# ANALYSIS AND SITE OF SYNTHESIS OF RIBOSOMAL PROTEINS FROM YEAST MITOCHONDRIA

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## 1. Introduction

Eukaryotic cells contain in addition to the cytoplasmic protein synthesizing machinery, other semi-autonomous protein synthesizing systems localized in the mitochondria and/or chloroplasts. These systems have been identified by menas of their selective sensitivity to anti-bacterial antibiotics, such as chloramphenicol or erythromycin [1–4]. The characterization of mitochondrial ribosomes has only been achieved recently; those from animal cells sediment at 55–60 S while in lower eukaryotic cells such as *Neurospora crassa* and yeast, mitochondria contain 72–74 S ribosomes [5, 8].

The mitochondrial rRNA from yeast is transcribed from the genome of the organelle [5] and has a peculiar base composition (about 30% G+C) [9–12]. While we had reported that proteins from mitochondrial ribosomes are different from their cytoplasmic counterparts [13] only a few proteins band at different positions on polyacrylamide gels, in the work of Halvorson's group [11].

In order to obtain a final answer to these contradictory results, we have now compared the proteins from mitochondrial ribosomes by cochromatography on CMC** columns with cytoplasmic ribosomal proteins.

We have also investigated the site of synthesis of mitochondrial ribosomal proteins by means of specific inhibitors of the protein synthesizing systems of cytoplasm and mitochondria.

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** Carboxymethyl cellulose.

## 2. Materials and methods

For labelling the proteins, yeast strain D261 $\rho+$, was grown with a doubling time of 5 hr 20 min on minimal medium containing per l: 15 g ethanol; 0.5 g $MgCl_2$; 1 g $KH_2PO_4$; 0.1 g NaCl; 0.1 g $CaCl_2$; 6 g $(NH_4)_2HPO_4$; 0.5 g boric acid; 0.04 mg CuCl; 0.1 mg KI; 0.5 mg $MnCl_2$; 0.4 mg $ZnCl_2$; 0.2 mg $FeCl_3$; 0.2 mg $Na_2MoO_4$; 2 mg pantothenate; 2 mg thiamine; 2 mg inositol; 2 mg nicotinic acid; 0.02 mg biotine and 0.1 mM $Na_2SO_4$.

The starting cultures were grown on 50 ml minimal medium inoculated with 5 ml of a culture growing on rich medium.

Yeast cells were labelled with 20 mCi $Na_2SO_4$ (CEA-CEN, Mol) or 6 mCi [$^3$H]lysine (7.5 Ci/mmole, Amersham).

The labelled cells were collected at mid-exponential phase of growth and diluted with carrier cells grown on rich medium as previously described [7].

Lysis of yeast cells and isolation of ribosomes or ribosomal subunits were carried out as previously described [13].

Isolated ribosomal subunits were 95% pure as checked by analysis on sucrose gradients. The protein:RNA ratio of ribosomal subunits from mitochondria was the same as that of purified ribosomal subunits from the cytoplasm. ($A_{238}$:$A_{260}$ = $A_{280}$:$A_{260}$ = 0.51–0.53).

Ribosomal subunits were precipitated from sucrose solutions by the addition of polyethylene glycol 6000 to a final conc. of 10% (w/v) followed by $MgCl_2$ to a final conc. of 10 mM.

After 1 hr at 0°, the precipitate was centrifuged

and redissolved in 30 mM acetic acid–methylamine (pH 5.6), 6 M urea, 3 mM β-mercaptoethanol, 3 mM EDTA.

The ribosomes were precipitated and recovered quantitatively using this method (1 mg carrier cold cytoplasmic ribosomes were added to the subunits from mitochondrial ribosomes before precipitation).

The two samples to be cochromatographed were mixed, diluted with cold cytoplasmic ribosomes, (if necessary) to a final quantity of 4 mg ribosomes. RNAase $T_1$ and pancreatic RNAase (Worthington) were added to a final concentration of 10 μg per ribosome [14]. The mixture was incubated for 45 min at 37°.

The ribosomal proteins (about 100,000 cpm) were then chromatographed at 4°, on CM 32 Whatman columns (0.6 × 30 cm) prewashed with 0.03 M acetic acid–methylamine (pH 5.6), 3 mM mercaptoethanol, 6 M urea using a 2 × 200 ml linear gradient starting from this buffer to 0.5 M Na acetate pH 5.6, 3 mM mercaptoethanol, 6 M urea [14, 15]. Flow rates were in the range of 1 ml/45 min.

1 ml fractions were collected, mixed with 1 ml water and 20 ml scintillation fluid (toluene, 4 g/l omnifluor:triton, 2:1) and counted in a Packard liquid scintillation counter.

There was 20% contamination of $^{35}S$ counts in the $^{3}H$ channel and no $^{3}H$ contamination in the $^{35}S$ channel under the conditions used.

### 2.1. *Sucrose gradient analysis of mitochondrial ribosomes*

Mitochondrial ribosomes were layered on 15–30% sucrose gradients in 10 mM Tris-HCl (pH 7.5) 10 mM $MgCl_2$ and spun 15 hr at 21 000 rpm, at 2°, in Spinco SW 27 rotor. Fractions were collected and counted in a Packard liquid scintillation counter.

## 3. Results

### 3.1. *Comparison of the ribosomal proteins from mitochondria and from cytoplasm*

Fig. 1 shows the pattern of cochromatography of the ribosomal proteins from mitochondria and cytoplasm of yeast.

The mitochondrial ribosomes were labelled with $^{35}S$, and the cytoplasmic ribosomes with [$^{3}H$]lysine. The two patterns, therefore, cannot be compared quantitatively; one should consider only the position of the peaks, not their size. One of the proteins of the 38 S mitochondrial subunit and one from the 50 S subunit do not incorporate $^{35}S$; their position is indicated by arrows.

It will be noticed that most of the proteins from the 50 S mitochondrial subunit appear in the first half of the chromatogram, whereas the proteins from cytoplasmic 60 S subunits are on the average less basic than those from the 60 S cytoplasmic subunits; this may explain in part their lower rate of migration in polyacrylamide gel electrophoresis [13].

The proteins from the 38 S subunits of mitochondrial and cytoplasmic origins give quite different patterns of elution; the positions of a few peaks only may coincide, most are clearly different.

### 3.2. *Site of synthesis of the proteins of mitochondrial ribosomes*

In order to find out whether the proteins from mitochondrial ribosomes are made by the mitochondrial or by the cytoplasmic ribosomal system, we studied the incorporation of $^{35}S$ into the mitochondrial ribosomes in the presence of either chloramphenicol or cycloheximide, which are known to inhibit, respectively, the mitochondrial and the cytoplasmic systems of protein synthesis.

A suspension of yeast growing exponentially in minimal medium at 25° is divided into three batches. The first serves as the control, the second receives 4 mg chloramphenicol per ml, the third 50 μg cycloheximide per ml.

Thirty minutes later, 6 mCi of $Na_2{}^{35}SO_4$ (carrier free) are added per 300 ml to each flask. After 3 hr, casamino acid (final conc. 4%) and unlabelled $Na_2SO_4$ (final conc. 1.4%) are added to each flask, and incubation is continued for a 10 min chase.

All protein synthesis is then interrupted by adding both cycloheximide and chloramphenicol to the control flask, cycloheximide to the flask which already contained chloramphenicol, and chloramphenicol to the third flask, which already contained cycloheximide.

The three $^{35}S$ cell suspensions were centrifuged; to each was added an equal amount of cells which had been grown in minimal medium containing 3 mCi [$^{3}H$]lysine, in 1000 ml, and of cold carrier cells grown on rich medium. Both chloramphenicol and cyclo-

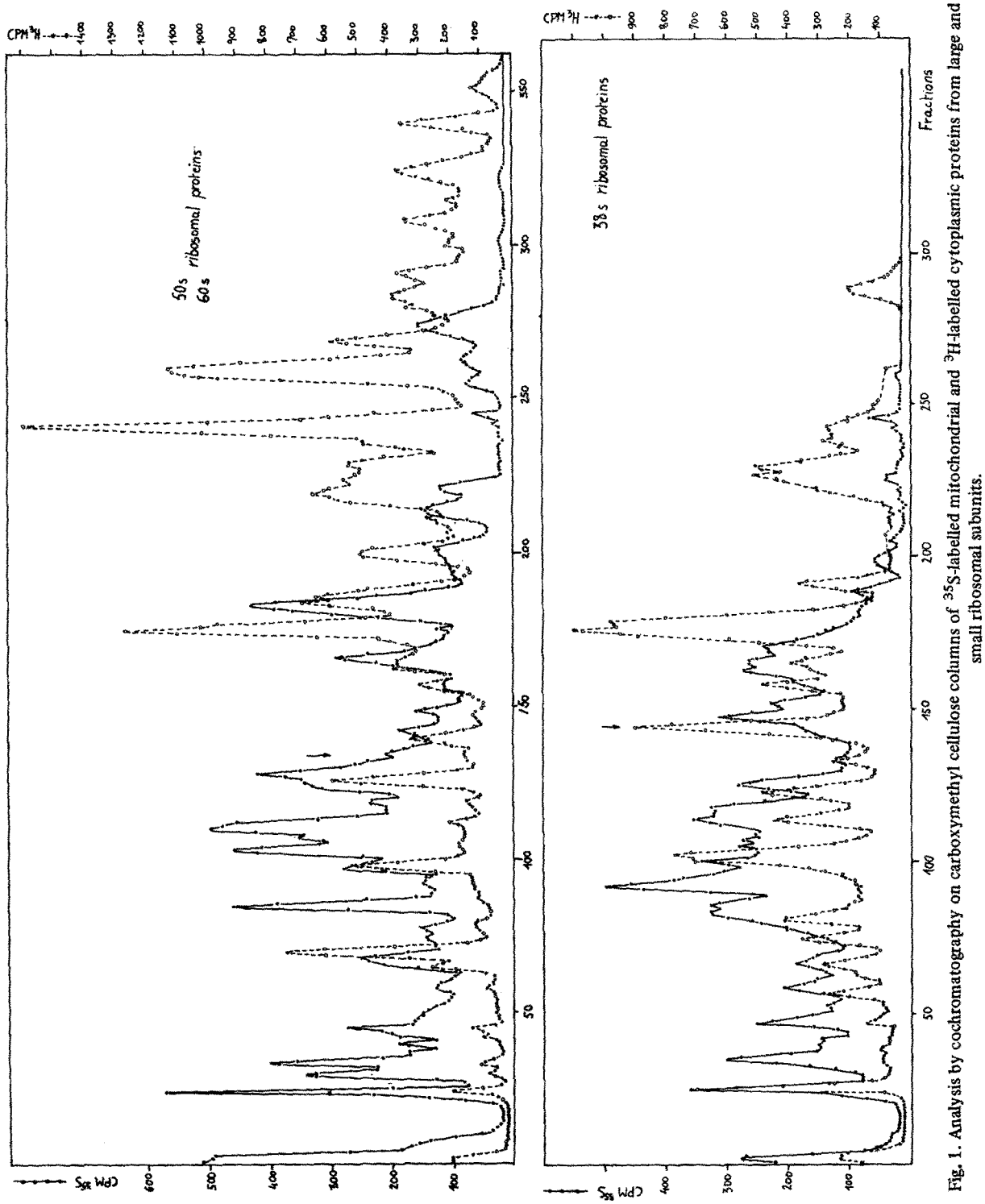


Fig. 1. Analysis by cochromatography on carboxymethyl cellulose columns of $^{35}S$-labelled mitochondrial and $^{3}H$-labelled cytoplasmic proteins from large and small ribosomal subunits.

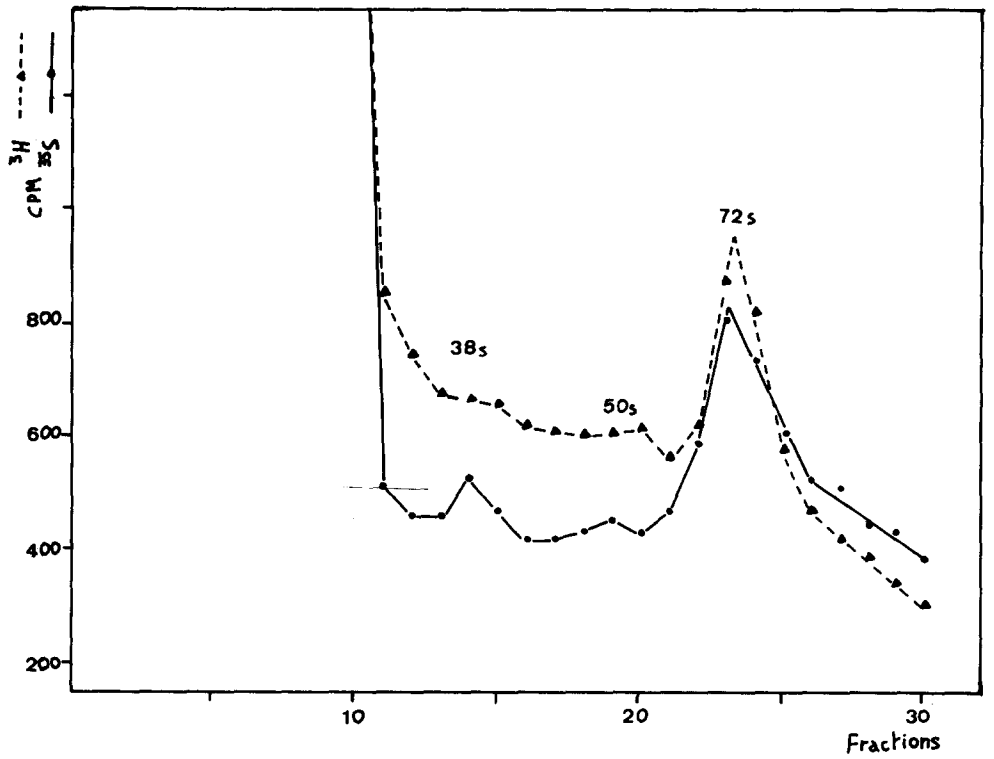


Fig. 2. Sucrose gradient analysis of mitochondrial ribosomes labelled with $^{35}SO_4$ without antibiotics in cell culture. The $^{3}H$-curve indicates the position of the particles labelled with [$^{3}H$]lysine in exponentially growing cells.

heximide were added to the mixture, and mitochondrial ribosomes were isolated as previously described [7]. The ribosomes were submitted to sucrose gradient centrifugation and the distribution of $^{3}H$ and $^{35}S$ was determined. Fig. 2 is the control. Fig. 3 shows that in presence of cycloheximide no $^{35}S$ was incorporated into the ribosomes or ribosomal subunits of mitochondria. It can be concluded therefore that inhibition of the cytoplasmic system of protein synthesis prevents the formation of the proteins of mitochondrial ribosomes, or their incorporation into newly formed ribosomal particles. This means that at least some (and possibly all) of the proteins of the mitochondrial ribosomes are made by the cytoplasmic system of protein synthesis.

Table 1
Incorporation of $^{35}SO_4$ into cytoplasmic ribosomal proteins in presence or absence of cycloheximide or chloramphenicol.

| Sample | Specific radioactivity (cpm $^{35}S$/100 000 cpm $^{3}H$) | Inhibition (%) |
|---|---|---|
| Control | 30 500 | 0 |
| + Cycloheximide | 710 | 97.7 |
| + Chloramphenicol | 19 700 | 35 |

Fig. 4 shows that chloramphenicol exerts a partial inhibition on the incorporation of $^{35}S$ into mitochondrial ribosomes; this can probably be explained by the known inhibition exerted by chloramphenicol on the formation of the mitochondrial respiratory chain and the associated phosphorylation system, as a similar inhibition is observed on the incorporation of label into cytoplasmic ribosomes (table 1).

Our results establish clearly that at least part of the ribosomal proteins of yeast mitochondria are made in the cytoplasm. This confirms preliminary results obtained by Kuntzel [6] and by Davey et al. [16].

The extrachromosomal localization of chloramphenicol and erythromycin resistance in some mutant yeast strains had brought their authors to infer that

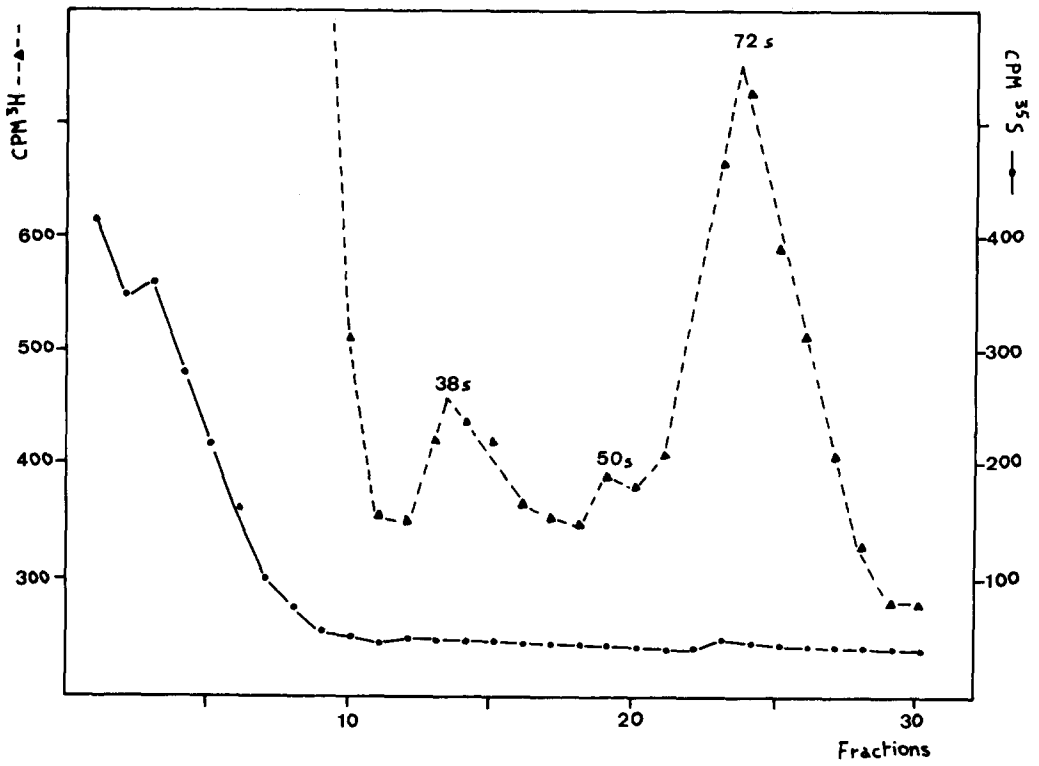


Fig. 3. Sucrose gradient analysis of mitochondrial ribosomes labelled with $^{35}SO_4$ in presence of cycloheximide.

mitochondrial genetic information could code for mitochondrial ribosomal proteins [1–5].

Our results do not completely exclude this possibility as mitochondrial mRNA could be exported and read on cytoplasmic ribosomes [17].

It seems however that an alteration in rRNA would better explain antibiotic resistance in those strains where ribosomes are the cause of resistance.

Mutations of rRNA are uncommon in antibiotic-resistant bacterial cells; this is probably due to the redundancy of their cistrons. Mitochondrial DNA on the contrary contain only one copy of each rRNA gene [5].

Recently bacterial mutants resistant to kasugamycin and erythromycin have been shown to be affected in rRNA methylation [18, 19], so that modification of a methylase could also be a cause of antibiotic resistance in yeast mitochondrial ribosomes.

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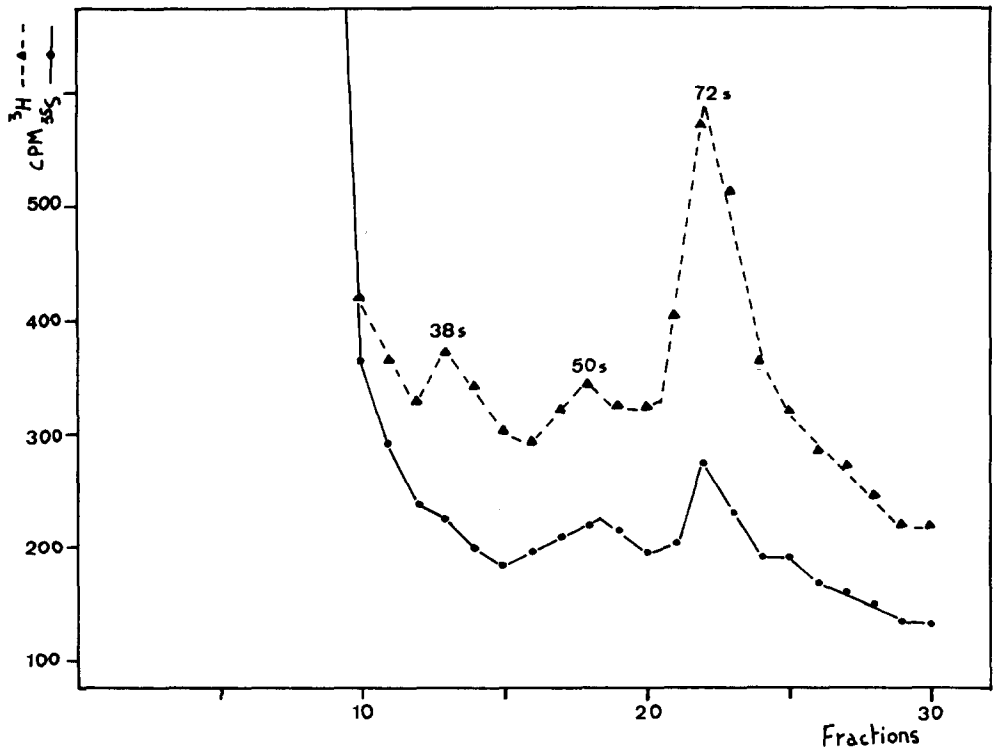


Fig. 4. Sucrose gradient analysis of mitochondrial ribosomes labelled with $^{35}SO_4$ in presence of chloramphenicol.

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