

Cloning and characterization of nuclear genes for two mitochondrial ribosomal proteins in *Saccharomyces cerevisiae*

Madoka Kitakawa*, Lutz Grohmann[1], Hanns-Rüdiger Graack[1] and Katsumi Isono
Department of Biology, Faculty of Science, Kobe University, Rokkodai, Nada-ku, Kobe 657, Japan and [1]Max-Planck-Institut für Molekulare Genetik, Abteilung Wittmann, Ihnestrasse 73, D-1000 Berlin 33, FRG

Received November 28, 1989; Revised and Accepted January 29, 1990

ABSTRACT

The genes for two large subunit proteins, YmL8 and YmL20, of the mitochondrial ribosome of *Saccharomyces cerevisiae* were cloned by hybridization with synthetic oligonucleotide mixtures corresponding to their N-terminal amino acid sequences. They were termed *MRP-L8* and *MRP-L20*, respectively, and their nucleotide sequences were determined using a DNA sequencer. The *MRP-L8* gene was found to encode a 26.8-kDa protein whose deduced amino acid sequence has a high degree of similarity to ribosomal protein L17 of *Escherichia coli*. The gene *MRP-L20* was found to encode a 22.3-kDa protein with a presequence consisting of 18 amino acid residues. By Southern blot hybridization to the yeast chromosomes separated by field-inversion gel electrophoresis, the *MRP-L8* and *MRP-L20* genes were located on chromosomes X and XI, respectively. Gene disruption experiments indicate that their products, YmL8 and YmL20 proteins, are essential for the mitochondrial function and the absence of these proteins causes instability of the mitochondrial DNA.

INTRODUCTION

The ribosome in mitochondria of *S. cerevisiae* consists of two subunits which contain two species of RNA encoded in the mitochondrial DNA (1) and about 70 different proteins (r-proteins) (2,3) all except one of which are nuclear coded (1). The mitochondrial ribosomes are more closely related to eubacterial than to cytoplasmic ribosomes (4). Of the seven nuclear genes for the mitochondrial r-proteins of *S. cerevisiae* that had been cloned and characterized at the nucleotide sequence level (5−9), two were found to have a high degree of sequence similarity to bacterial r-proteins. Similarly, two nuclear genes for mitochondrial r-proteins In *Neurospora crassa* showed sequence similarity to *E. coli* r-proteins (10, 11).

Several lines of evidence suggest that the mitochondrion is a descendant of an endosymbiotic procaryote (12, 13). The cases mentioned above appear to be in favor of this idea. However, if this endosymbiont hypothesis is correct, then most of the genes for mitochondrial r-proteins must have moved from the mitochondrial genome to the nucleus during the course of mitochondrial evolution. It is interesting to note in this respect that the genes for some of the r-proteins which show sequence similarity to bacterial r-proteins have been found in the mitochondrial genome in plants (14−17) the mitochondrial genomes of which are much larger than those in fungi and animal cells (18). One problem that emerges with this hypothesis is, how the genes that were translocated from the mitochondrial genome to the nucleus have become able to correctly direct their protein products synthesized in the cytoplasm into mitochondria. It has been shown that the presequence of a protein plays a role in defining its location within the cell. However, it is not readily clear how the mitochondrial r-proteins have acquired their presequences. Furthermore, at least one mitochondrial r-protein was found to lack such a presequence (9). Another problem is that, in contrast to the cases described above, at least five mitochondrial r-proteins of *S. cerevisiae* so far characterized show no sequence similarity to any known bacterial r-proteins (5, 7−9). It could be that they were of nuclear origin and were adapted to mitochondrial ribosomes during the course of mitochondrial evolution. More systematic studies at the molecular level of the genes for mitochondrial r-proteins and their products will provide further insights in the evolution of mitochondria.

MATERIALS AND METHODS

Yeast strains and media

Strains DC5 (**a**; *leu2−3, leu2−112, his3, can1*) and YNN27[*rho°*] (α; *trp1, ura3*, [*rho°*]) are generous gift from A. Toh-e and N. Gunke, respectively. Strain 07173 was previously described (3). Cells were grown in YPD medium which contains 2% peptone, 1% yeast extract and 2% glucose. The respiration ability was examined on YPG agar plates (2% peptone, 1% yeast

* To whom correspondence should be addressed

extract, 3% glycerol, 2% bacto agar). Prototrophs were selected on minimal medium SD plates (0.67% yeast nitrogen base without amino acids (Difco), 2% glucose, 2% bacto agar).

Cloning and nucleotide sequencing of ribosomal protein genes

Cloning of the mitochondrial ribosomal protein genes were performed essentially as described previously (8). The total chromosomal DNA was digested with *Eco* RI and *Pst* I and separated by agarose gel electrophoresis. The DNA of the size hybridizing to the oligonucleotide mixture was eluted by the method of Tautz and Renz (19) and ligated with pUC118 vector (20). Positive transformants of DH5α (F⁻ *endA1, hsdR17*(r_k^-, m_k^+), *supE44, thi-1, recA1, gyrA, relA1*, φ80*lacZ*ΔM15, Δ(*lacZYA-argF*)$_{U169}$) were selected by colony-hybridization with oligonucleotide mixtures.

Nucleotide sequencing was performed using a DNA sequencer, GENESIS 2000 of DuPont, with the operating programm version 2.2. The sequence reaction was carried out following the procedure provided by DuPont using either Sequenase (USB) or T7 DNA polymerase (Pharmacia). The plasmid DNA was denatured and annealed with the primer according to Chen and Seeburg (21). When an oligonucleotide mixture was used as a primer, it was used at a concentration 50−100 times that of the standard primer. Subcloning and isolation of single-stranded DNA for sequencing was performed using strain XL-1 blue (F′ *proAB, lacI*^{q}ZΔM15, Tn10; *endA1, hsdR17, supE44, thi-1*, λ-, *recA1, gyrA96, relA*, Δ(lac)) and helper phage KO7 (20). Deletions were obtained either by the method of Henikoff (22) or by the method of Burton et al. (23) with several modifications. To create various lengths of partial polymerization products in the latter method, we adopted unidirectional polymerase chain reaction (PCR) in a 50 μl reaction mixture which contained 4 μg of single stranded DNA, 50 pmol of M13 universal primer, 1.25 mM dNTP, 1× Taq polymerase buffer (24) and 2.5 U Taq polymerase (Cetus). The reaction was repeated 30 times under the conditions of denaturation at 90°C for 1 min, annealing and polymerization at 42°C for 3 min. The reaction mixture was extracted once with chloroform and the DNA was sedimented with ethanol, dissolved in 50 μl S1 buffer (25) and treated with 80 U of S1 nuclease at 37°C for 30 min. After phenol/chloroform extraction and ethanol precipitation, the DNA was treated with T4 polymerase to make the ends blunt, purified through a Sephadex G50 spin column and then digested with restriction enzyme *Hin*d III. Resultant DNA fragments were size fractionated and purified by agarose gel electrophoresis, ligated with pUC119 vector DNA and used to transform XL-1 blue cells to ampicillin resistance.

Alignment and analysis of the sequencing data and comparison of the translated protein sequences with the compiled sequence data of ribosomal proteins were performed using computer programs DATBAS, NUCDAT and AACOMP (26).

Field-inversion gel electrophoresis of yeast chromosomes

The samples were embedded in agarose according to Carle and Olson (27) and electrophoresis was performed essentially as described (28) on 1% agarose in 1×TBE buffer. The duration of the electrophoresis for the forward direction was increased linearly from 9 to 60 seconds and that for the reverse direction from 3 to 20 seconds during a run for 20 hr at 170 V constant.

Gene disruption analysis

Derivatives of plasmids pL8EP and pL20EP carrying disrupted ribosomal protein genes were constructed as follows. The yeast vector YEp13 (29) was digested with *Sal* I and *Xho* I and a 2.2 kb fragment containing the *LEU2* gene was purified. The ends of the fragment were filled in with T4 polymerase and ligated with plasmid pL8EP or pL20EP which was digested with *Hpa* I and dephosphorylated by calf intestine alkaline phosphatase (Boehringer). They were then used to transform *E. coli* HB101 (F⁻; *proA, leuB, thr, lacY, rpsL, hsdR, hsdM, endI, recA*) cells to ampicillin resistance and Leu⁺, making use of the fact that the yeast *LEU2* gene can complement the *E. coli leuB* mutation (30, 31). Plasmids were then isolated from resultant transformants, linearized and then introduced into yeast cells by the lithium-acetate method (32) with a slight modification. Detection of the disrupted genes by Southern hybridization (33) was performed using a nonradioactive DNA labelling and detection kit, Geneus™ (Boehringer). The labeling of purified fragment by random primed incorporation of digoxigenin-labeled dUTP and subsequent procedures were performed as recommended by the supplier.

Miscellaneous methods

The purification and amino acid sequence of ribosomal proteins have been described (3). The standard procedures for transformation of *E. coli* cells, plasmid preparation and *in vitro* DNA manipulation (25) were used. For rapid plasmid preparation the CTAB method (34) was also used. The DNA for Southern hybridization analysis was prepared essentially by the method of Niederacher et al. (35). Oligonucleotides were synthesized using a DNA synthesizer from Applied Biosystems, Model 381A, and purified by reverse phase HPLC.

RESULTS

Cloning of the genes for YmL8 and YmL20

The mitochondrial ribosomal proteins YmL8 and YmL20 (3) were isolated from the purified mitochondria of *S. cerevisiae* strain 07173 and their N-terminal amino acid sequences were determined. As shown in Fig.1, 17- or 20-mer oligonucleotide mixtures were synthesized based on the resultant amino acid sequence data and used as probes to clone the nuclear genes encoding these proteins. From the Southern hybridization analyses of total chromosomal DNA digested with various restriction enzymes, it was found that a 2.3 kb *Eco* RI-*Pst* I fragment hybridized with oligonucleotide mixtures L8−1 and L8−2. Similarly, a 1.8 kb *Eco* RI-*Pst* I fragment was found to hybridize with oligonucleotide mixtures L20−1 and L20−3. Therefore, these fragments were cloned into plasmid vector pUC118 and analyzed. Two clones which gave strong hybridization signals to the oligonucleotide probes were obtained and termed pL8EP and pL20EP, respectively. We performed DNA sequencing of these recombinant plasmids using the oligonucleotide mixture L8−1 or L20−3 as the primer in the sequencing reactions and found that the nucleotide sequences matched with the amino acid sequences shown in Fig. 1. Thus we concluded that the two plasmid contained the genes for proteins YmL8 and YmL20 and termed the genes *MRP-L8* and *MRP-L20*, respectively. To define the coding regions, we performed restriction enzyme mapping and Southern hybridization analysis. A 0.5 kb *Hpa* I-*Acc* I fragment and a 0.3 kb *Pst* I-*Hpa* I fragment shown in Figs. 2A and 2B were found to hybridize with oligonucleotides L8−1 and L20−3, respectively.

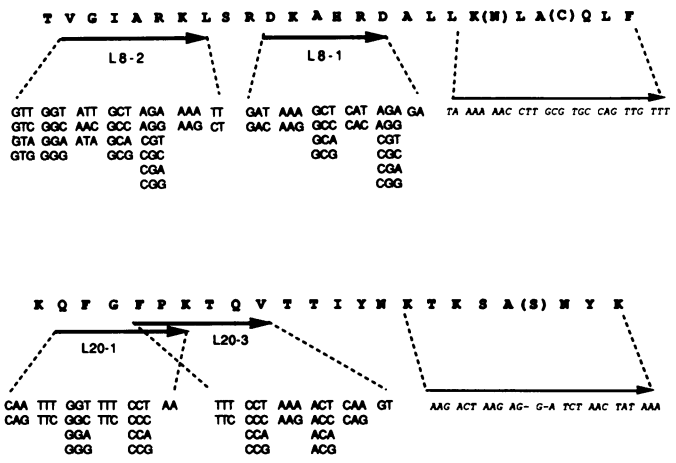

Figure 1. The N-terminal amino acid sequences of proteins YmL8 (**A**) and YmL20 (**B**). Amino acid residues in parentheses are slightly ambiguous. Thick arrows indicate the regions of the amino acid sequences chosen for the synthesis of oligonucleotide mixtures the sequences of which are shown underneath in the 5′ to 3′ direction. Thin arrows show the nucleotide sequences determined using the oligonucleotide mixtures as primers (see text).

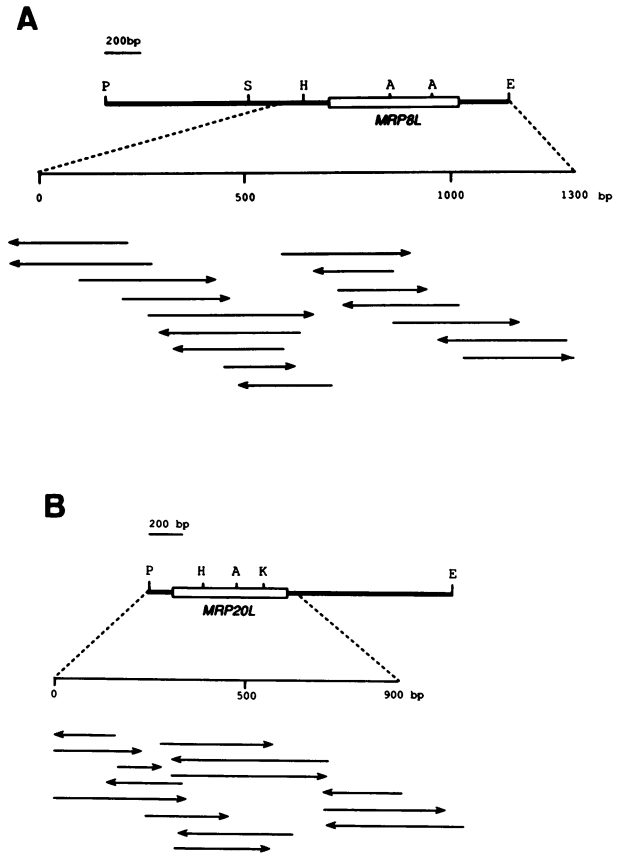

Figure 2. Restriction map and sequencing strategy for *MRP-L8* (**A**) and for *MRP-L20* (**B**) and their flanking regions. Abbreviations for restriction enzyme cleavage sites are: A, *Acc* I; E, *Eco*RI; H, *Hpa* I; K, *Kpn* I; P,*Pst* I; and S, *Sac* I.

Nucleotide sequence of the gene *MRP-L8*

The nucleotide sequence of the 1.2 kb *Hpa* I-*Eco* RI fragment and its upstream region were determined by the dideoxy chain termination method (36) as described in Materials and Methods. As illustrated in Fig.2A, the *Hpa* I-*Eco* RI fragment was sequenced in both directions to various degrees and the sequence thus determined is shown in Fig. 3. An open reading frame (ORF) capable of encoding a protein with 238 amino acid residues was found. The protein encoded by this ORF is quite basic (a net charge of +14) and has a calculated molecular weight of 26,822 daltons. This value is consistent with the value, 28-kDa, for protein YmL8 which was estimated by SDS polyacrylamide gel electrophoresis. Comparison of the nucleotide sequence of the gene and the N-terminal amino acid sequence of the protein indicates that protein YmL8 contains no presequence. The sequence surrounding the predicted initiator codon shows the presence of an A at position −3 and another A at position +4, a feature suggested to be favorable for efficient initiation of translation (37). The TATA sequences are found at 67 and 166 bp upstream of the translation initiation site and the sequences proposed to be important for the efficient termination of transcription in yeast, TAG . . . TAGT . . . TTT (38), are present downstream of the coding region.

The amino acid sequence of the protein encoded by the gene was then compared with *E. coli* and yeast cytoplasmic ribosomal proteins. The computer search revealed that two stretches in the N-terminal half of this protein had a high degree of similarity to the *E. coli* r-protein L17 (EL17) (39), although this protein is much smaller than protein YmL8. As shown in Fig. 4, 36% of the amino acid residues are identical in a stretch from positions 5 to 70 and 53% in another stretch from positions 89 to 128. The sequence between the two stretches is 12 amino acids longer and not similar to the corresponding sequence of EL17. Interestingly, comparison between EL17 and its *Bacillus stearothermophilus* homologue, BL17 (40), shows also a break in their sequence similarity in this region. Thirty eight and 59% of amino acid residues in protein BL17 are identical with YmL8 in the first and second aligned regions. In addition, a short stretch in the C-terminal region of protein YmL8 starting from position 173 was found to have a significant degree of similarity with a region of *E. coli* S13 (41) (Fig. 4).

Nucleotide sequence of the gene *MRP-L20*

According to the scheme shown in Fig. 2B, a 900 bp nucleotide sequence of clone pL20EP was determined. The sequence is shown in Fig. 5. A single ORF capable of coding for a protein with a calculated molecular weight of 22,219 and a net charge of +20 was found. The comparison of the amino acid sequence of this protein with the N-terminal sequence of purified protein YmL20 suggested the existence of an 18 amino acid residue long presequence. Therefore, the calculated molecular weight of the mature protein, 20,626 dalton, is in good agreement with the value of 19-kDa for YmL20 determined from SDS gel electrophoresis. That the predicted presequence has a positive net charge and contains two serines and no acidic amino acids is consistent with the feature proposed for leader sequences of mitochondrial matrix targeting proteins (42, 43). The initiator codon AUG of this ORF is also preceded by an A at position −3 and succeeded by another A at position 4 as in the case of the *MRP-L8* gene. However, neither a typical TATA sequence nor a transcriptional termination sequence that was found in *MRP-L8* was present in the upstream and downstream regions so far

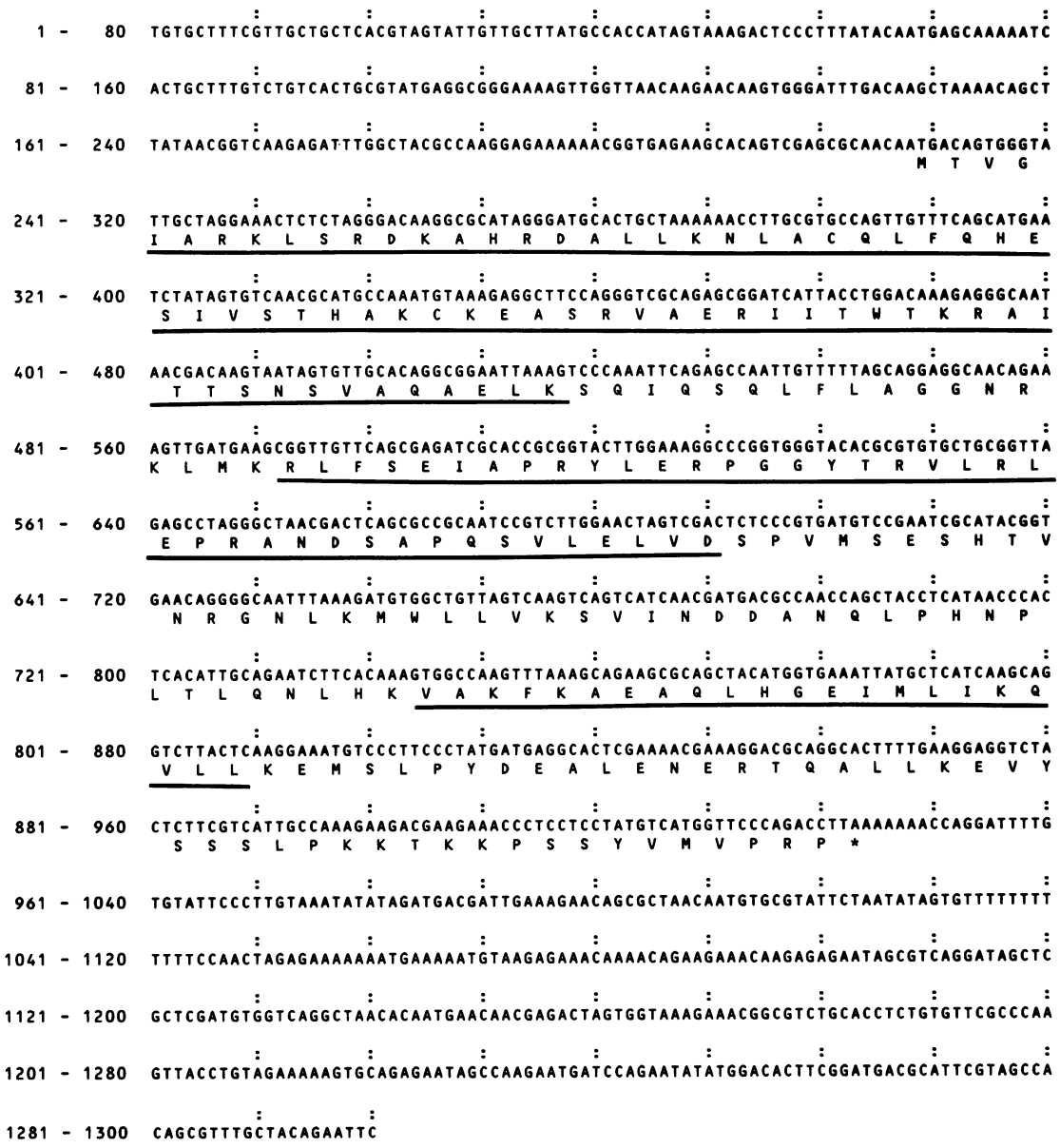

Figure 3. Nucleotide sequence of the *MRP-L8* gene and its flanking regions. The open reading frame was translated into single letter amino acid codes and the regions showing a high degree of similarity to the ribosomal proteins of *E. coli* and *B stearothermophilus* are underlined (for detail, see Fig. 4).

sequenced. It may be that the TATA sequence for *MRP-L20* is located farther away from the coding region as in the case of some other mitochondrial ribosomal protein genes (5–7,9) unlike other yeast genes in which a TATA sequence occurs 40 to 120 bp upstream of the transcriptional initiation site (44). Additional experiments are necessary to clarify these points.

Codon usage and chromosomal locations of *MRP-L8* and *MRP-L20*

The codon usage patterns in the MRP-*L8* and *MRP-L20* genes were examined (data not shown). As in other mitochondrial ribosomal protein genes so far analyzed, no biased codon usage towards a particular subset of codons which are recognized by most abundant cognate tRNAs in the cell (45, 46) was observed. This implies that the expression of the genes for mitochondrial ribosomal protein is not high (47). One notable observation is that among mitochondrial r-protein genes so far analyzed, UGA is preferred as a stop codon: six out of 9 genes (67%) including the *MRP-L20* use this codon whereas its occurrence in the nuclear genes for other mitochondrial proteins is 16 out of 44 examined (36%) (8).

To locate the *MRP-L8* and *MRP-L20* genes on the yeast chromosome, the cloned fragments containing the genes were used as probes in Southern hybridization experiments with the chromosomes separated by field inversion gel electrophoresis. As shown in Fig. 6, the 2.3 kb *Eco* RI-*Pst* I fragment containing the *MRP-L8* gene was found to hybridize with chromosome X. Similarly, the *MRP-L20* gene in the 1.8 kb *Eco* RI-*Pst* I fragment was located on chromosome XI.

Gene disruption analysis of *MRP-L8* and *MRP-L20*

To examine whether ribosomal proteins YmL8 and YmL20 are

Figure 4. Similarity between the protein encoded by the *MRP-L8* gene and ribosomal proteins EL17 (39) and ES13 (41) from *E. coli* and BL17 (40) from *B. stearothermophilus*. The amino acid residues identical with YmL8 are boxed and similar amino acid residues are marked with colons. Dashes indicate gaps introduced to maximize the matching.

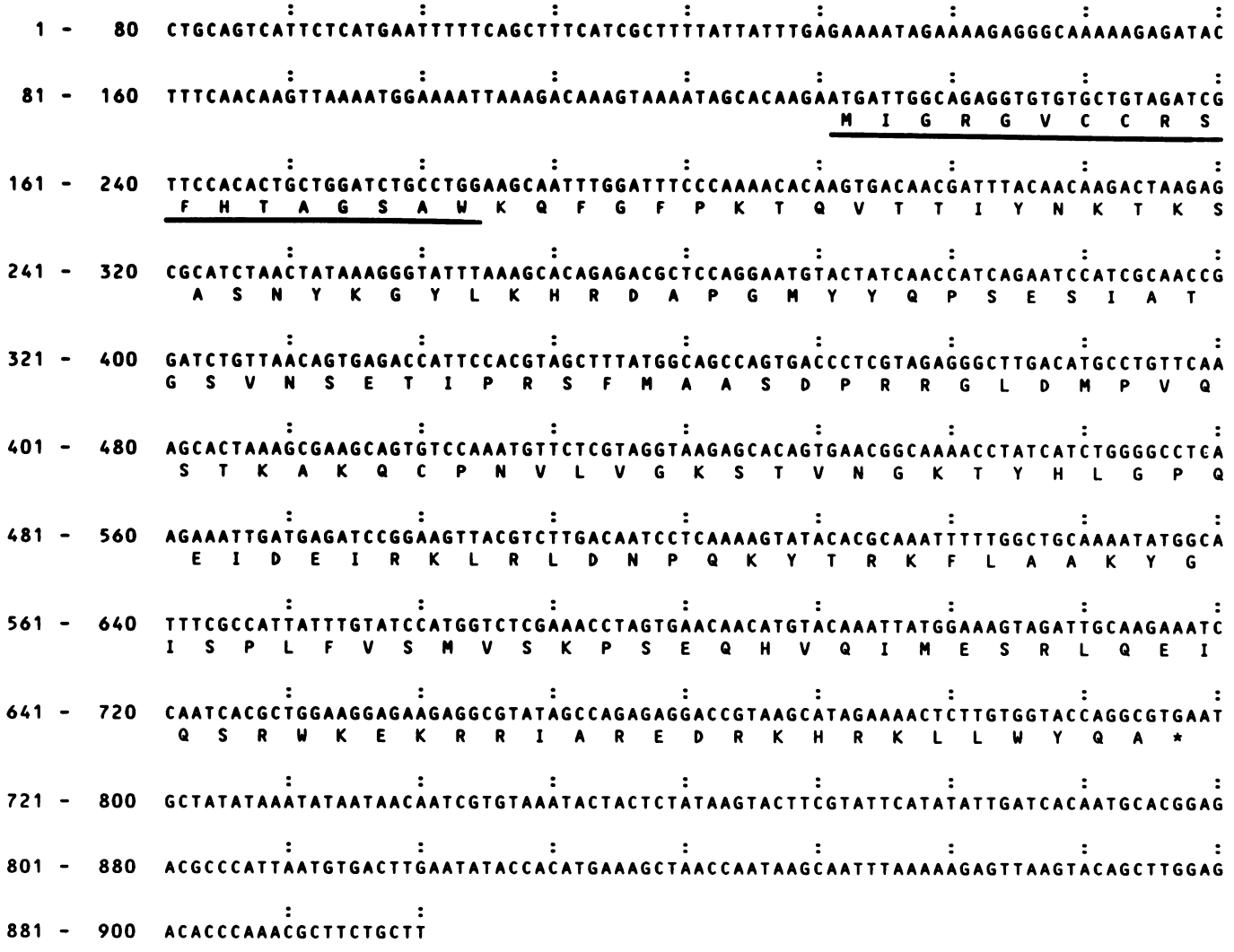

Figure 5. Nucleotide sequence of the *MRP-L20* gene and its flanking regions. The open reading frame was translated into single letter amino acid codes. A likely presequence of the gene product, YmL20, is underlined.

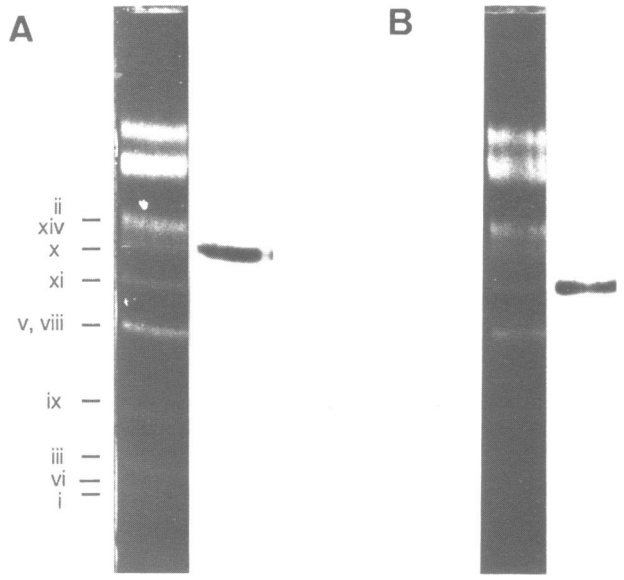

Figure 6. Chromosomal locations of the *MRP-L8* and *MRP-L20* genes. The chromosomes of strain DC5 [rho°] was separated by field inversion gel electrophoresis, transferred onto nitrocellulose membrane and hybridized with a fragment containing *MRP-L8* (**A**) or *MRP-L20* (**B**). The left hand side panels show the stained gel strips and the right hand side panels show the autoradiograms after Southern hybridization. The chromosome numbers were assigned according to Carle and Olson (28).

essential for the mitochondrial function in yeast cells, strains with null mutations for these ribosomal proteins were constructed and analyzed. As shown in Fig. 7, derivatives of plasmids pL8EP and pL20EP were first constructed by inserting *LEU2* as a selecting marker and thereby disrupting the genes on them and termed pL8EP-L and pL20EP-L, respectively. The *Hpa* I site of pL8EP is located outside of the coding region, but within the region necessary for its expression (see below). A haploid strain DC5 *leu2* was then transformed with the plasmid derivatives thus constructed and LEU$^+$ transformants were selected. Among 120 transformants obtained with pL8EP-L, 112 were found to be respiration deficient (RD), judging from their poor growth on fermentable carbons and inability to grow on non-fermentable carbons. Similarly, when pL20EP-L DNA was used 26 out of 30 LEU$^+$ transformants showed an RD phenotype. These results suggest that proteins YmL8 and YmL20 are essential for the mitochondrial function. To confirm that their genes, *MRP-L8* and the *MRP-L20*, in the RD transformants are indeed disrupted, the genomic DNA of some of these transformants were analyzed by Southern hybridization using the *Eco* RI-*Pst* I fragment of pL8EP and the *Kpn* I-*Pst* I fragment of pL20EP as probes. All three LEU$^+$ RD transformants obtained with plasmid pL8EP-L showed the restriction pattern expected for the interrupted *MRP-L8* gene as depicted in Fig7A and 7C. In the case of the transformants obtained with pL20EP-L, however, only one out of four transformants (Fig.7D, lane1 and 2) showed an expected restriction pattern for the disrupted gene. The others (Fig.7D, lane3 and 4) showed a restriction pattern indistinguishable from that for the undisrupted *MRP-L20* gene. A possible explanation for the latter results may be that the length of the 5′ sequence upstream of the inserted *Sal* I-*Xho* I fragment is too short and in most of the transformants homologous recombination was not completed in the initial stage of transformation. However, partial loss of the mitochondrial genome occurred during the initial stage and a stable RD phenotype resulted. In the subsequent stages, homologous recombination at the *MRP-L20* gene was aborted, while LEU$^+$ phenotype was established by the homologous recombination of *LEU2* gene with its mutant allele. An evidence supporting this explanation is the observation of one transformant which initially showed a clear RD phenotype but later reverted to respiration proficient. Its *MRP-L20* gene was indistinguishable from the wild type allele as judged by restriction map analysis of the chromosome. Perhaps, the postulated partial loss of the mitochondrial genome did not occur in this clone, and hence it was able to revert to respiration proficient. This phenomenon might be due to the nature of the fragment containing the *LEU2* gene, because difficulties to obtain transformants carrying a disrupted gene were encountered in other cases as well, when *LEU2* was used as a selection marker for the gene disruption (A.Toh-e, personal communication). It could be that the difficulties with *LEU2* were caused by a portion of a Ty element located near it (our unpublished results). To examine whether the RD transformants obtained still contained functional mitochondrial genome, we performed complementation analysis between them and strain YNN27 [rho°]. Two RD transformants with pL8EP-L and three with pL20EP-L (two showing an apparently undisrupted *MRP-L20* and one showing clearly disrupted *MRP-L20*) were examined. However, all failed to complement the RD phenotype of YNN27 [rho°]. These results indicate that deficiencies in the *MRP-L8* and *MRP-L20* genes cause an instability of the mitochondrial genome as postulated above and as reported previously for the genes responsible for mitochondrial protein synthesis (48).

DISCUSSION

In this work two nuclear genes, *MRP-L8* and *MRP-L20*, for mitochondrial ribosomal proteins YmL8 and YmL20, respectively, were cloned and their nucleotide sequences determined. The screening procedure for recombinant plasmids containing these mitochondrial ribosomal protein genes was based on the hybridization with the oligonucleotides corresponding to the N-terminal amino acid sequences of the proteins. This approach requires rather time-consuming steps of purification and partial amino acid sequence of individual proteins, but it is certainly a more generally applicable method for the cloning of the ribosomal protein genes than other methods such as isolation and use of nuclear *PET* mutants. Thus, the genes for eleven mitochondrial ribosomal proteins have so far been cloned by this method (8, 9; our unpublished results). An advantage of this approach is that it is possible to deduce the presence, if any, of a presequence in the protein in question by comparing the nucleotide sequence of a gene and the amino acid sequence of its product. As described above, protein YmL20 was found to possess a presequence of 18 amino acid residues, while protein YmL8 contained no such presequence. Table 1 summarizes the putative presequences of mitochondrial ribosomal proteins in yeast so far reported. They have properties proposed for the presequence of mitochondrial matrix targeted proteins; a net positive charge, a relatively high content of hydroxylated amino acids, serine and threonine, and no or very few acidic amino acids (43). However, their length and the amino acid sequences at the cleavage sites on them are variable and no apparent consensus can be drawn. Our preliminary data shows that one of the mitochondrial r-protein has two different N-terminal

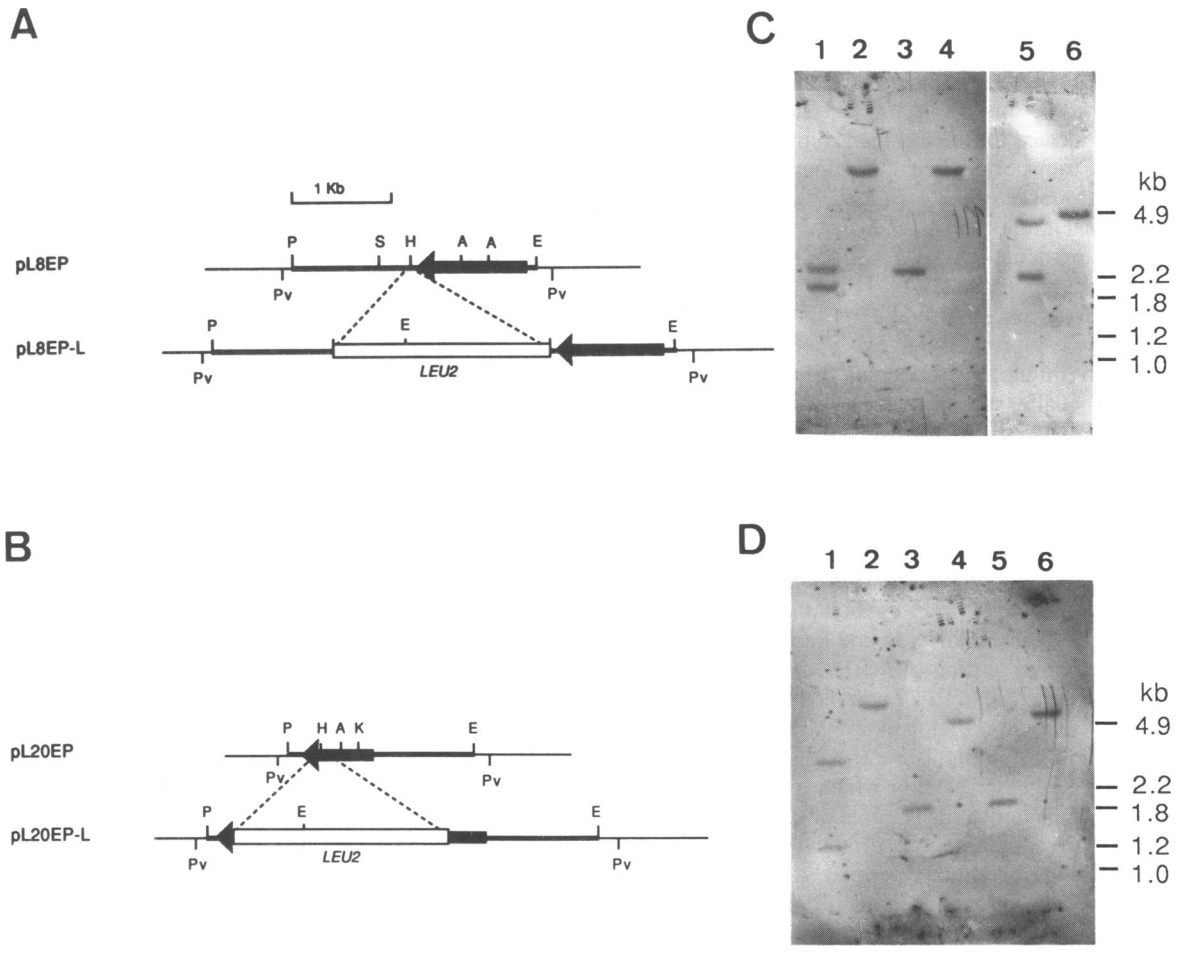

Figure 7. Gene disruption analysis of *MRP-L8* and *MRP-L20*. Plasmids pL8EP (**A**) and pL20EP (**B**) were disrupted at the *Hpa* I sites by the insertion of a 2.2 kb fragment containing the *LEU2* gene which contained an *Eco*RI site. DC5 cells were transformed with the *Pst* I-*Pvu* II fragments of the resultant plasmids. Arrows indicate the genes (ORF) with their initiating codons situated at arrowheads. **C:** The chromosomal DNAs of a LEU$^+$ RD transformant containing plasmid pL8EP-L and of its parent DC5 were completely digested with *Eco*RI and *Pst* I (lanes 1 and 3), *Hin*dIII (lanes 2 and 4), or *Eco*RI (lanes 5 and 6), respectively, and analyzed by Southern hybridization with the 2.3 kb EcoRI-*Pst* I fragment of pL8EP as a probe as described in Materials and Methods. **D** The DNAs of LEU$^+$ RD transformants (lanes 1−4) and DC5 (lanes 5 and 6) were analyzed as in **C** with 0.7 kb *Pst* I-*Kpn* I fragment of pL20EP. The DNAs were digested with *Eco*RI and *Pst* I (lanes 1, 3 and 5) or *Hin*dIII (lanes 2, 4 and 6). The *Hin*dIII fragments which contain undisrupted *MRP-L8* and *MRP-L20* are 10 and 5 kb, respectively.

Table 1. Presequences of mitochondrial ribosomal proteins [a].

Protein	Presequence | Sequence [b]	Reference
MRP2 [c]	(M) GNFRFPIKTKLPPG | FINARILRDN	5
MRP7	(M) WNPILLDTSSFSFQKHVSGVPLQVRN | ATKRAAGSRT	6, Our data [d]
YmL31	(M) FGPFKLTSPVA | GGLLWKIPWR	8
YMR31	(M) IATPIRL | AKSAYEPMIK	9
YMR44	(M) | ITKYFSKVIV	9
YmL8	(M) | TVGIARKLSR	This work
YmL20	(M) IGRGVCCRSFHTAGSAW | KQFGFPKTQV	This work

[a] The presequences and the N-terminal ten amino acid residues of mature ribosomal proteins so far examined are listed in single letter codes.

[b] Vertical bars (\|) indicate the cleavage sites.

[c] The presequence of this protein was deduced from the comparison to the homologous proteins in *E. coli* and chloroplast.

[d] The existence of a presequence in in this protein was confirmed by our N-terminal amino acid sequence data (unpublished) of a purified protein identical to it.

sequences, suggesting the existence of two alternative cleavage sites or the occurrence of partial cleavage. Moreover, since several of the mitochondrial ribosomal proteins in *S. cerevisiae* lack a presequence (9, this work and our unpublished data), search must be performed for the mechanism by which they, too, can be effectively transported into mitochondria.

The nucleotide sequence of the gene *MRP-L8* revealed that the predicted N-terminal amino acid sequence of its product, protein YmL8, is highly related to *E. coli* r-protein L17 and its *B. stearothermophilus* homologue BL17. However, protein YmL8 is twice as large as its bacterial counterparts. In *S. cerevisiae* several multifunctional proteins have been found and it was postulated that they might have arisen as the results of fusions of the genes for smaller proteins (49). In this respect it is interesting to note that a short stretch in the C-terminal region of YmL8 has a sequence similarity to protein ES13 of *E. coli*. The gene for ES13 is located in the same operon as the gene for EL17 (50). Recently, it was reported that one of the mitochondrial r-proteins in *S. cerevisiae*, MRP7, has a significant degree of sequence similarity to an *E. coli* r-protein, EL27, in its N-terminal region. Protein MRP7 is about three times as large as EL27 and no sequence similarity to any known r-protein was found for the C- terminal portion of MRP7. It appears as if the coding region of the gene for an EL27 homologue was transferred from the original mitochondrial genome to the nucleus and thereby disrupted an existing nuclear gene, thus forming a new fused gene. There is another case in which a mitochondrial r-protein of *S. cerevisiae* shows a high degree of sequence similarity to an *E. coli* r-protein; namely, protein MRP2 of yeast mitochondrial ribosome is highly similar to *E. coli* ES14. Interestingly, the size of the two proteins is comparable to each other except for the putative presequence of MRP2 (5). Comparative analysis of the mitochondrial ribosomal proteins of *S. cerevisiae* and those of other organisms will provide clues with respect to the origin and evolution of mitochondrial proteins.

The alignment of the amino acid sequences of YmL8 and the above mentioned bacterial homologues shows a break in the sequence similarity (Fig. 4). This might imply that these proteins contain two functional domains. *E. coli* protein EL17 is an RNA binding protein and has been shown to be important for the assembly of large subunit of *E. coli* ribosome (51), although further details of its function is not known. It will be interesting to construct mutant strains with various alterations in YmL8 and examine their effects on the structure and function of ribosomes. In this connection it should be noted that the disruption of the genes for YmL8 and YmL20 caused a lesion in the mitochondrial DNA. A similar observation has been reported for other proteins which participate in the protein synthesis in mitochondria (48). Therefore, to investigate the effects of ribosomal protein alterations on the function of mitochondrial ribosomes, it seems necessary to establish a system in which such alterations do not result in lesions in the mitochondrial DNA. This approach will require the knowledge as to how the DNA replication and the ribosomal functions are linked in yeast mitochondria.

The ribosomes of yeast mitochondria apparently contain more proteins than those of bacteria. Some of them are most likely homologous to bacterial r-proteins, while some others are not. The function of proteins of the latter category as well as their possible origins are difficult to assign. In *E. coli*, several proteins have been shown to be non-essential at least for cellular viability (52). A question is, then, how many r-proteins are non-essential for the protein synthesis and why they exist there. Among the eight mitochondrial r-proteins in *S. cerevisiae* so far examined, two proteins, MRP13 (7) and YMR31 (our unpublished data) appeared not to be essential for the mitochondrial function, whereas the rest of proteins including YmL8 and YmL20 described here are essential. It will be interesting to establish the minimum number of r-proteins that are necessary for the protein synthesis in yeast mitochondria and then to examine whether the addition of non-essential proteins can enhance the fidelity of translation, the stability of the resultant ribosome, and so on. With *S. cerevisiae*, it is easier to investigate these points compared to *E. coli*, since it is relatively easy to replace the wild type gene on the chromosome with a plasmid-borne mutant allele constructed *in vitro*. Furthermore, we can exploit the fact that the mitochondria in yeast are not essential for growth under certain conditions.

In bacteria, most of the r-proteins genes are clustered at a few loci on the chromosome (50). In contrast, the nuclear genes for mitochondrial r-proteins of *S. cerevisiae* so far reported are located on different chromosomes (5–9). As described above, we located the genes for YmL8 and YmL20 on chromosomes X and XI, respectively. The gene for protein YmL31, *MRP-L31*, has been located also on chromosome XI (8). We have no evidence at the moment with respect to the proximity of *MRP-L20* and *MRP-L31* on this chromosome. However, there could be a chromosome(s) in *S. cerevisiae* on which many mitochondrial r-protein genes are located and some of them are close to each other (our unpublished data and personal communication from Abraham, P. R.). This point is currently investigated further.

In this work, the nucleotide sequences of the *MRP-L8* and *MRP-L20* genes were determined using a DNA sequencer from DuPont. When the cost spent for the sequencing were taken into consideration, the use of a DNA sequencer at present cannot be regarded as a better alternative to manual sequencing. However, it has several advantages over conventional manual sequencing; (a) we can avoid hazardous radioisotopes, (b) the reaction procedure is simpler, (c) sequence ambiguities caused by non-specific 'stops' during the polymerase reaction can be alleviated, because the newly synthesized DNA fragments are labelled only at their 3'-termini by dideoxynucleotides, and (d) the data created are in a computer-readable form and can be analyzed directly with various computer programs. To confirm the data obtained using the DNA sequencer, we determined some part of the nucleotide sequence by the conventional manual method as well. with the sequencer the purity of sample DNA was critical, because bases were identified by calculating the ratios of fluorescence measured at two wave lengths. When the purity of DNA was high enough, then the accuracy of base assignment by the sequencer with additional editings was comparable to the manual sequencing. In some instances a stretch containing several consecutive Gs or Cs was more correctly determined by the sequencer than the manual sequencing, presumably because of the presence of rather large chromophores on dideoxy-nucleotides which alleviated electrophoretic compression.

ACKNOWLEDGEMENT

We are grateful to H. G. Wittmann for support, to T. Choli for help in performing amino acid sequencing, to A. Yoshikawa for carrying out hybridization analysis of the cloned genes with FIGE-separated yeast chromosomes. This work was partly supported

by the grant for scientific research, Nos. 62480465 and 01780299 from the Ministry of Education, Culture and Science of Japan.

REFERENCES

1. Dujon, B. (1981) In Strathern, J. N., Jones, E. W. and Broach, J. R. (eds) The molecular biology of the yeast *Saccharomyces* : Life cycle and inheritance, Cold Spring Harbor Labolatory, Cold Spring Harbor, NY, pp 505–635.
2. Faye, G. and Sor, F. (1977) Mol. Gen. Genet. **155**, 27–34.
3. Graack, H-R., Grohmann, L. and Choli, T. (1988) FEBS Lett. **242**, 4–8.
4. Borst, P. and Grivell, L. A. (1971) FEBS Lett. **13**, 73–88.
5. Myers, A.M., Crivellone, M.D. and Tzagoloff, A. (1987) J. Biol. Chem. **262**, 3388–3397
6. Fearon, K. and Mason, T.L. (1988) Mol. Cell. Biol. **8**, 3636–3646.
7. Partaledis, J. A. and Mason, T. L. (1988) Mol. Cell. Biol. **8**, 3647–3660.
8. Grohmann, L., Graack, H-R. and Kitakawa, M. (1989) Eur. J. Biochem. **183**, 155–160.
9. Matsushita, Y., Kitakawa, M. and Isono, K. (1989) Mol. Gen. Genet. **219**, 119–124.
10. Kuiper, M. T. R., Akins, R. A., Holtrop, M., de Vries, H. and Lambowitz, A. M. (1988) J. Biol. Chem. **263**, 2840–2847.
11. Kreader, C. A., Langer, C. S. and Heckman, J. E. (1989) J. Biol. Chem. **264**, 317–327.
12. Schwartz, R. M. and Dayhoff, M. O. (1978) Science **199**, 395–403.
13. Gray, M. W. and Doolittle, W. F. (1982) Microbiol. Rev. **46**, 1–42.
14. Bland, M. M., Levings, III, C. S. and Matzinger, D. F. (1986) Mol. Gen. Genet. **204**, 8–16.
15. Bonen, L. (1987) Nucl. Acids Res. **15**, 10393–10404.
16. Wahleithner, J. A. and Wolstenholme, D. R. (1988) Nucl. Acids Res. **16**, 6897–6913.
17. Gualberto, J. M., Wintz, H., Weil, J-H. and Grienenberger, J-M. (1988) Mol. Gen. Genet. **215**, 118–127.
18. Newton, K. J. (1988) Ann. Rev. Plant Physiol. Plant Mol. Biol. **39**, 503–532.
19. Tautz, D. and Renz, M. (1983) Anal. Biochem. 132, 14–19.
20. Vieira, J. and Messing, J. (1987) Meth. Enzymol. **153**, 3–11.
21. Chen, E. Y. and Seeburg, P. H. (1985) DNA **4**, 165–170.
22. Henikoff, S. (1984) Gene 28, 351–359.
23. Burton, F. H., Loeb, D. D., McGraw, R. A., Edgell, M. H. and Hutchison III, C. A. (1988) Gene **67**,159–168.
24. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. and Erlich, H. A. (1988) Science **239**, 487–491.
25. Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
26. Isono, K. (1984) Nucl. Acids Res. **12**, 101–112.
27. Carle, G. F. and Olson, M. V. (1985) Proc. Natl. Acad. Sci. USA **82**, 3756–3760.
28. Carle, G. F., Frank, M. and Olson, M V. (1986) Sience **232**, 65–68.
29. Broach, J. R., Strathern, J. N. and Hicks, J. B. (1979) Gene **8**, 121–133.
30. Ratzkin, B. and Carbon, J. (1977) Proc. Natl. Acad. Sci. USA **74**, 487–491.
31. Hicks, J. and Fink, G. R. (1977) Nature **269**, 265–267.
32. Ito, H., Fukuda, Y., Murata, K. and Kimura, A. (1983) J. Bacteriol. **153**, 163–168.
33. Southern, E. M. (1975) J. Mol. Biol. **98**, 503–517.
34. Del Sal, G., Manfioletti, G. and Schneider, C. (1988) Nucl. Acids Res. **16**, 9878.
35. Niederacher, D. and Entian, K. D. (1987) Mol. Gen. Genet. **206**, 505–509.
36. Sanger, F., Nicklen, S. and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. USA **74**, 5463–5467.
37. Kozak, M. (1981) Nucl. Acids Res. **9**, 5233–5252.
38. Zaret, K. S. and Sherman, F. (1982) Cell **28**, 563–573.
39. Rombauts, W., Feytons, V. and Wittmann-Liebold, B. (1982) FEBS Lett. **149**, 320–327.
40. Kimura, M. and Chow, C. K. (1984) Eur. J. Biochem. **139**, 225–234.
41. Lindemann, H. and Wittmann-Liebold, B. (1976) FEBS Lett. **71**, 251–255.
42. Douglas, M. G., McCammon, M. T. and Vassarotti, A. (1986) Microbiol. Rev. **50**, 166–178.
43. Roise, D. and Schatz, G. (1988) J. Biol. Chem. **263**, 4509–4511.
44. Struhl, K. (1987) Cell **49**, 295–297.
45. Bennetzen, J. L. and Hall, B. D. (1982) J. Biol. Chem. **257**, 3026–3031.
46. Sharp, P. M., Tuohy, T. M. F. and Mosurski, R. (1986) Nucl. Acids Res. **14**, 5125–5143.
47. Sharp, P. M., Cowe, E., Higgins, D. G., Shields, D. C., Wolfe, K. H. and Wright, F. (1988) Nucl. Acids Res. **16**, 8207–8211.
48. Myers, A. M., Pape, L. K. and Tzagoloff, A. (1985) EMBO J. **4**, 2087–2092.
49. Jones, E. W. and Fink, G. R. (1982) In Strathern, J. N., Jones, E. W. and Broach, J. R. (eds),The molecular biology of the yeast *Saccharomyces* : Metabolism and gene expression, Cold Spring Harbor Labolatory, Cold Spring Harbor, NY, pp181–299.
50. Nomura, M. and Post, L. E. (1980) In Chambliss, G., Craven, G. R., Davies, J., Davis, K., Kahan, L. and Nomura, M. (eds), Ribosomes, University Park Press, Baltimore, pp. 671–691.
51. Röhl, R. and Nierhaus, K. H. (1982) Proc. Natl. Acad. Sci. USA **79**, 729–733.
52. Dabbs, E. R. (1986) In Hardesty, B. and Kramer, G. (eds) Structure, Function and Genetics of Ribosomes, Springer-Verlag, New York, pp. 733–749.