears that the genes for mitochondrial ribosomal proteins are not clustered in the yeast nucleus, unlike the ribosomal protein genes in eubacteria such as *E. coli*. These facts might reflect that transfer of mitochondrial ribosomal protein genes from mitochondria to the nucleus occurred via reverse transcription of mRNA rather than direct transfer of genes themselves.

The genes for ribosomal proteins S13 and L17 in *E. coli* are encoded in the α-operon and flank three other genes (S13-S11-S4-α-L17). The gene for YmL8 in

S. cerevisiae has regions showing similarity to L17 and S13 in this order as shown in Fig.22 (Kitakawa et al. 1990). Therefore, the order of the two "genes" is different from that in *E. coli*. It is not known whether the two proteins are located next to each other in the order of L17-S13 in the *E. coli* ribosomal particles or not. If this is the case, then the organization of two domains in the gene for YmL8 showing similarity to the two *E. coli* protein genes can possibly be explained as a consequence of this structural and/or functional constraint.

Mitochondrial ribosomal protein MRP2 in *S. cerevisiae* shows similarity to ribosomal protein S14 of *E. coli*. They are nearly the same in size. In contrast, mitochondrial ribosomal proteins MRP7 and YmL33 are two to three times as large as ribosomal proteins L27 and L30 of *E. coli* to which they are similar. This raises the possibility that mitochondrial ribosomal protein genes inserted themselves into pre-existing genes in the nucleus to acquire a signal sequence into mitochondria and fused with them. Consequently, they became utilized as mitochondrial ribosomal proteins.

Recently, it was reported that the genes for ribosomal proteins L2, L14, S12 and S14 exist in the mitochondrial genome in *Paramecium* (Pritchard et al. 1990). However, they are not present there in animals and fungi (Anderson et al. 1981, 1982, Zamaroczy and Bernardi 1986, Dujon 1983). Some of these genes exist in the mitochondrial genome in plants (Ohyama et al. 1990). The *MRP2* gene which encodes a protein similar to S14 exists in the nucleus in *S. cerevisiae* (Myer et al. 1987). The fact that the genes for mitochondrial ribosomal proteins are located in different genomes in different organisms is interesting and perhaps it is important for the understanding of the mechanism for gene transfer from mitochondria to the nucleus.

5. Expression of mitochondrial ribosomal protein genes

To understand how the expression of mitochondrial ribosomal protein genes is regulated in the nucleus and mitochondria, the transcripts from some of the genes were examined in ρ^+ cells and ρ^0 cells. The results showed that all genes examined are transcribed in ρ^0 cells despite that rRNAs were not supplied in ρ^0 cells. In addition, the level of transcription was observed to be slightly different between ρ^+ and ρ^0 cells. Their response to different carbon sources in medium was also different. These suggest that the presence or absence of mitochondrial DNA influences the transcription of the genes in the nucleus. However, it is not known whether this phenomenon is caused by the expression of mitochondrial rRNA or the respiratory competence of mitochondria. Further examination in mit- strain or *PET* mutant strain is required to distinguish between the two possibilities.

Among the mitochondrial ribosomal protein genes so far characterized, it was reported that the expression of *MRP7* is regulated at the translational level while the expression of *MRP13* is regulated at the transcriptional level (Fearon and Mason 1988, Partaledis and Mason 1988). In these reports, it was also shown that the transcription of *MRP7* was hardly repressed by glucose while that of *MRP13* was repressed by glucose. The transcription of all genes examined in this work were repressed by glucose as in the case of *MRP13*. This may reflect a common mechanism of gene expression. However, it is necessary to investigate further the amount of proteins using antibodies against individual proteins in order to establish at which level the expression of these genes is mainly regulated.

6. Transport of ribosomal proteins into mitochondria

Most of proteins transported into mitochondria have a leader sequence in their N-terminus by which they are directed into there. Comparison of the N-

terminal amino acid sequences of mitochondrial ribosomal proteins in *S. cerevisiae* with their gene structures indicates that some of them contain signals for mitochondrial transport in their C-terminus. The results of deletion experiments in the C-terminus of YmL8 protein showed that, when the 3'-terminus of the *MRP-L8* gene was deleted so that the mutant YmL8 would lack ten amino acid residues in the C-terminus, such a gene in a single copy plasmid could not function. However, when it was placed in a high copy plasmid, it could function. Since it was confirmed that the gene with a 3'-terminus deletion in the single copy plasmid was transcribed at the same level as the wild type gene, the results of this deletion experiments could be explained as follows. One is that YmL8 proteins with deleted C-terminus cannot be transported into mitochondria. Another explanation is that such a protein is deficient in assembly into mitochondrial ribosomes. A third explanation is that it is transported and assembled but can not function in mitochondrial ribosomes. Of these, the first or second explanation seems more likely the case, since a high dosage of the gene could recover the function of the protein. To clarify whether the observed deficiency is due to its transport or not, it is required to further examine, for example, using antibodies against to YmL8. Such experiments are now in progress.

It is known that an ADP/ATP carrier protein of *S. cerevisiae* does not contain a leader sequence in the N-terminus but contains an import signal between amino acid residues 72 and 111 (Smagula and Douglas 1988). Recently, Söllner et al. (1990) reported that the ADP/ATP carrier protein was recognized by a receptor protein MOM72 which is different from the receptor (MOM19) for proteins containing pre-sequence in their N-terminus. Moreover, the ADP/ATP carrier protein was most likely not present in the putative prokaryotic ancestor of

mitochondria (Klingenberg 1985) but probably introduced after endosymbiosis event had taken place. Therefore, it is possible that mitochondrial ribosomal proteins without a presequence in their N-terminus may also have evolved from non-related protein and have acquired particular targeting sequences in their C-terminus.

It is known that the tripeptide Ser-Lys-Leu (or its conservative variants; Ser/Ala-Lys/Arg/His-Leu) in the C-terminus functions as a peroxisomal targeting signal (Gould et al. 1987, Miyazawa et al. 1989). In addition, it was reported that the C-terminal 12 amino acid residues of peroxisomal protein PMP20 in yeast could direct chloramphenicol acetyltransferase (CAT) into peroxisome in mammalian cells (Gould et al. 1990). These data suggest that a targeting signal into a certain organelle could be located in C-terminal position. To elucidate the role in mitochondrial transport of the amino acid sequence in the C-terminus of mitochondrial ribosomal proteins shown in Table 5, such fusion experiments remain to be carried out.

As described in the previous section, some of mitochondrial ribosomal proteins including the YMR31 protein contain relatively short leader sequences in their N-terminus. It is not known that these sequences indeed contain targeting information into mitochondria. Furthermore, these proteins also contain putative mitochondrial transport signals as shown in Fig. 23. Therefore, it is interesting from an evolutionary point of view of mitochondrial ribosomal protein genes to think the relationship between the length of the leader sequence in the N-terminus and the existence of targeting information into mitochondria in the C-terminal region.

In summary, I investigated the structure and expression of some mitochondrial ribosomal protein genes from an evolutionary point of view to

establish how their expression are regulated in the nucleus and mitochondria, and the results raised several features. The results obtained in this work will be useful for further understanding of mitochondrial evolution.

References

Altaba, A. R. and D. A. Melton. (1990) Axial patterning and the establishment of polarity in the frog embryo. Trends in Genetics 6: 57-64

Anderson, S., A. T. Bankier, B. G. Barrell, M. H. L. de Bruijn, A. R. Coulson, J. Drouin, I. C. Eperson, D. P. Nierlich, B. A. Roe, F. Sanger, P. H. Schreier, A. J. H. Smith, R. Staden, and I. G. Young. (1981) Sequence and organization of the human mitochondrial genome. Nature 290: 457-465

Anderson, S., M. H. L. De Bruijn, A. R. Coulson, I. C. Eperson, F. Sanger, and I. G. Young. (1982) Complete sequence of bovine mitochondrial DNA conserved features of the mammalian mitochondrial genome. J. Mol. Biol. 156: 683-717

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. (1987) Current protocols in molecular biology. Vol. II (chap. 13) Green Publishing Associates and Wiley-Interscience.

Baldauf, S. L., J. R. Manhart, and J. D. Palmer. (1990) Different fates of the chloroplast *tufA* gene following its transfer to the nucleus in green algae. Proc. Natl. Acad. Sci. USA 87:5317-5321

Baldauf, S. and J. D. Palmer. (1990) Evolutionary transfer of the chloroplast *tufA* gene to nucleus. Nature 344:262-265

Beauchamp, P. M., E. W. Horn, and S. R. Gross. (1977) Proposed involvement of an internal promoter in regulation and synthesis of mitochondrial and cytoplasmic leucyl-tRNA synthetases of *Neurospora*. Proc.

Natl. Acad. Sci. USA 74:1172-1176

Beltzer, J. P., L.-F. L. Chang, A. E. Hinkkanen, and G. B. Kohlhaw. (1986) Structure of yeast *LEU4* : The 5' flanking region contains features that predict two modes of control and two productive translation starts. J. Biol. Chem. 261: 5160-5167

Beltzer, J. P., S. R. Morris, and G. B. Kohlhaw. (1988) Yeast *LEU4* encodes mitochondrial and nonmitochondrial forms of α-isopropylmalate synthase. J. Biol. Chem. 263:368-374

Bibb, M. J., R. A. Van Etten, C. T. Wright, M. W. Walberg, and D. A. Clayton. (1981) Sequence and gene organization of mouse mitochondrial DNA. Cell 26: 167-180

Bielecki, J., P. Youngman, P. Connelly, and D. A. Portnoy. (1990) *Bacillus subtilis* expressing a haemolysis gene from *Listeria monocytogenes* can grow in mammalian cells. Nature 345:175-176

van den Broeck, G., M. P. Timko, A. P. Kausch, A. R. Cashmore, M. van Montagu, and L. H.-Estrella. (1985) Targeting of a foreign protein to chloroplasts by fusion to the transit peptide from the small subunit of ribulose 1,5-bisphosphate carboxylase. Nature 313:358-363

Brown, T. A., R. B. Waring, C. Scazzocchio, and R. W. Davies. (1985) The *Aspergillus nidulans* mitochondrial genome. Current Genetics 9: 113-117

Carle, G. F., M. Frank, and M. V. Olson. (1986) Electrophoretic separations of large DNA molecules by periodic inversion of the electric field. Science 232:65-68

Ceretti, D. P., D. Davis, G. R. Davis, D. M. Bedwell, and M. Nomura.

(1983) The *spc* ribosomal protein operon of *Escherichia coli:* sequence and cotranscription of the ribosomal protein genes and a protein export gene. Nucleic Acids Res. 11:2599-2616

Chatton, B., P. Walter, J.-P. Ebel, F. Lacroute, and F. Fasiolo. (1988) The yeast *VAS1* gene encodes both mitochondrial and cytoplasmic valyl-tRNA synthetases. J. Biol. Chem. 263:52-57

Clary, D. O. and D. R. Wolstenholme. (1985) The mitochondrial DNA molecule of *Drosophila yakuba* : nucleotide sequence, gene organization, and genetic code. J. Mol. Evol. 22: 252-271

Cooke, J. and J. C. Smith. (1990) Measurement of developmental time by cells of early embryos. Cell 60:891-894

Cummings, D. J., and J. M. Domenico. (1988) J. Mol. Biol. 204: 815-839

Dihanich, M., D. Najarian, R. Clark, E. C. Gillman, N. C. Martin, and A. K. Hopper. (1987) Isolation and characterization of *MOD5*, a gene required for isopentenylation of cytoplasmic and mitochondrial tRNAs of *Saccharomyces cerevisiae.* Mol. Cell. Biol. 7:177-184

Doonan, S., D. Barra, and F. Bossa. (1984) Structural and genetic relationships between cytosolic and mitochondrial isoenzymes. Int. J. Biochem.16:1193-1199

Dujon, B. (1983) In R. J. Schweyen et al. (eds.). Mitochondria 1983. Walter de Gruyter, Berlin, pp. 1-24

Ellis, S. R., A. K. Hooper, and N. C. Martin. (1987) Amino-terminal expression generated from an upstream AUG codon is not required for mitochondrial import of yeast N^2, N^2-dimetylguanosine-specific tRNA methyltransferase. Proc. Natl. Acad. Sci. USA 84:5172-5176

Faye, A. and F. Sor. (1977) Analysis of mitochondrial ribosomal proteins of *Saccharomyces cerevisiae* by two dimensional polyacrylamide gel electrophoresis. Mol. Gen. genet. 155:27-34

Fearon, K. and T. L. Mason. (1988) Structure and regulation of a nuclear gene in *Saccharomyces cerevisiae* that specifies MRP7, a protein of the large subunit of the mitochondrial ribosome. Mol. Cell. Biol. 8:3636-3647

Feinberg, A. P. and B. Vogelstein. (1983) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132:6-13

Flurkey, W., Y. S. Kim, and P. E. Kolattukudy. (1982) Precursor of a mitochondrial enzyme accumulates in the cytoplasm of preen glands for a specialized function. Biochem. Biophys. Res. Commun. 106:1346-1352

Gallwitz, D. and R. Seidel. (1980) Molecular cloning of the actin gene from yeast *Saccharomyces cerevisiae.* Nucleic Acids Res. 8:1043-1059

Geyl, D., A. Böck, and K. Isono. (1981) An improved method for two-dimensional gel electrophoresis: analysis of mutationally altered ribosomal proteins in *Escherichia coli.* Mol. Gen. Genet. 181:309-312

Gould, S. J., G. A. Keller, and S. Subramani. (1987) Identification of a peroxisomal targeting signal at the carboxy terminus of firefly luciferase. J. Cell Biol. 105:2923-2931

Gould, S. J., G. A. Keller, M. Schneider, S. H. Howell, L. J. Garrard, J. M. Goodman, B. Distel, H. Tabak, and S. Subramani. (1990) Peroxisomal protein import is conserved between yeast, plants, insects and mammals. EMBO. J. 9:85-90

Graack, H-R., L. Grohmann, and T.Choli. (1988) Mitochondrial ribosomes

of yeast: isolation of individual proteins and N-terminal sequencing. FEBS Lett. 242:4-8

Graack, H.-R. (1990) Charakterisierung kern-kodierter proteine der mitochondrialen ribosomen aus der hefe *Saccharomyces cerevisiae* und untersuchungen zur struktur und organisation ihrer gene. PhD thesis, Freien Universität Berlin

Gray, M.W. and W. F. Doolittle. (1982) Has the endosymbiont hypothesis been proven? Microbiol. Rev. 46:1-42

Gray, M. W., D. Sankoff, and R. J. Cedergren. (1984) On the evolutionary descent of organisms and organelles: a global phylogeny based on a highly conserved structural core in small subunit ribosomal RNA. Nucleic Acids Res. 12:5837-5852

Gray, M. W. (1989) The evolutionary origins of organelles. Trends in Genetics 5: 294-299

Grohmann, L., H-R. Graack, and M. Kitakawa. (1989) Molecular cloning of the nuclear gene for mitochondrial ribosomal protein YmL31 from *Saccharomyces cerevisiae*. Eur. J. Biochem. 183:155-160

Grohmann, L. (1989) Genetische und biochemische untersuchungen an ribosomalen proteinen aus mitochondrien der hefe *Saccharomyces cerevisiae*. PhD thesis, Freien Universität Berlin

Grunstein, M. and J. Wallis. (1979) Colony hybridization. Methods Enzymol. 68:379-389

Guérin, B., P. Labbe, and M. Somlo. (1979) Preparation of yeast (*Saccharomyces cerevisiae*) mitochondria with good P/O and respiratory control ratios. Methods Enzymol. 55:149-159

Guiard, B. (1985) Structure, expression and regulation of a nuclear gene encoding a mitochondrial protein: the yeast L(+)-lactate cytochrome c oxidoreductase (cytochrome b2). EMBO J. 4:3265-3272

Haffter, P., T. W. McMullin, and T. D. Fox. (1990) A genetic link between an mRNA-specific translational activator and the translation system in yeast mitochondria. Genetics 125:495-503

Hiratsuka, J., H. Shimada, R. Whittier, T. Ishibashi, M. Sakamoto, M. Mori, C. Kondo, Y. Honji, C. R. Sun, B. Y. Meng, Y. Q. Li, A. Kanno, Y. Nishizawa, A. Hirai, K. Shinozaki, M. Sugiura. (1989) The complete sequence of rice (*Oryza sativa*) chloroplast genome: intermolecular recombination between distinct tRNA genes accounts for a major plastid DNA inversion during the evolution of cereals. Mol. Gen. Genet. 217:185-194

Hoffman, C. S., and F. Winston. (1987) A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli.* Gene 57:267-2

Horwich, A. L., F. Kalousek, W. A. Fenton, R. A. Pollock, and L. E. Rosenberg. (1986) Targeting of pre-ornitine transcarbamylase to mitochondria: Definition of critical regions and residues in the leader peptide. Cell 44:451-459

Hudspeth, M. E. S., W. M. Ainley, D. S. Shumard, R. A. Butow, and L. I. Grossman. (1982) Location and structure of the *var1* gene on yeast mitochondrial DNA: Nucleotide sequence of the *40.0* allele. Cell 30:617-626

Hülskamp, M., C. Schröder, C. Pfeifle, H. Jäckle, and D. Tautz. (1989) Posterior segmentation of the *Drosophira* embryo in the absence of a maternal posterior organizer gene. Nature 338: 629-632

Hurt, E., B. Pesold-Hurt, G. Schatz. (1984) The cleavable prepiece of an imported mitochondrial protein is sufficient to direct cytosolic dehydrofolate reductase into the mitochondrial matrix. FEBS Lett. 178:306-310

Hurt, E., U. Muller, and G. Schatz. (1985) The first twelve amino acids of a yeast mitochondrial outer membrane protein can direct a nuclear encoded cytochrome oxidase subunit to the mitochondrial inner membrane. EMBO J. 4:3509-3518

Isono, K. (1984) A computer package for storing and retrieving DNA/RNA and protein sequence data. Nucleic Acids Res. 12:101-112

Ito, H., Y. Fukuda, K. Murata, and A. Kimura. (1983) Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. 153:163-168

Jacobs, H. T., D. J. Elliott, V. B. Math, and A. Farquharson. (1988) J. Mol. Biol. 202: 185-217

Kang, W. K., Y. Matsushita, and K. Isono. (1991) Cloning and analysis of *YMR26*, the nuclear gene for a mitochondrial ribosomal protein in *Saccharomyces cerevisiae*. Mol. Gen. Genet. (in press)

Kang, W. K., Y. Matsushita, L. Grohmann, H.-R. Graack, M. Kitakawa, and K. Isono. (1991) Cloning and analysis of the nuclear gene for YmL33, a protein of the large subunit of the mitochondrial ribosome in *Saccharomyces cerevisiae*. J. Bacteriol. (submitted)

Kang, W. K. (1991) On the mitochondrial ribosome synthesis and genetic interactions between the nucleus and mitochondria in *Saccharomyces cerevisiae*. PhD thesis, Kobe University

Kenyon, C. and A. Kamb. (1989) Cellular dialogs during development. Cell 58:607-608

Kimura, M. (1984) Proteins of the *Bacillus stearothermophilus* ribosome: the amino acid sequencing of proteins S5 and L30. J. Biol. Chem. 259:1051-1055

Kitakawa, M., L. Grohmann, H-R. Graack, and K. Isono. (1990) Cloning and characterization of nuclear genes for two mitochondrial ribosomal proteins in *Saccharomyces cerevisiae*. Nucleic Acids Res. 18:1521-1529

Klingenberg, M. (1985) Principles of carrier catalysis elucidated by comparing two similar membrane translocators from mitochondria, the ADP/ATP carrier and the uncoupling protein. Ann. NY Acad. Sci. 456:279-288

Kreader, C. A., C. S. Langer, and J. E. Heckman. (1988) A mitochondrial protein from *Neurospora crassa* detected both on ribosomes and membrane fractions; Analysis of the gene, the message, and the protein. J. Biol. Chem. 264:317-3

Kuiper, M. T. R., M. Holtrop, and H. de Vries. (1988) Integration and expression of 3'-truncated derivatives of the *Neurospora crassa cyt-21+* gene, encoding a mitochondrial ribosomal protein, in *Neurospora* transformants. Mol. Gen. Genet. 213:519-528

Laemmli, U. K. (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227:680-685

Lambowitz, A. M. (1979) Preparation and analysis of mitochondrial ribosomes. Methods Enzymol. 59:421-433

van Loon, A. P. G. M., A W. Brändli, and G. Schatz. (1986) The presequences of two imported mitochondrial proteins contain information for intracellular and intramitochondrial sorting. Cell 44:801-812

Maniatis, T., E. F. Fritsh, and J. Sambrook. (1982) Molecular cloning : a

laboratory manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Manseau, L. J. and T. Schüpbach. (1990) The egg came first, of course ! ; Anterior-posterior pattern formation in *Drosophila* embryogenesis and oogenesis. Trends in Genetics 5: 400-405

McMullin, T. W., P. Haffter, and T. D. Fox. (1990) A novel small-subunit ribosomal protein of yeast mitochondria that interacts functionally with an mRNA-specific translational activator. Mol. Cell. Biol. 10:4590-4595

Miyazawa, S., T. Osumi, T. Hashimoto, K. Ohno, S. Miura, and Y. Fujiki. (1989) Peroxisome targeting signal of rat liver acyl-coenzyme A oxidase resides at the carboxy terminus. Mol. Cell. Biol. 9:83-91

Myers, A. M., M. D. Crivellone, and A. Tzagoloff. (1987) Assembly of the mitochondrial membrane system : *MRP1* and *MRP2* , two yeast nuclear genes coding for mitochondrial ribosomal proteins. J. Biol. Chem. 262:3388-3397

Najarian, D., M. E. Dihanich, C. N. Martin, and A. K. Hopper. (1987) DNA sequence and transcript mappimg of *MOD5* : Features of the 5' region which suggest two translational starts. Mol. Cell. Biol. 7:185-191

Natsoulis, G., F. Hilger, and G. R. Fink. (1986) The *HTS1* gene encodes both the cytoplasmic and mitochondrial histidine tRNA synthetase of *S. cerevisiae.* Cell 46:235-241

Niederacher, D., and K. Entian. (1987) Isolation and characterization of the regulatory *HEX2* gene necessary for glucose repression in yeast. Mol. Gen. Genet. 206:505-509

Ohyama, K., H. Fukuzawa, T. Kohchi, H. Shirai, T. Sano, S. Sano, K. Umesono, Y. Shiki, M. Takeuchi, Z. Chang, S. Aota, H. Inokuchi, and H. Ozeki. (1986) Chloroplast gene organization deduced from

complete sequence of liverwort *Marchantia polymorpha* chloroplast DNA. Nature 322:572-574

Ohyama, K., Y. Ogura, K. Oda, K. Daiwa, K. Kanegae, H. Ohta, Y. Nakamura, and M. Takemura. (1990) Structural analysis of mitochondrial genome in liverwort *Marchantia polymorpha*. Congress of molecular biology, Kyoto in Japan (1990).

Partaledis, J. A., and T. L. Mason. (1988) Structure and regulation of a nuclear gene in *Saccharomyces cerevisiae* that specifies MRP13, a protein of the small subunit of the mitochondrial ribosome. Mol. Cell. Biol. 8:3647-3660

Pfanner, N. and W. Neupert. (1990) The mitochondrial protein import apparatus. Annu. Rev. Biochem. 59:331-353

Pritchard, A. E., C. L. Sable, S. E. Venti, and D. J. Cummings. (1990) Analysis of NADH dehydrogenase proteins, ATPase subunit 9, cytochrome b, and ribosomal protein L14 encoded in the mitochondrial DNA of *Paramecium.* Nucleic Acids Res. 18: 173-180

Pritchard, A. E., J. J. Seilhamer, R. Mahalingam, C. L. Sable, S. E. Venuti, and D. J. Cummings. (1990) Nucleotide sequence of the mitochondrial genome of *Paramecium.* Nucleic Acids Res. 18: 173-180

Roe, B. A., M. Din-Pow, R. K. Wilson, and J. Wong. (1985) The complete nucleotide sequence of the *Xenopus laevis* mitochondrial genome. J. Biol. Chem. 260: 9759-9774

Roth, S., D. Stein, and C. N. Volhard. (1989) A gradient of nuclear localization of the *dorsal* protein determines dorsoventral pattern in the Drosophila embryo. Cell 59: 1189-1202

Sambrook, J., E. F. Fritsh, and T. Maniatis. (1989) Molecular cloning : a

laboratory manual, Second Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Sanger, F., S. Nicklen, and A. R. Coulson. (1977) DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463-5467

Schatz, G. (1967) Stable phosphorylating submitochondrial particles from baker' yeast. Methods Enzymol. 10:197-202

Sherman, F., G. R. Fink, and J. B. Hicks. (1983) Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Shinozaki, K., M. Ohme, M. Tanaka, T. Wakasugi, N. Hayashida, T. Matsubayashi. N. Zaita, J. Chunwongse, J. Obokata, K. S. Yamaguchi, C. Ohto, K.Torazawa, B.Y. Meng, J. Kusuda, F. Takaiwa, A. Kato, N. Tohdoh, H. Shimada, and M. Sugiura (1986) The complete nucleotide sequence of the tobacco chloroplast genome: its gene organization and expression. EMBO J. 5:2043-2049

Simpson, L., N. Neckelman, V. F. de la Cruz, A. M. Simpson, J. E. Feagin, D. P. Jasmer, and K. Stuart. (1987) Comparison of the maxicircle (mitochondrial) genomes of *Leishmania tarentolae* and *Trypanosoma brucei* at the level of nucleotide sequence. J. Biol. Chem. 262: 6182-6196

Smagula, C. S. and M. G. Douglas. (1988) ADP-ATP carrier of *Saccharomyces cerevisiae* contains a mitochondrial import signal between amino acids 72 and 111. J. Cellular Biochem. 36:323-327

Söllner, T., G. Griffiths, G. Pfaller, N. Pfanner, and W. Neupert. (1989) MOM19, an import receptor for mitochondrial precursor proteins. Cell 59:1061-1070

Söllner, T., G. Pfaller, G. Griffiths, N. Pfanner, and W. Neupert. (1990) A mitochondrial import receptor for the ADP/ATP carrier. Cell 62:107-115

Southern, E. M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517

Sprague, G. F. Jr., R. Jensen, and I. Herskowitz. (1983) Control of yeast cell type by the mating type locus: positive regulation of the a-specific *STE3* gene by *MATa1* product. Cell 32:409-415

Strub, K. and P. Walter. (1989) Isolation of a cDNA clone of the 14-kDa subunit of the signal recognition particle by cross-hybridization of differently primed polymerase chain reactions. Proc. Natl. Acad. Sci. USA 86:9747-9751

Takeda, M., A. Vassarotti, and M. G. Douglas. (1985) Nuclear genes coding the yeast mitochondrial adenosine triphosphatase complex: primary sequence analysis of ATP2 encoding the F_1-ATPase β-subunit precursor. J Biol. Chem. 260:15458-15465

Teem, J. L., N. Abovich, N. F. Kaufer, W. F. Schwidinger, J. R. Warner, A. Levy, J. Woolford, R. J. Leer, M. M. C. van Ramsdonk-Duin, W. H. Mager, R. J. Planta, L. Schultz, J. D. Friesen, H. Fried, and M. Rosbash. (1984) A comparison of yeast ribosomal protein gene DNA sequence. Nucleic Acids Res. 12:8295-8312

Thorsness, P. E. and T. D. Fox. (1990) Escape of DNA from mitochondria to the nucleus in *Saccharomyces cerevisiae*. Nature 346:376-379

Tzagoloff, A., G. Macino, and W. Sebald. (1979) Mitochondrial genes and translation products. Ann. Rev. Biochem. 48:419-441

Tzagoloff, A., and A. M. Myers. (1986) Genetics of mitochondrial biogenesis. Ann. Rev. Biochem. 55:249-285

Warner, J. R. (1989) Synthesis of ribosomes in *Saccharomyces cerevisiae.* Microbiol. Rev. 53:256-271

Wood, W. I., J. Gitschier, L. A. Lasky, and R. M. Lawn. (1985) Base composition-independent hybridization in tetramethyl ammonium chloride: a method for oligonucleotide screening of highly complex gene libraries. Proc. Natl. Acad. Sci. USA 82:1585-1588

Woodland, H. R. (1989) Mesoderm formation in Xenopus. Cell 59:767-770

de Zamaroczy, M. and G. Bernardi. (1986) The primary structure of the mitochondrial genome of *Saccharomyces cerevisiae.* Gene 47: 155-177

Wu, R. S., J. D. Stedman, M. H. P. West, P. Pantazis, and W. M. Bonner. (1982) Discontinuous agarose electrophoresis system for the recovery of stained proteins from polyacrylamide gels. Anal. Biochem. 124:264-271

Wu, M. and A. Tzagoloff. (1987) Mitochondrial and cytoplasmic fumarases in *Saccharomyces cerevisiae* are encoded by a single nuclear gene *FUM1.* J. Biol. Chem. 262:12275-12282

Yoshikawa, A. and K. Isono. (1990) Chromosome III of *Saccharomyces cerevisiae:* an ordered clone bank, a detailed restriction map and analysis of transcripts suggest the presence of 160 genes. Yeast 6:383-401

de Zamaroczy, M. and G. Bernardi. (1986) The primary structure of the mitochondrial genome of *Saccharomyces cerevisiae.* Gene 47:155-177

Zinn, B., D. S. Kerr, and C. L. Hoppel. (1986) Fumarase deficiency: A new cause of mitochondrial encephalomyopathy. The New England Journal of Medicine 315:469-475