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# Mitochondrial ribosomes of yeast: isolation of individual proteins and N-terminal sequencing

Hanns-Rüdiger Graack, Lutz Grohmann and Theodora Choli

*Max-Planck-Institut für Molekulare Genetik, Abt. Wittmann, D-1000 Berlin 33, Ihnestr. 73, Germany*

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Proteins of the small and large subunits of mitochondrial ribosomes from the yeast *Saccharomyces cerevisiae* were isolated and characterized by two-dimensional gel electrophoresis. Ribosomal proteins of the large subunit were separated by reverse-phase HPLC and up to 37 amino acid residues of the N-terminal sequences of L3, L4, L9 and L31 were determined. No significant homology to ribosomal protein sequences so far determined from other organisms was found.

Ribosomal protein; Amino acid micro-sequencing; Mitochondria; *(Saccharomyces cerevisiae)*

## 1. INTRODUCTION

Ribosomal proteins from various organisms have been characterized by amino acid and/or nucleotide sequencing. These data, together with ribosomal RNA sequences, have been used for comparative studies on homology, conservation and evolution of ribosomal constituents [1,2]. Much less is known about mitochondrial ribosomes. Sequences of mitochondrial rRNAs and ribosomal proteins encoded by the mitochondrial DNA alone do not give sufficient information about the evolution of the mitochondrial translational machinery [3,4]. Indeed, in yeast almost all of the mitochondrial ribosomal proteins are encoded by the nuclear DNA [5]. Recently the first genes of such nuclear coded proteins have been determined. These authors used a complementation assay [6] or immunological screening of an expression library [7,8] for gene identification.

Alternatively, we started a programme to obtain sequence data by direct micro-sequencing of the proteins. We isolated mitochondrial ribosomes on a large scale and employed reverse-phase HPLC for the purification of individual proteins. Amino acid sequence data from very small amounts of these proteins were obtained by micro-sequencing. Here we present the isolation and the N-terminal amino acid sequences of four proteins derived from the large subunit of the mitochondrial ribosome of the yeast *Saccharomyces cerevisiae*.

*Correspondence address:* H.-R. Graack and L. Grohmann, Max-Planck-Institut für Molekulare Genetik, Abt. Wittmann, D-1000 Berlin 33, Germany

*Abbreviations:* βME, β-mercaptoethanol; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; TP50, total protein of the 50 S mitochondrial ribosomal subunit; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate

## 2. MATERIALS AND METHODS

### 2.1. *Isolation of mitochondria*

Mitochondria were prepared as described [9] with some alterations: the wild-type *S. cerevisiae* strain 07173 (a/α; obtained from Professor Stahl, Institut für Gärungsgewerbe, Berlin) was grown aerobically in 30 l of YEPGly$_3$-medium (1% yeast extract, 2% bacto peptone, 3% glycerol), and cells were harvested at a density of 8 g/l. After washing they were incubated in 7.5 ml/g wet wt of prewarmed buffer A (0.1 M Tris/$H_2SO_4$, pH 9.4; 20 mM βME) for 15 min at 30°C, washed once with buffer B (1.2 M sorbitol; 20 mM potassium phosphate, pH 7.4) and resuspended in the same buffer up to a concentration of 0.1 g wet wt/ml. Zymolyase 100T (0.4 mg/g cells) was added, and the suspension was incubated for 60 min at 30°C. Formation of spheroplasts was monitored



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photometrically. Spheroplasts were centrifuged (2000 × g for 3 min), washed once with buffer B and resuspended in 7.5 ml/g wet wt of cold buffer C (0.6 M sorbitol, 20 mM Hepes/KOH, pH 7.4, 1 mM PMSF). All further steps were carried out at 4°C. Cells were homogenized by 12 strokes in a tight fitting Dounce homogenizer, diluted 1:1 with buffer C and centrifuged at 3000 × g for 5 min. The supernatant was stored, and the pellet was re-homogenized and centrifuged at 3300 × g for 10 min. Supernatants were combined and centrifuged at 17000 × g for 15 min. The resuspended mitochondria were centrifuged briefly to remove the remaining cell debris, again sedimented and washed 4 times with buffer D (0.6 M sorbitol, 20 mM Hepes/KOH, pH 7.4, 1 mM EDTA) and once with buffer E (0.6 M sorbitol, 20 mM Hepes/KOH, pH 7.4). Finally they were resuspended in buffer F (350 mM $NH_4Cl$, 20 mM Mg-acetate, 1 mM EDTA, 2 mM βME, 20 mM Tris-HCl pH 7.5) as described [10].

2.2. *Isolation of mitochondrial ribosomes and their proteins*

Mitochondria were lysed with 1/20 volume of 26% Triton X-100 in buffer F. The lysate was cleared by centrifugation at 30000 × g for 10 min, and ribosomes were pelleted through a sucrose cushion as described [10]. Ribosomes were resuspended in buffer F and centrifuged at 30000 × g for 10 min. Ribosomal subunits were separated by sucrose gradient centrifugation in a Beckman SW 27 rotor (10–30% in buffer F) and pelleted overnight at 110000 × g. Ribosomal proteins were extracted as described [11], dialysed intensively against 5% acetic acid and either lyophilized or subjected directly to HPLC.

2.3. *Isolation of cytoplasmic ribosomes*

Yeast cells were grown on $YEPD_8$-medium (1% yeast extract, 2% bacto peptone, 8% glucose) to midlog-phase and harvested, then washed once with water and once with buffer G (10 mM Tris-HCl, pH 7.4, 5 mM βME, 10 mM $MgCl_2$, 50 mM $NH_4Cl$). The cells were resuspended in buffer G and broken in a French press cell. The homogenate was subjected to a series of centrifugation steps (1000 × g for 10 min, 20000 × g for 20 min, 36000 × g for 20 min), and the ribosomes were pelleted from the supernatant through a sucrose cushion in buffer H (25 mM $NH_4Cl$, 10 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.4, 5 mM βME). Sucrose gradient centrifugation and protein extraction were carried out as described above.

2.4. *PAGE*

Proteins from 37 S, 50 S or 80 S ribosomal particles were separated by 2D-PAGE according to [12], and analyses of HPLC fractions were performed by SDS gel electrophoresis [13] of dried aliquots. The SDS gels were stained by silver staining [14]. The molecular masses of the mitochondrial ribosomal proteins were estimated by comparison with standard molecular mass markers. For sequencing, proteins were blotted from SDS gels as described [15].

2.5. *Reverse-phase HPLC*

Ribosomal proteins were separated by reverse-phase HPLC on a Vydac $C_4$ column as described [16]. Up to 1 mg of TP50 in 3–7 ml 5% acetic acid was applied to the column by multiple injections and eluted with a gradient of 0.1% TFA/90% acetonitrile/10% water vs 0.1% TFA/0.001% βME in water at 35°C (see legend to fig.2).

2.6. *Amino acid micro-sequencing*

After HPLC separation, 200–1000 pmol of pure dried proteins were dissolved in 20 μl 100% TFA, applied to a glass filter (1 cm diameter) which had been pre-treated with 2 mg Biobrene (purchased from Applied Biosystems, Foster City, CA) and subjected to two filter cycles prior to application of the sample. Proteins blotted on PVDF membranes were pre-treated as described [15]. Degradation was performed in a pulsed-liquid gas-phase sequencer (model 477A) and a PTH-analyzer (model 120) both from Applied Biosystems. The PTH-amino acids were monitored at 269 nm using the standard gradient program provided with the analyzer.

## 3. RESULTS

Subunits of mitochondrial ribosomes from *S. cerevisiae* wild-type strain 07173 were isolated from cells grown on medium containing glycerol as a non-fermentable carbon source, to avoid the effects of glucose repression and to increase the biosynthesis of mitochondria. A typical preparation yielded 50–100 $A_{260}$ of ribosomes per 100 g of cells. As a result of the relatively high $NH_4^+$ content of the buffers used for cell lysis and sucrose gradients, we obtained 37 S and 50 S ribosomal subunits and no monosomes.

Compared to the 50 S subunits, the 37 S subunits were isolated in variable amounts, indicating their less stable character. Total proteins of both ribosomal subunits were separated by 2D-PAGE, and 34 proteins of the small subunit and 34 proteins of the large subunit could be discerned (fig.1). Some additional spots, which were stained very weakly and not present in all preparations, were not counted. A comparison of the respective 2D gel patterns showed that there was no cross-contamination of ribosomal proteins from mitochondria with those from the cytoplasm (data not shown).

TP50 protein mixtures were subjected to separation by reverse-phase HPLC (fig.2), and the eluted proteins were analysed for their molecular masses and purity by SDS-PAGE. Individual proteins were identified by 2D-PAGE. Four of them were subjected to N-terminal micro-sequencing (table 1). For L3, 26 amino acids with 5 basic, 5 acidic and 6 hydrophobic residues were determined. L4 was blotted from the SDS gels after HPLC separation and directly sequenced on the PVDF membrane. 26 residues could be identified, including 5 hydrophobic and 8 basic amino acids, but only 1

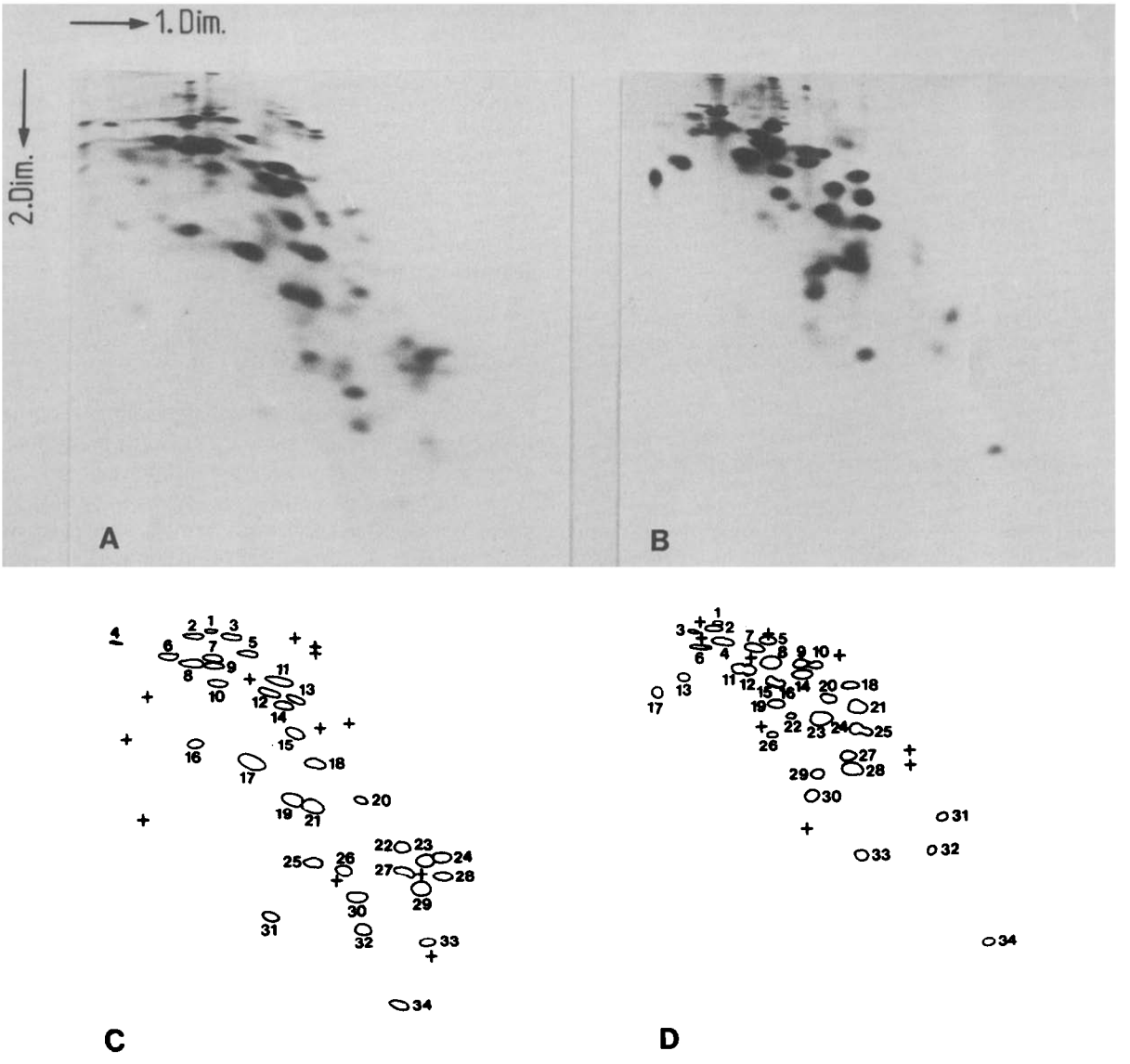


Fig.1. 2D-PAGE separation of ribosomal proteins from yeast mitochondria. (A) Proteins from the small 37 S and (B) from the large 50 S subunit. 160 $\mu$g and 200 $\mu$g of protein were applied to the gel in A and B, respectively. (C,D) Schematic diagram of the protein patterns shown in panels A and B. Proteins are numbered in an analogous manner to that of Kaltschmidt and Wittmann [23]. Faintly visible and not reproducibly detected spots are marked with (+).

acidic residue. Remarkably, 5 of these 26 residues are prolines. For L9, 33 amino acid residues could be sequenced and only 6 of them are charged, whereas 8 are hydrophobic. L31 provided a sequence of 37 residues. In this case 10 amino acids are positively and two negatively charged. Interestingly, 12 hydrophobic amino acids are concentrated into two stretches separated by a peptide of 11 amino acids with 6 charged residues.

Using the computer programs ALIGN and RELATE [17,18] these sequences were compared with those of all known ribosomal protein se-

Table 1

N-terminal sequences and molecular masses of ribosomal proteins from yeast mitochondria

| | |
|---|---|
| | 10 |
| L3 (36 kDa) | E X K R F L X E S E L A K Y K E Y Y Q |
| | 20 |
| | G L K S T V N E I |
| | 10 |
| L4 (35 kDa) | A R T K F T K P K P K Q P V L P K D K |
| | 20 |
| | I R P X T Q L T |
| | 10 |
| L9 (27.5 kDa) | S V T R P F L V A P S I A N S I T T E |
| | 20 30 |
| | A P A I N H S P E L A N A R K |
| | 10 |
| L31 (14.5 kDa) | G G L L W K I P W R M S T H Q K T R Q |
| | 20 30 |
| | R E R L R N V D Q V I K Q L T L G X H |

quences collected in the database RIBO:SEQ (Köpke and Wittmann-Liebold, unpublished). No significant similarities were found.

## 4. DISCUSSION

For the isolation of pure mitochondrial ribosomal proteins the avoidance of contamination by cytoplasmic ribosomal proteins and ribosome-associated factors is one of the most important requirements. Accordingly, we employed stringent conditions for the washing of mitochondria and for the isolation of ribosomal subunits. Thus, the number of mitochondrial ribosomal proteins given in fig.1 reflects a minimum, and it is not surprising that the number of proteins in the 2D pattern differs from previous reports [19,20].

For the majority of mitochondrial proteins it has been shown that an amino-terminal presequence is necessary for their transport into mitochondria, and that this leader peptide is cleaved off the precursor protein during that process [21]. Therefore, one would expect that presequences also exist for the mitochondrial ribosomal proteins, to direct them into the mitochondrial matrix. Indeed for mitochondrial ribosomal protein L31 a DNA region, located upstream of the mature protein and corresponding to a leader peptide with 12

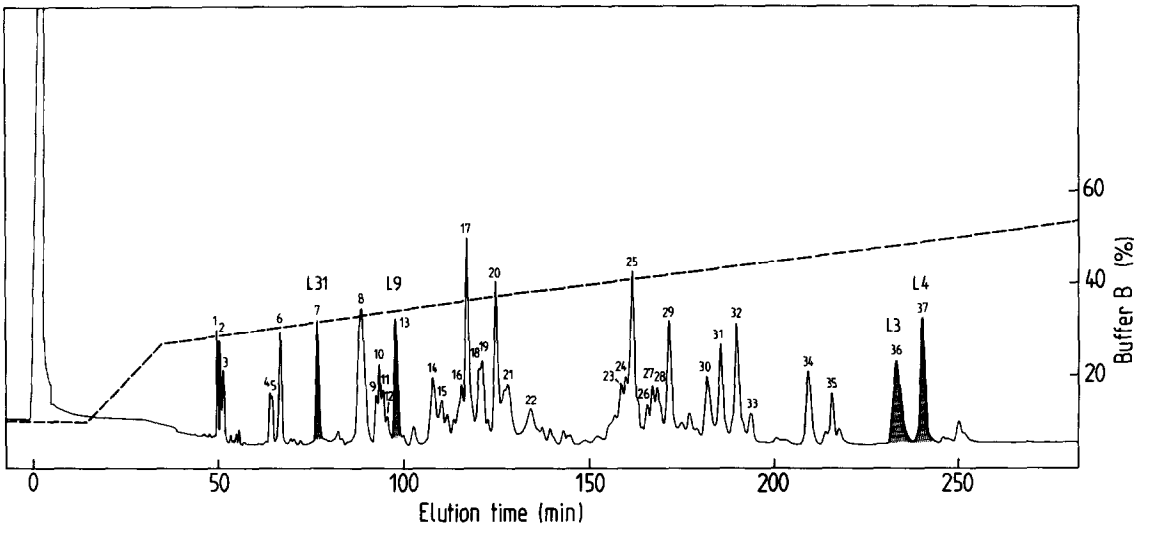


Fig.2. Reverse-phase HPLC separation profile of proteins from the large ribosomal subunit of yeast mitochondria. TP50 proteins were extracted from 4 $A_{260}$ 50 S subunits and directly applied to a $C_4$ Vydac precolumn (4 × 30 mm) and a column (4 × 250 mm) containing particles of 5 $\mu$m size and 30 nm pore diameter. The columns were packed in this laboratory. Solvent A: 0.1% TFA, 0.001% $\beta$ME in water; solvent B: 0.1% TFA in 90% acetonitrile/10% water. Flow rate: 0.4 ml/min. Gradient % B: for 15 min 10%, in 20 min to 27%, in 80 min to 36%, in 110 min to 47%, in 55 min to 53%, in 30 min to 65%, and in 20 min to 10% B. Column temperature was 35°C, O.D. range 0.32 at 220 nm. Fractions were collected every minute.

residues, has recently been identified (Grohmann et al., to be published).

No significant similarity between the amino acid sequences presented in this paper and those of all other ribosomal proteins sequenced so far has been found. However, one has to consider that only a relatively small part of proteins L3, L4 and L9 has so far been sequenced, whereas for protein L31 about 30% of the total sequence has been determined. It remains to be seen by the determination of more complete sequences whether or not the lack of homology to ribosomal proteins from other sources holds true. The nucleotide sequences of five nuclear encoded proteins of mitochondrial ribosomes have so far been determined [6–8,22], and three show homologies to *E. coli* ribosomal proteins whereas two of them (MRP1, MRP13) do not. For the isolation of these genes the method of genetic complementation of nuclear mutants defective in mitochondrial functions [6,22], or screening of a genomic library with monoclonal antibodies raised against ribosomal proteins [7,8] has been used. However, the complementation assay is not specific for ribosomal proteins, and both methods give no information about leader peptides.

The most direct way for obtaining amino acid sequences of ribosomal proteins from mitochondria is first to isolate the mitochondria and their ribosomes in pure form by stringent washing procedures in order to remove non-ribosomal contaminants, and then to isolate and sequence the ribosomal proteins. The first successful application of this direct method, which requires a large-scale preparation of pure mitochondrial ribosomes, is described in this paper.

On the basis of the data presented it should be possible to synthesize oligonucleotide probes for hybridization with yeast chromosomal DNA, and by cloning of the corresponding genes complete sequences of ribosomal proteins from yeast mitochondria should become available. Comparison of the DNA sequences with the N-terminal peptide data will provide information about the existence and length of leader peptides. Work using this strategy is in progress in our laboratory.

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