# ALTERED MITOCHONDRIAL RIBOSOMES IN A CYTOPLASMIC MUTANT OF YEAST

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## 1. Introduction

Yeast mitochondrial protein synthesis is normally sensitive to a wide spectrum of antibiotics effective on bacterial ribosomes [1, 2]. Mutant strains, resistant to one or several of these antibiotics have been isolated [3] and in some of these mutants resistance is inherited cytoplasmically [4–8], implying that mitochondrial DNA specifies the component changed. Such mutants, therefore, provide a promising approach for the identification of mitochondrial genes.

Bunn et al. [9] have shown that two types of cytoplasmic mutants can be distinguished. The first type is isolated as resistant to mikamycin, but is cross-resistant to several other unrelated antibiotics. The mitochondria of this class of mutants lose resistance to the antibiotics on isolation. They are therefore thought to possess an altered inner membrane, impermeable to the antibiotics *in vivo*, but made leaky by the process of isolation. The second type of mutant is isolated as resistant to erythromycin, but often shows cross-resistance to several macrolides and lincomycin [5, 7], a phenomenon also observed for some bacteria [10]. Mitochondria isolated from these mutants retain resistance *in vitro*, even if the mitochondrial membrane is damaged by freezing and thawing and this led Linnane et al. [5] to propose that resistance in these mutants is due to an altered mitochondrial ribosome. This experiment is not conclusive, however, because amino acid incorporation of frozen-thawed mitochondria is very low (cf. [5]) and this low residual activity could merely reflect the presence of intact organelles remaining after the treatment rather than the presence of resistant ribosomes. Unambiguous proof that the ribosome itself is changed requires, therefore, the isolation of mitochondrial ribosomes with good catalytic activity.

We have recently succeeded in isolating mitochondrial ribosomes from yeast that are highly active in catalyzing poly U-directed polyphenylalanine synthesis [11]. Using such ribosomes, we show in this paper that resistance to erythromycin in mutant 6–81c is due to a change, not in the mitochondrial membrane, but in the mitochondrial ribosome itself.

## 2. Methods and materials

6-81c, a strain of *Saccharomyces cerevisiae* isolated as a spontaneous cytoplasmic mutant resistant to greater than 4 mg/ml erythromycin (cf. [7]) was, together with the wild-type strain D-6 *arg me*, generously donated by Drs. Thomas and Wilkie**. Yeast was grown and mitochondria were prepared essentially according to the method of Ohnishi, Kawaguchi and Hagihara [12]. A four-times washed mitochondrial fraction (cf. [13]) was lysed in 50 mM $NH_4Cl$, 10 mM magnesium acetate, 10 mM tris-Cl (pH 7.5), 6 mM 2-mercaptoethanol (AMT), containing 0.3%

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sodium deoxycholate and ribosomes were isolated and purified by a method to be described fully elsewhere [14].

Assay of amino acid incorporation by intact mitochondria *in vitro* was as described by Grivell [15]. The incubation medium contained mitochondria at a final concentration of approximately 1 mg/ml, 150 mM KCl, 44–60 mM mannitol, 20 mM tris base, 10 mM $KH_2PO_4$, 10 mM $MgCl_2$, 5 mM 2-oxoglutarate, 2 mM ATP, 2 mg/ml bovine plasma albumin. A further, small amount of tris base was used to adjust the medium to pH 6.7. $^{14}$C-L-Leucine (62 mCi per mmole; Radiochemical Centre, Amersham, England) was 16 $\mu$M. Incubation was for 30 min at 30° in a total volume of 0.5 ml.

The measurement of poly U-directed polyphenylalanine synthesis was carried out as described by Hosokawa, Fujimura and Nomura [16] in the presence of a supernatant fraction prepared from *Escherichia coli* by alumina grinding, centrifugation at 105,000 *g* for 4 hr and overnight dialysis versus AMT. The incubation mixture contained 15 mM magnesium acetate (instead of 10 mM), 660 $\mu$g protein of the supernatant fraction per ml; mitochondrial ribosomes corresponding to an $A_{260\ nm}$ of 6 per ml and 5 $\mu$M $^{14}$C-phenylalanine (U) (477 mCi per mmole; Radiochemical Centre, Amersham, England).

The peptidyl transferase activity of mitochondrial and *E. coli* ribosomes was assayed at 0° as described by De Vries, Agsteribbe and Kroon [17]. The assay is a modification of the fragment reaction of Monro, Cerna and Marcker [18] and uses acetyl $^{3}$H-leucyl-tRNA as substrate in place of CACCA-$^{3}$H-Leu-Ac. The specific activity of the $^{3}$H-4,5-leucine used was 10 mCi per mmole.

Ribosomes were prepared from *E. coli* Q13 as described by Nirenberg [19].

Erythromycin base was obtained from Abbott and lincomycin hydrochloride monohydrate from Upjohn.

## 3. Results

Growth of strain 6-81c on glycerol-containing media is resistant to concentrations of erythromycin greater than 4 mg/ml, while that of the parent strain, D-6, is sensitive to less than 100 $\mu$g/ml (D. Thomas and D. Wilkie, personal communication). This difference between the strains is also observed when incorporation of $^{14}$C-leucine by intact mitochondria *in vitro* is measured (fig. 1A). Similar results have been reported for other, independently isolated mutants [5, 7].

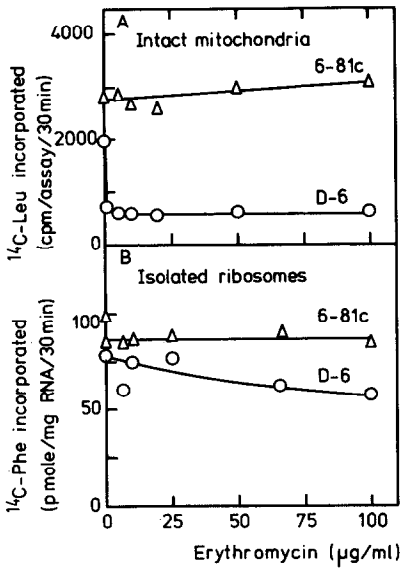


Fig. 1. Effects of erythromycin on protein synthesis by (A) intact mitochondria and (B) isolated mitochondrial ribosomes. The erythromycin-resistant and wild-type strains are denoted by 6-81c and D-6, respectively.

In order to determine whether resistance was the result of a change in the mitochondrial ribosome, isolated ribosomes were tested for erythromycin sensitivity. Mitochondrial ribosomes were combined with a supernatant fraction obtained from *E. coli* (see sect. 2) and the effect of erythromycin on poly U-directed phenylalanine incorporation was measured. The rate of amino acid incorporation in this system was 80–100 pmoles per mg RNA per 30 min and the degree of stimulation by poly U was 20-fold.

Polyphenylalanine synthesis by ribosomes from 6-81c, the resistant mutant, was completely insensitive to erythromycin (fig. 1B). However, activity of ribosomes from the wild-type strain, D-6, was only slightly inhibited even at concentrations of the antibiotic as high as 100 $\mu$g/ml. A similar insentivity to erythromycin of poly U-directed incorporation has been reported for wild-type ribosomes from *E. coli* [20]. It was clear, therefore, that this system could not distinguish in a conclusive manner between antibiotic resistant and sensitive ribosomes, even though the difference in sensitivity shown in fig. 1 was quite reproducible in different ribosome preparations.

Experiments in which poly A or poly C were used as synthetic messenger were also not satisfactory: although incorporation directed by these polynucleotides is highly sensitive to erythromycin when bacterial ribosomes are used, the activity of mitochondrial ribosomes was too low with these messengers to permit response to antibiotics to be tested reliably.

Two other approaches were successful, however. First, use was made of the observations that resistance to many macrolide antibiotics and lincomycin is often linked [4, 5, 10] and that poly U-directed phenylalanine incorporation is sensitive to lincomycin [21]. Analysis of the response of mitochondria isolated from the two strains indeed showed that amino acid incorporation by the wild-type strain, D-6, was completely inhibited by $10^{-3}$ M lincomycin, whereas incorporation by mitochondria from the mutant strain, 6-81c, was only partially inhibited (fig. 2A). This difference in sensitivity was retained when the isolated ribosomes were analysed. Polyphenylalanine synthesis by the wild-type ribosomes was totally inhibited by $10^{-5}$ M lincomycin, but even $10^{-4}$ M lincomycin failed to inhibit the mutant ribosomes by more than 50% (fig. 2B). The results strongly suggest that resistance

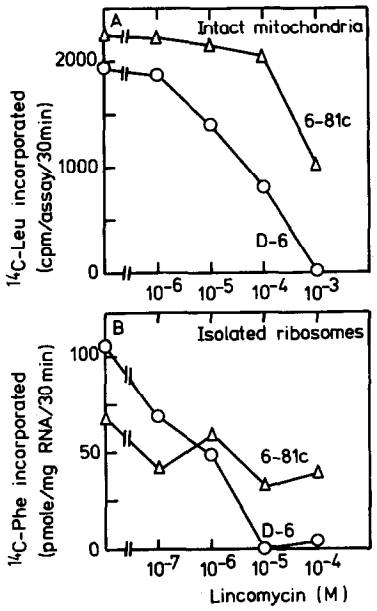


Fig. 2. Effects of lincomycin on protein synthesis by (A) intact mitochondria and (B) isolated mitochondrial ribosomes.

to erythromycin in strain 6-81c is associated with a change in the mitochondrial ribosome and that this change is also involved in determining resistance to lincomycin.

This conclusion was confirmed by direct assay of the effects of the two antibiotics on ribosomal peptidyl transfer, measured by the fragment reaction [18]. This reaction is completely inhibited by chloramphenicol, but not by many macrolide antibiotics [22]. It is possible, however, to test the effect of these macrolides in an indirect way, by their ability to compete effectively with chloramphenicol for binding to the ribosome. The inhibitory effect of chloramphenicol on the formation of acetyl-phenylalanylpuromycin by wild-type ribosomes from *E. coli* can be completely reversed by erythromycin [23].

Table 1 presents characteristics of the peptidyl transferase activity of mitochondrial ribosomes from D-6 and 6-81c as assayed by a modified form of the fragment reaction, using acetyl $^{3}$H-leucyl-tRNA as substrate [17]. Data obtained with *E. coli* ribosomes are included for comparison. In agreement with the observations of De Vries, Agsteribbe and Kroon [17] on the activity of mitochondrial ribosomes from *Neurospora crassa* in the fragment reaction, yeast mitochondrial ribosomes were highly sensitive to chloramphenicol, but only slightly affected by anisomycin, an inhibitor of peptidyl transferase activity of eukaryotic ribosomes. Wild-type ribosomes from strain D-6 resemble *E. coli* ribosomes in that inhibition by chloramphenicol is completely reversed by erythromycin. In contrast, erythromycin cannot reverse the chloramphenicol inhibition with mutant ribosomes.

Erythromycin alone markedly stimulated the fragment reaction by wild-type yeast ribosomes. This effect may result from an induced alteration in the ability of ribosomes to bind acetyl-leucyl-tRNA (cf. ref. [24]). It is of interest that significant stimulation is also observed with the ribosomes from the erythromycin-resistant strain 6-81c. This suggests that erythromycin still binds to these ribosomes but not strongly enough to inhibit protein synthesis or prevent binding of chloramphenicol.

Lincomycin inhibits the fragment reaction directly and this fact could be used to verify the cross-resistance of the ribosomes to this antibiotic (table 1). Activity of ribosomes from the wild type, D-6, was

Table 1
Antibiotic sensitivity of peptidyl transferase activity by mitochondrial and *E. coli* ribosomes.

| Additions or omissions | Acetyl $^3$H-Leucyl-puromycin formed by ribosomes from | | |
|---|---|---|---|
| | D-6 | 6-81c | *E. coli* |
| Control | 100 | 100 | 100 |
| Puromycin omitted | 4 (2–6) | 3 (2–4) | 3 (1.5–4.5) |
| Chloramphenicol 67 μg/ml | 7 (4–10) | 8 (5–10) | 18 (11–22) |
| Anisomycin 27 μg/ml | 101* | 93* | 95 (91–97) |
| Erythromycin 6.7 μg/ml | 163 (155–170) | 124 (121–126) | 109 (101–121) |
| Erythromycin 13.3 μg/ml | 163 (160–167) | 134 (125–142) | 99 (92–103) |
| Chloramphenicol + erythromycin 6.7 μg/ml | 102 (100–103) | 15 (10–20) | 100 (91–107) |
| Chloramphenicol + erythromycin 13.3 μg/ml | 100 (93–107) | 15 (10–20) | 107 (106–107) |
| Lincomycin $10^{-5}$ M | 10 (7–12) | 71 (59–82) | 40 (30–50) |
| Lincomycin $10^{-4}$ M | 2* | 46 (41–51) | 10 (9–11) |

* Results of a single determination.

Peptidyl transferase activity was measured using acetyl-$^3$H-leucyl tRNA as substrate. Values given are the mean (range) of two experiments with mitochondrial ribosomes and five experiments with *E. coli* ribosomes. Data have been corrected for blanks without ribosomes (107–126 cpm). In incubations with *E. coli* ribosomes approximately 60% of added acetyl-$^3$H-leucyl tRNA (22,000 dpm) was converted to acetyl-leucyl-puromycin. For mitochondrial ribosomes this value was approximately 50%.

totally inhibited by $10^{-4}$ M lincomycin. In contrast, ribosomes from 6-81c were only partially inhibited.

## 4. Discussion

Our experiments clearly show that a cytoplasmic mutation involving resistance to erythromycin results, at least in this mutant, in a change in the mitochondrial ribosome. We are at present investigating the nature of the component responsible for resistance. Although it would be reasonable to assume that this component, as in many bacterial mutants examined [25, 26], is a protein of the large ribosomal subunit, we have not excluded the possibility that it is the ribosomal RNA which is changed (see [11] for discussion).

Analysis of mutants was initially held up by our inability to find conditions that would allow the isolated mitochondrial ribosomes to effectively use mRNAs other than poly U. This complicated the analysis of resistance, because poly U-directed polyphenylalanine synthesis is rather insensitive to many antibiotics of interest. The results presented here show that the fragment reaction provides a convenient way of overcoming this problem for erythromycin and chloramphenicol and it may also be useful with other antibiotics that act on the 50 S ribosomal subunit.

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