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Cloning and Analysis of the Nuclear Gene for YmL33, a Protein of the Large Subunit of the Mitochondrial Ribosome in *Saccharomyces cerevisiae*

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The N-terminal amino acid sequence of a large subunit protein, termed YmL33, of the mitochondrial ribosome of the yeast *Saccharomyces cerevisiae* was determined. The data were obtained to synthesize two kinds of oligonucleotide primers, which were used in the polymerase chain reaction to amplify and clone the nuclear gene for this protein. By nucleotide sequencing, the cloned gene, *MRP-L33*, was found to encode a basic protein of 11 kDa with 98 amino acid residues. The protein encoded by this gene appears to have no leader sequence at its N terminus. The N-terminal two-thirds of the deduced amino acid sequence showed a significant degree of sequence similarity to ribosomal protein L30 of *Escherichia coli* and *Bacillus stearothermophilus*. In addition, the C-terminal one-third showed sequence similarity, though to a lesser extent, to a yeast cytoplasmic ribosomal protein termed L16. By hybridization with the yeast chromosomes and their restriction enzyme fragments, the *MRP-L33* gene was concluded to exist on chromosome XIII as a single-copy gene. Disruption of the gene by insertion of a *HIS3*-containing fragment showed that *MRP-L33* was essential for mitochondrial function. The transcriptional level of *MRP-L33* in strains with different mitochondrial genetic backgrounds was analyzed in the presence of glucose, galactose, or glycerol.

The mitochondrial ribosome of *Saccharomyces cerevisiae* consists of two RNA molecules and as many as about 90 proteins (8a). The two RNAs are encoded in the mitochondrial DNA, while all except one of the protein components are synthesized from nuclear genes (34). The biogenesis of the mitochondrial ribosome, therefore, depends on the activity of two physically separated sets of genes, one in the nucleus and the other in the mitochondrion itself. Evidence has accumulated that mitochondria have descended from prokaryotic symbionts. A notable example in support of this notion is that the secondary structure of the mitochondrial rRNAs from several organisms resembles that of *Escherichia coli* rRNA (33). Therefore, it will be interesting to investigate how the cooperative interactions between the two sets of genes have been achieved during the course of mitochondrial evolution.

Recently, several nuclear genes for mitochondrial ribosomal proteins have been cloned and characterized in several organisms. It has been shown that some of the mitochondrial ribosomal proteins show significant sequence similarity to *E. coli* ribosomal proteins. For example, mitochondrial ribosomal proteins MRP2, MRP7 and YmL8 of *S. cerevisiae* are considerably similar to *E. coli* ribosomal protein S14, L27, and L17, respectively (6, 18, 22). On the other hand, other yeast mitochondrial ribosomal proteins that have been characterized show no such sequence similarity (10, 16a, 21, 24). These data suggest that there may be three classes of mitochondrial ribosomal proteins. One consists of those derived from the original prokaryote, the putative mitochondrial ancestor. The proteins of this class still contain several conserved sequences due most likely to some functional constraints. Another class of proteins consists of those which are derived from the original prokaryote but have undergone extensive amino acid changes during the evolution of mitochondria so that their sequence similarity to *E. coli* ribosomal proteins can no longer be traced. A third class of proteins comprises those derived from the host eukaryote which were originally unrelated to mitochondrial ribosomes. At present, however, it is not possible to distinguish between the second and third classes of mitochondrial proteins.

In addition to these structural features, the expression of yeast mitochondrial protein genes has been characterized as well. For example, the transcription of mitochondrial ribosomal protein genes was shown to be affected by catabolites such as glucose (16a, 22, 24). The level of transcription in cells growing in medium with nonfermentable carbon sources is significantly higher than that in cells growing with a fermentable carbon source such as glucose. Moreover, it was found that such catabolite effects were observed only in [*rho*$^+$] strains (16a, 24).

To investigate mitochondrial ribosomal proteins and their genes from an evolutionary point of view and to establish how the genes are organized and regulated in the nucleus, we have undertaken cloning and molecular characterization of the nuclear genes for mitochondrial ribosomal proteins of *S. cerevisiae*. For this purpose, we purified most of the large-subunit proteins of the mitochondrial ribosomes from cells of strain 07173 and several small-subunit proteins from commercial yeast cells, determined their N-terminal amino acid sequences, and used the data for cloning of the corresponding genes. In this report, we describe the isolation and molecular characterization of *MRP-L33*, the gene for a

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11-kDa protein, termed YmL33, of the large subunit of the yeast mitochondrial ribosome. The cloning was performed after amplification of a DNA fragment by polymerase chain reaction (PCR). The data demonstrate that the N-terminal region of the protein encoded by this gene shows a high degree of sequence similarity to *E. coli* ribosomal protein L30.

MATERIALS AND METHODS

Strains and media. The yeast strains used were DC-5 (**a** *leu2-3,112 his3 can1* [*rho*$^+$]), DC-5 [*rho*^{0}] (a [*rho*^{0}] derivative of DC-5), DC-5-Δ*MRP-L33* (a derivative of DC-5 in which *MRP-L33* is disrupted), AB972 (α *trp1-O* [*rho*^{0}]; from A. M. Trayler), and 07173 (**a**/α, wild type) (9). The culture media used were YPD (1% yeast extract, 2% peptone, 2% glucose), YPG (1% yeast extract, 2% peptone, 3% glycerol), and SD (0.67% yeast nitrogen base without amino acid, to which 2% glucose and required amino acids and bases were added). All media were solidified, when necessary, with 2% agar (30).

Purification and amino acid sequencing of YmL33. The isolation and N-terminal sequencing of mitochondrial ribosomal protein YmL33 from *S. cerevisiae* 07173 were performed as described previously (9).

PCR and cloning of the product. Oligonucleotide mixtures (17-mers) were synthesized on a model 381A DNA synthesizer (Applied Biosystems). PCR was performed as described by Saiki et al. (25) in a Thermal Cycler (Perkin-Elmer Cetus). The reaction mixture (total of 50 μl) contained 5 nmol of each of the oligonucleotide mixtures shown in Fig. 1, 1 U of *Taq* DNA polymerase (Takara Shuzo), and 300 ng of the genomic DNA prepared from strain 07173. It was subjected to 20 cycles of 1 min at 94°C, 2 min at 43°C, and 1.5 min at 72°C, 20 cycles of 1 min at 94°C, 2 min at 38°C, and 1.5 min at 72°C, and 20 cycles of 1 min at 94°C, 2 min at 33°C, and 1.5 min at 72°C. The product was then purified by agarose gel electrophoresis, treated with T4 polymerase and T4 kinase (Takara Shuzo), and ligated into the *Sma*I site of pUC118. The vector DNA was treated with calf intestine phosphatase (Sigma) before ligation.

Procedures for screening of clones. The genomic DNA was prepared as described by Niederacher and Entian (23), and rapid isolation of plasmid DNA was carried out according to Hoffman and Winston (14). Digested DNA was separated by agarose gel electrophoresis and transferred to a nitrocellulose filter as described by Southern (31). DNA fragments used as hybridization probes were recovered by electroelution after electrophoresis in an agarose gel and radioactively labeled by the random primer method (7). For screening of colonies by hybridization, colonies were transferred to a nitrocellulose filter and treated by the method of Grunstein and Wallis (11).

Construction of deletion derivatives and nucleotide sequencing. To introduce deletions into the cloned segment of plasmid pL33, an enzyme kit containing exonuclease III, mung bean nuclease, *E. coli* DNA polymerase Klenow fragment, and T4 DNA ligase (Takara Shuzo) was used according to the procedure of Henikoff (13). Nucleotide sequencing was performed according to Sanger et al. (27), using T7 DNA polymerase (Pharmacia). The sequence data obtained were stored and analyzed by using the computer programs DATBAS, NUCDAT, and AACOMP (15). The National Biomedical Research Foundation (NBRF) protein data base (release 25.0) was used to search for sequence similarity.

Miscellaneous methods. Matings were carried out according to Sherman et al. (30). Transformation of yeast cells was performed by the lithium acetate method (16), with minor modifications: we added 15% glycerol to 0.1 M lithium acetate solution in Tris-EDTA (pH 8.0) for treating yeast cells. Pulse-field gel electrophoresis of chromosomal DNA was performed essentially as described by Carle et al. (2), using a CHEF-DRII electrophoresis system (Bio-Rad). Other routine methods for the manipulation of plasmids and *E. coli* were as described by Sambrook et al. (26).

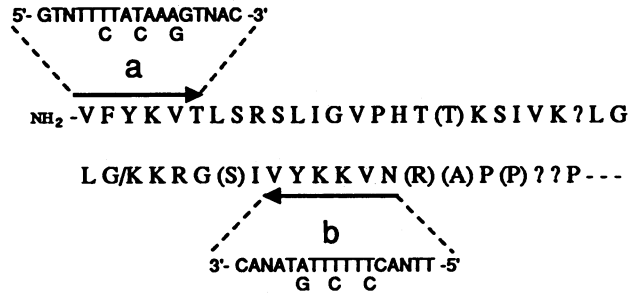

FIG. 1. N-terminal amino acid sequence (single-letter code) of mitochondrial ribosomal protein YmL33. Amino acid residues in parentheses are slightly ambiguous. Question marks indicate that the residues at the corresponding positions were undeterminable. Two stretches of the sequence indicated by horizontal arrows were chosen, and oligonucleotide mixtures a and b were synthesized as indicated.

Nucleotide sequence accession number. The sequence data reported here have been assigned GenBank accession no. D90217.

RESULTS

Cloning of the gene for YmL33. Protein YmL33 was purified along with other mitochondrial ribosomal proteins from cells of *S. cerevisiae* 07173 (9). Its molecular mass was determined to be 11 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). The amino acid sequence of the purified protein was determined for the N-terminal 43 residues (Fig. 1). Two stretches of the sequence data shown in Fig. 1 were chosen for the synthesis of oligonucleotide mixtures. The oligonucleotide mixtures were used as primers in PCR to amplify the region of the yeast genomic DNA flanked by them. Two bands, approximately 110 and 40 bp in length, were detected upon electrophoresis of the PCR products. The size of the 110-bp-long band coincided well with the expected size (114 bp) deduced from the amino acid sequence data. Therefore, this PCR product was cloned into the *Sma*I site of pUC118. The amino acid sequence deduced from its nucleotide sequence matched perfectly the N-terminal amino acid sequence of protein YmL33. Therefore, we concluded that the PCR product was derived from the gene for this protein. Accordingly, the cloned segment was isolated by digestion with *Eco*RI and *Bam*HI, labeled, and used as a probe for the identification of the cloned nuclear gene for YmL33.

When the genomic DNA of strain 07173 was digested with various restriction enzymes, subjected to electrophoresis, and hybridized with the above-mentioned probe, a single band was observed in each lane (data not shown). A parallel experiment performed with the genomic DNA of strain DC-5 [*rho*^{0}] showed that the two strains were indistinguishable from each other. Digestion with *Hin*dIII was found to yield a hybridizing fragment of 1.9 kb in length, which was the

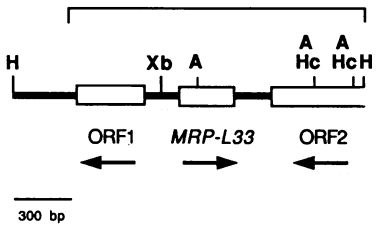

FIG. 2. Restriction map of plasmid pL33. Open boxes represent the coding regions of the *MRP-L33* gene and the two other open reading frames (ORF1 and ORF2) found. Arrows indicate the direction of transcription. The sequenced region shown in Fig. 3 is bracketed. Abbreviations for restriction enzyme sites: A, *Acc*I, H, *Hin*dIII; Hc, *Hin*cII; Xb, *Xba*I. No sites were found for *Bam*HI, *Cla*I, *Eco*RI, *Kpn*I, *Pst*I, *Pvu*II, *Sac*I, and *Sma*I.

shortest among all restriction enzyme fragments analyzed. Therefore, *Hin*dIII fragments of approximately 1.8 to 2.0 kb in length were prepared from the genomic DNA of strain DC-5 [*rho*^{0}], ligated with pUC119, and used to transform *E. coli* XL-1 to Amp^r. Two positive clones were obtained by colony hybridization of the resultant transformants. Double-strand nucleotide sequencing of these clones using oligonucleotide mixture **a** (Fig. 1) as a sequencing primer showed that the sequence corresponded to an amino acid sequence which completely agreed with the N-terminal amino acid sequence of protein YmL33 shown in Fig. 1. Therefore, we concluded that the two clones contained the gene for YmL33 and named the gene *MRP-L33*. One of them was designated pL33 and analyzed further.

Nucleotide sequence of the *MRP-L33* gene. A detailed restriction map analysis was performed with pL33 (Fig. 2). The nucleotide sequence of the cloned segment in pL33 was accordingly determined (Fig. 3). We found an open reading frame capable of encoding a basic protein (net charge of +10) harboring 99 amino acid residues and a calculated molecular weight of 11,012. The amino acid sequence deduced from the nucleotide sequence of this open reading frame agreed with that of protein YmL33 except for the N-terminal Met. The open reading frame is preceded by the sequence TATATC, a possible candidate for a TATA box, which is situated 72 bases upstream of the translational initiator codon (Fig. 3). Furthermore, the open reading frame is followed by the sequence TAG...TATGT...TTT (underlined in Fig. 3), similar to the one proposed for a transcrip-

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                                                  * Q E A E H L S L F T K Q Y F A A R A P W
  1 -  100  AAGCAATTATAAGTCATTAGCTTGAATCTAAATCCAGTTACTGCTCTGCCTCATGTAGAGATAGGAAGGTCTTCTGATAAAATGCAGCTCTGGCGGGCCA

              D P D V G Q A L M A D R K A K R M A E R E A R V K A I H E P T V I
101 -  200  GTCCGGATCAACACCTTGAGCCAACATTGCATCTCTTTTAGCCTTTCTCATAGCTTCCCTTTCGGCACGAACCTTAGCGATATGTTCTGGGGTCACTATG

              P K S K K G K K G K K K H Q L L S D D N T Q H K A D K V S K N I E V
201 -  300  GGCTTCGATTTTTTACCTTTCTTGCCCTTCTTTTTATGTTGAAGTAACGAATCATCATTGGTTTGGTGTTTTGCATCTTTTACAGATTTATTAATTTCTA

                E Q D P A V D P A V V K V E K D Q T M
301 -  400  CTTCTTGGTCGGGCGCAACATCTGGGGCAACCACTTTGACTTCTTTGTCTTGTGTCATATTTACTTAATATTCTTAACTACAATTAAGAAGCTATAAGGA

401 -  500  GACTACAATCTCATCTTCTTGCAACACTACTTTTCCTTTCCTCTTGCTTTAGTGCAACCATTTTTTGAAAAATGAATGGTCTTTCTGCACGAAATATCTC

501 -  600  TCGAAGAGCTTTTCTAGATATTAAGGATAAGTATATCATCCCAAAAGTTAGGTGATGGGATCAATATTGATGGACCAGCTAAAACACGTTGAAAGCAGAT

601 -  700  ATCAGGAATGGTGTTTTACAAAGTCACGTTGAGTAGGTCGCTTATCGGTGTGCCACACACAACGAAGAGCATTGTCAAAAGTTTGGGTTTAGGTAAAAGA
                    M V F Y K V T L S R S L I G V P H T T K S I V K S L G L G K R

701 -  800  GGGTCCATAGTCTACAAAAAAGTAAACCCGGCCATAGCTGGCTCCCTAGCTAAAGTGAAAGAGTTAGTCAAAGTAGAAGTAACAGAACATGAATTAACAC
               G S I V Y K K V N P A I A G S L A K V K E L V K V E V T E H E L T

801 -  900  CATCGCAGCAACGAGAGTTGAGAAAGTCAAACCCTGGGTTTATAGTTGGAGAAGAGAACCATCGACTGAAACAGCGAAATAAGGCACTGGATTTCCTTTC
               P S Q Q R E L R K S N P G F I V G E E N H R L K Q R N K A L D F L S

901 - 1000  TTCTTGATATTCCTGACTTATATATGTATATAGTATTATTTTTTCCTGCATAGGCACATATGTTAATAGTAATTTATGCAATTTTTGATACAAAGAAATC
                 S *

1001 - 1100  AATACACTATCTCGATTTCATATTTTATATATGTATATATTTACAAGAAGAAAATTATGCATGGTCTATTGAATTTTATATTTGGAAAAAGAGTCCCTTT

              * L K E L E L M G E L C D I K S I K C N K V I D G I M P Y H M S S
1101 - 1200  TTACAGCTTTTCCAACTCTAACATTCCTTCTAAACAGTCAATTTTTGAAATTTTACAATTCTTCACGATATCTCCAATCATCGGATAATGCATAGAACTT

              P H L K L T G R A F S L D P F V C G T F G N Q P V S T V M V M V D F
1201 - 1300  GGATGCAATTTTAATGTTCCTCTAGCGAACGATAAGTCTGGAAAAACACATCCAGTAAATCCGTTCTGCGGTACTGATGTTACCATTACCATTACATCGA

               T R E P E L K T L K K L Y N L T W Y T S S N R S A R K L I D A R S
1301 - 1400  AAGTCCTTTCTGGCTCTAGTTTGGTTAGCTTTTTTAAATAATTCAGTGTCCAGTACGTCGACGAATTACGGCTAGCCCTTTTTAAAATATCCGCTCGAGA

              Q I T W I L S D I Q T Q N F C L P K K Q L H R H I Q L H N I L D P
1401 - 1500  TTGAATTGGCCAGATCAAACTGTCGATTTGTGTTTGATTAAAACATAGAGGCTTTTTCTGCAAATGACGGTGAATTTGTAGATGATTTATCAAGTCTGGA

              F R R L P S T V T L Y Q K A G I M E H R F P R G T Y F S S N L L S S
1501 - 1600  AAACGACGAAGAGGGGATGTTACTGTCAAATATTGTTTAGCTCCAATCATTTCATGTCTAAAAGGTCGACCTGTATAAAAACTCGAGTTCAACAATGAAG

               L K
1601 - 1607  AAAGCTT
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FIG. 3. Nucleotide sequence of the chromosomal segment containing the *MRP-L33* gene. The nucleotide sequence was determined as described in the text and numbered arbitrarily. The predicted amino acid translation (in single-letter code) of the coding region of the *MRP-L33* gene is shown below the nucleotide sequence, and those of the two open reading frames are shown above it in the reverse direction. A likely TATA sequence is boxed, and sequences matching the consensus terminator sequences (see text) are underlined. The right-hand *Hin*dIII site shown in Fig. 2 is positioned at the end (doubly underlined).

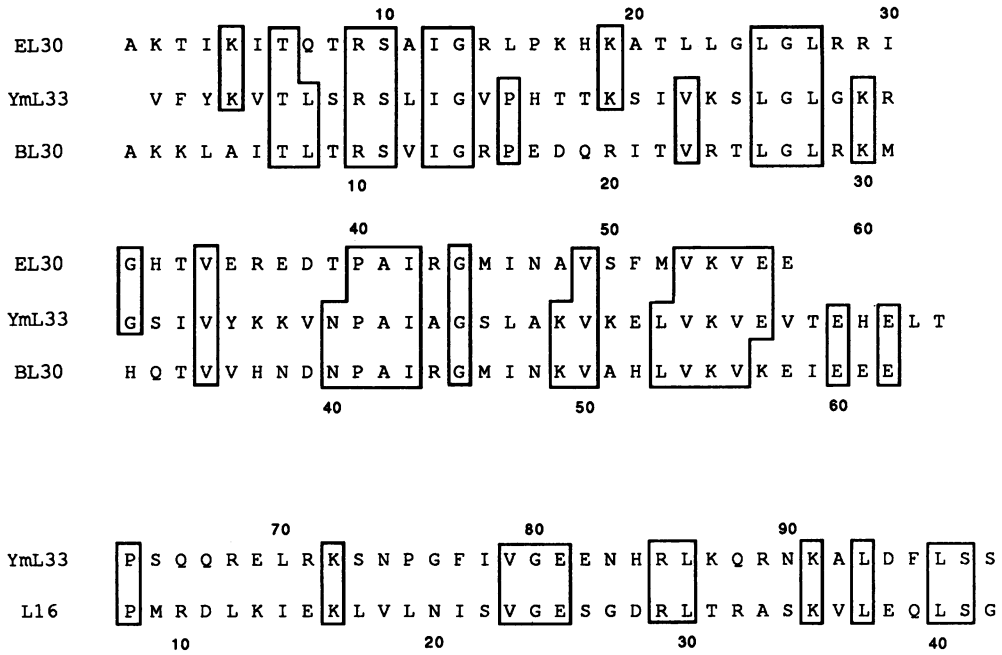

FIG. 4. Similarity of YmL33 to other ribosomal proteins. EL30 is L30 protein from *E. coli* (3), and BL30 is an EL30 homolog of *B. stearothermophilus* (17). L16 is a cytoplasmic ribosomal protein from *S. cerevisiae* (32). Identical residues are boxed. The residue numbers above the amino acid sequence are for YmL33, and those below the sequence are for the proteins being compared.

tional terminator (35). This sequence was also found to be present downstream of five other mitochondrial ribosomal protein genes, *MRP7* (6), *MRP-L8* (18), *MRP13* (24), *YMR26* (16a), and *MRP-L31* (10), although the spaces between the proposed consensus sequences are different among these genes. Thus, the open reading frame was concluded to be the coding region of the gene for protein YmL33 and was termed *MRP-L33*. From the amino acid sequence data for protein YmL33 and the nucleotide sequence data of its gene, *MRP-L33*, protein YmL33 apparently contains no leader sequence.

The deduced amino acid sequence of the *MRP-L33* gene was then compared with the sequence data contained in the NBRF protein sequence data base. The computer search revealed that the N-terminal two-thirds of this protein has a high degree of sequence similarity to *E. coli* ribosomal protein L30 (EL30) (3) and its *Bacillus stearothermophilus* homolog BL30 (17). As shown in Fig. 4, 39.6 and 45.6% of the amino acid residues were found to be identical with EL30 and BL30, respectively. Interestingly, its C-terminal region showed sequence similarity, though to a lesser extent (26.2% identical), with the N-terminal sequence of yeast cytoplasmic ribosomal protein L16 (32).

In addition to the open reading frame corresponding to *MRP-L33*, the sequence was found to contain two other open reading frames flanking it. Both of them are transcribed in the direction opposite that of *MRP-L33*. One of them, termed ORF1, is situated upstream of *MRP-L33* and is capable of encoding a basic protein (net charge of +9) with a calculated molecular weight of 19,396. The other open reading frame (ORF2) was found to start outside of the sequenced region shown in Fig. 3 and to end 192 bp downstream of *MRP-L33*. Whether the two open reading frames encode active genes remains to be investigated further.

Chromosomal location and copy number of *MRP-L33*. To establish the chromosomal location of the *MRP-L33* gene, a 1.1-kb *Hin*dIII-*Xba*I fragment containing the gene (Fig. 2) was radioactively labeled and used to hybridize with the yeast chromosomes separated by pulse-field gel electropho-

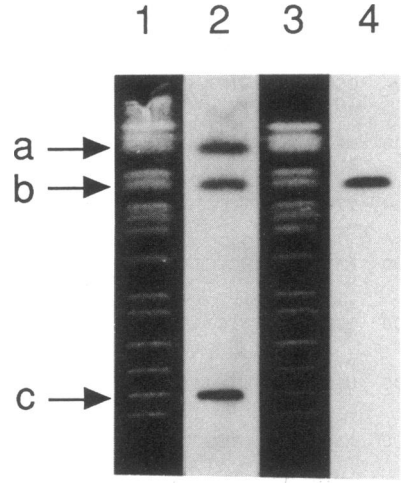

FIG. 5. Chromosomal location of the *MRP-L33* gene. The chromosomal DNA of strain YNN295 was separated by pulse-field electrophoresis in a 1% agarose gel in a CHEF-DRII electrophoresis system (Bio-Rad). After electrophoresis, the gel was stained with ethidium bromide (1 μg/ml) to visualize the DNA bands (lanes 1 and 3). The DNA bands were transferred to a nylon membrane and hybridized with the 1.1-kb *Hin*dIII-*Xba*I fragment of plasmid pL33 containing the *MRP-L33* gene (lane 4). DNA probes containing the *YMR26* gene located on chromosome XV or VII (16a) and the *YMR31* gene located on chromosome VI (21) were used to identify the hybridizing chromosomal band (lane 2). Arrows a, b, and c point to the bands corresponding to chromosomes XV or VII, XIII, and VI, respectively.

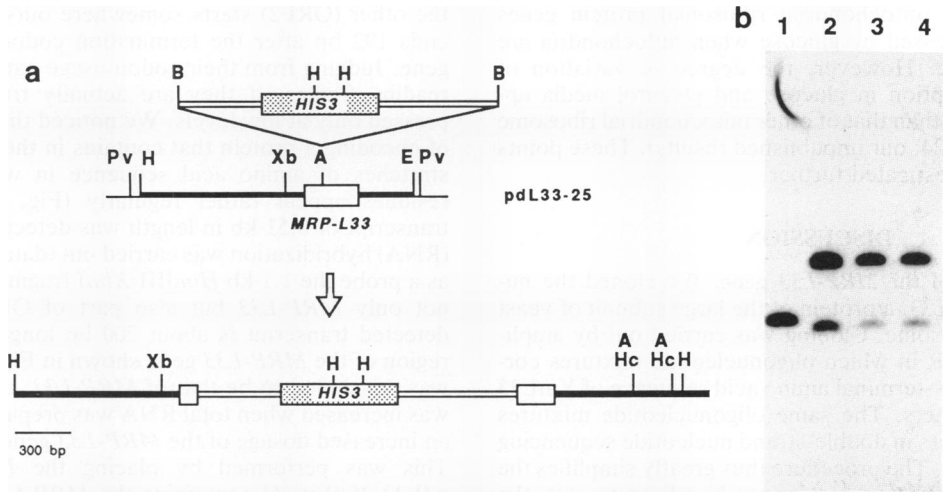

FIG. 6. Gene disruption analysis of *MRP-L33*. (a) Plasmid pdL33-25, a deletion derivative of pL33, was digested with *Acc*I and treated with Klenow fragment to make the restricted ends blunt. A 1.75-kb *Bam*HI fragment containing the *HIS3* gene (hatched box) derived from YIp1 (28) was purified, treated with Klenow fragment to make the ends blunt, and ligated with pretreated pdL33-25. A 3.3-kb *Pvu*II fragment of the resultant plasmid in which the *MRP-L33* gene (open box) was disrupted by the *HIS3*-containing segment was then used to transform DC-5 cells to His^+. Consequently, the chromosomal *MRP-L33* gene was expected to be replaced by the disrupted allele as shown at the bottom. (b) Disruption of *MRP-L33* was confirmed by Southern hybridization after digestion with *Hin*dIII and *Xba*I of the chromosomal DNA from three His^+ transformants (lanes 2 to 4) and that from the parental strain DC-5 as an undisrupted control (lane 1). The probe used was the 1.1-kb *Hin*dIII-*Xba*I fragment of pL33 (see Fig. 2). Abbreviations for restriction enzymes: A, *Acc*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Hc, *Hin*cII; Pv, *Pvu*II; Xb, *Xba*I.

resis. As shown in Fig. 5, positive hybridization was observed with chromosome XIII. The copy number of *MRP-L33* in the yeast genome was then estimated by genomic Southern hybridization. For this purpose, the genomic DNA of strain DC-5 [rho^0] was digested with *Bam*HI, *Eco*RI, *Hin*dIII, or *Pst*I, subjected to electrophoresis, transferred, and hybridized with the fragment used in the analysis described above. A single band was observed in all cases (data not shown), indicating that the *MRP-L33* gene most likely exists as a single-copy gene in the yeast genome.

Gene disruption analysis. To determine whether the product of *MRP-L33* is essential for mitochondrial function in yeast cells, the gene was disrupted and its effects were analyzed. For this purpose, a 1.75-kb *Bam*HI fragment containing the yeast *HIS3* gene was inserted into the coding region of *MRP-L33* on plasmid pdL33-25 (Fig. 6a). A *Pvu*II fragment of the resultant plasmid was then excised and used to replace the chromosomal gene with the disrupted allele by homologous recombination. His^+ transformants were selected and examined for the ability to grow on a nonfermentable carbon such as glycerol. Of 22 transformants analyzed, 20 were found to be respiration deficient. Southern blot analysis of the genomic DNA of several representative transformants verified that the *MRP-L33* gene was indeed disrupted in respiration-deficient transformants. Three such examples are shown in Fig. 6b. In all cases (lanes 2 through 4), two fragments of the expected sizes (1.0 and 1.7 kb) were observed to hybridize with the probe (a 1.1-kb *Hin*dIII-*Xba*I fragment containing the *MRP-L33* gene), as opposed to only one band of 1.1 kb in an undisrupted control (lane 1). In contrast, the *MRP-L33* gene remained undisrupted in transformants that were not respiration deficient (data not shown). By Southern hybridization analysis, it was found that homologous recombination in the latter transformants occurred somewhere outside the *MRP-L33* gene, most likely at the *HIS3* locus. These results, therefore, indicate that the *MRP-L33* gene is essential for mitochondrial function in yeast cells.

Previously, it was reported that disruption of the yeast genes required for mitochondrial protein synthesis led to conversion of [rho^+] strains to [rho^-] or [rho^0] (22). Apparently, the mitochondrial genome became unstable upon disruption of such genes. Therefore, this possibility was investigated with *MRP-L33*. When one of the disrupted mutants (DC-5-Δ*MRP-L33*) described above was mated with a [rho^0] strain containing intact *MRP-L33*, the diploids generated were found to be all respiration deficient. This result can be best interpreted to indicate that DC-5-Δ*MRP-L33* strain was either [rho^-] or [rho^0], which in turn suggests that the observation by Myers et al. (22) mentioned above is also true for *MRP-L33*.

Regulation of transcription of the *MRP-L33* gene. To investigate whether the expression of *MRP-L33* would be affected by different carbon sources, the amounts of *MRP-L33* transcript were measured and compared in [rho^+] and [rho^0] cells growing on fermentable or nonfermentable carbon sources. Total RNAs were prepared from early-log-phase cultures of DC-5 [rho^+] and DC-5 [rho^0] growing in YP medium supplemented with either 6% glucose, 2% glycerol, or 2% galactose. Equal amounts of RNA isolated from each culture were blotted onto a nitrocellulose membrane after agarose gel electrophoresis and hybridized with a 1.1-kb *Hin*dIII-*Xba*I fragment containing *MRP-L33* (Fig. 2). Each band in the resultant autoradiograms was quantified by tracing with a densitometer. As a control, the transcript of the *ACT1* gene (8) was similarly measured and used to normalize the data obtained. In a [rho^+] strain, the amount of the *MRP-L33* transcript in cells growing in galactose medium was more abundant than that in cells grown in glucose medium (data not shown). In contrast, its transcription in the [rho^0] background was at a high level even in the presence of glucose. Thus, the transcription of *MRP-L33*,

like that of other mitochondrial ribosomal protein genes (16a, 18a), is repressed by glucose when mitochondria are functionally active. However, the degree of variation of *MRP-L33* transcription in glucose and glycerol media appeared to be lower than that of other mitochondrial ribosome protein genes (22, 24; our unpublished results). These points are now being investigated further.

DISCUSSION

Characteristics of the *MRP-L33* gene. We cloned the nuclear gene for YmL33, a protein of the large subunit of yeast mitochondrial ribosome. Cloning was carried out by amplifying DNA by PCR in which oligonucleotide mixtures corresponding to the N-terminal amino acid sequence of YmL33 were used as primers. The same oligonucleotide mixtures were used as primers in double-strand nucleotide sequencing of the cloned DNA. The procedure thus greatly simplifies the cloning and identification of a gene. Needless to say, the procedure depends on the quality of amino acid sequence data and the length of the sequenced stretch. In the case of protein YmL33, it was possible to obtain an N-terminal amino acid sequence for as long as 40 residues. Moreover, the sequenced region contained two stretches of amino acid residues harboring least degenerate codons which were situated reasonably distant from each other. This enabled us to obtain a PCR-amplified product of 114 nucleotides in length.

Nucleotide sequence analysis of the clone thus obtained revealed the presence of three open reading frames in the sequenced region. Of them, the middle one was found to be capable of encoding a protein with 99 amino acid residues and a calculated molecular weight of 11,012. Its N-terminal amino acid sequence completely matched that of protein YmL33. Thus, it was concluded to be the structural gene for YmL33 and was termed *MRP-L33*. Comparison of the amino acid sequence of protein YmL33 with that deduced from the DNA sequence of *MRP-L33* indicates that protein YmL33 has no leader sequence. An N-terminal leader sequence is considered to play an important role in targeting proteins into various subcellular structures, including mitochondria. However, several mitochondrial proteins, including some of the mitochondrial ribosomal proteins, lack an N-terminal leader sequence (16a, 18, 21) and yet are correctly transported into mitochondria. Therefore, a sequence or sequences other than the N-terminal leader sequence must be recognized as a mitochondrial targeting signal. We are currently investigating such a possibility.

On the basis of its primary structure, the product of *MRP-L33* can be regarded as homologous to protein L30 of the large ribosomal subunit of *E. coli* and *B. stearothermophilus*. Three of ten other yeast mitochondrial ribosomal protein genes characterized so far were found to encode a protein with a high degree of sequence similarity to *E. coli* ribosomal proteins: MRP2 to S14 (22), MRP7 to L27 (6), and YmL8 to L17 (18). In addition to these yeast genes, two nuclear genes for mitochondrial ribosomal proteins in *Neurospora crassa* showed sequence similarity to *E. coli* ribosomal proteins (19, 20). These data support the notion that mitochondria have indeed descended from an endosymbiotic prokaryote (29).

From the nucleotide sequence data, the *MRP-L33* gene was found to be flanked by two other open reading frames, both of which are capable of encoding basic proteins and are situated in opposite directions (Fig. 2). One of them (ORF1) starts and ends within the sequenced region (Fig. 2), while the other (ORF2) starts somewhere outside the region and ends 192 bp after the termination codon of the *MRP-L33* gene. Judging from their codon usage pattern, the two open reading frames, if they are actually transcribed, are expressed only at low levels. We noticed that ORF2 is capable of encoding a protein that contains in the C-terminal region stretches of amino acid sequence in which Leu and Ile residues appear rather regularly (Fig. 3). Only a single transcript of 0.51 kb in length was detected when Northern (RNA) hybridization was carried out (data not shown), using as a probe the 1.1-kb *Hin*dIII-*Xba*I fragment which contains not only *MRP-L33* but also part of ORF2 (Fig. 2). The detected transcript is about 200 bp longer than the coding region of the *MRP-L33* gene shown in Fig. 3. The transcript was concluded to be that of *MRP-L33*, because its amount was increased when total RNA was prepared from cells with an increased dosage of the *MRP-L33* gene (data not shown). This was performed by placing the *Pvu*II fragment of pdL33-25 (Fig. 6) containing the *MRP-L33* gene in a multicopy plasmid (the *Pvu*II fragment in question was, however, not expected to support the transcription of ORF2, since it lacks the transcriptional initiation site for this open reading frame, as shown in Fig. 3). Thus, ORF2 is likely to be either a gene that is expressed at a very low level or not a gene at all.

As described in Results, the *MRP-L33* open reading frame is followed by a sequence similar to the one proposed to be a consensus needed for efficient transcriptional termination in yeast cells (35). A similar sequence was found in the 3′ noncoding regions of *MRP7* (6), *MRP-L8* (18), *MRP13* (24), *YMR26* (16a), and *MRP-L31* (10). However, no such sequence was found in the 3′ noncoding regions of *MRP2* (22) and *MRP-L20* (18), although sequences partially matching the proposed consensus were found in the latter genes. It remains to be investigated further whether the sequence in question indeed functions in efficient termination of the transcription of these genes.

From the in vitro experiments in which ribosomes were reconstituted with all components except for one protein at a time and functionally tested (single protein omission experiments), protein L30 was implicated to be involved in the peptidyltransferase activity in *E. coli* (12) and *B. stearothermophilus* (1, 4). Nonetheless, an *E. coli* mutant lacking L30 was isolated and shown to be viable with a growth rate similar to that of a wild-type strain (5). Therefore, L30 appears to be not absolutely essential for ribosomal function in *E. coli*. In contrast, we found that YmL33, its putative homolog in yeast mitochondria, was an essential protein for yeast mitochondria, because *MRP-L33* null mutants constructed by gene disruption were respiration deficient. Almost all other mitochondrial ribosomal protein genes so far characterized are also essential for mitochondrial function, because mutants lacking their structural integrity were found to be respiration deficient as in the case of *MRP-L33* described here. The apparent difference between the *E. coli* and the yeast mitochondrial ribosomes with respect to the essentiality of protein L30 and its equivalent might reflect structural differences between the two ribosomes. In support of this notion, many yeast mitochondrial ribosomal proteins show no sequence similarity to any *E. coli* ribosomal proteins. Furthermore, many of the yeast mitochondrial proteins that show sequence similarity to *E. coli* ribosomal proteins are much larger than their *E. coli* counterparts (6, 18). It should also be noted that yeast mitochondrial ribosomes contain more proteins than do *E. coli* ribosomes (8a). Therefore, it is conceivable that the assembly or location of

individual ribosomal proteins are different in the two ribosomes and that the structural resemblance of proteins from the two ribosomes do not necessarily imply their functional resemblance.

We demonstrated that the *MRP-L33* gene is located on chromosome XIII (Fig. 5). Of the mitochondrial ribosomal proteins so far characterized, the *YMR44* gene was found to be located on either chromosome XIII or XVI (21). If the endosymbiont hypothesis is correct, then most of the genes for mitochondrial ribosomal proteins must have been transferred from the mitochondrial genome to the nucleus during the course of mitochondrial evolution. However, in contrast to the fact that the genes for ribosomal proteins in present-day prokaryotes are largely clustered with each other, the yeast mitochondrial ribosomal protein genes so far characterized are not clustered on a particular chromosome (16a, 18a). This finding seems to imply that the movement of the ancestral mitochondrial ribosomal protein genes occurred individually. It might be that the movement was achieved via incomplete reverse transcription of polycistronic mRNAs. If this was the case, then loss of the corresponding gene from the mitochondrial genome must have occurred afterward. In this connection, it seems interesting that the mitochondrial genome becomes unstable when its function is impaired by disrupting nuclear genes such as *MRP-L33*. It remains to be investigated what causes the instability of the mitochondrial genome and whether a particular region of the mitochondrial genome is more unstable than other regions.

Analysis of the level of the transcription of *MRP-L33* showed that the expression of this gene was repressed by glucose when mitochondria were functional. Similar transcriptional repression by glucose was observed for *MRP1* and *MRP2* (22), *MRP13* (24), and *YMR26* (16a), although the extent of glucose effects on these genes appears to vary. How the transcriptions of these mitochondrial ribosomal protein genes which are located on different chromosomes are similarly affected by glucose and what is mediating the molecular communication between mitochondria and the nucleus remain to be investigated further.

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