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Increased expression of F_1ATP synthase subunits in yeast strains carrying point mutations which destabilise the β subunit

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In yeast strains (*S. cerevisiae*) carrying a point mutation of the *ATP2* gene, which destabilizes the β subunit of F_1 ATP synthase in vitro, the growth rate was reduced significantly, demonstrating that the mutation is also deleterious in vivo. Immunoblots showed that levels of the mutated β, but also of the wild-type α subunit were increased in the mutated strains, together with levels of the corresponding mRNAs (approximately 1.6-fold). Northern analysis showed that this was due to both the appearance of new transcript species as well as upregulation of the cognate transcripts, strongly indicating that the increase was probably due to activation of transcription. Levels of other mitochondrial proteins, e.g. cytochrome *c* oxidase, were unaffected. We conclude that a specific signal communicates the actual performance of the ATP synthase inside the mitochondria to the nuclear genes encoding its subunits.

Mitochondrion; F_1 ATP synthase; Gene expression; Intracellular signaling; Regulation of transcription; Point mutation; *Saccharomyces cerevisiae*

1. INTRODUCTION

The F_1 complex of mitochondrial ATP synthase consists of five different subunits encoded by the nuclear genome (α, β, γ, δ and ε) in a 3 : 3 : 1 : 1 : 1 stoichiometry, with a molecular weight of about 360,000 Da. The γ subunit seems to function as a proton gate, wheras the highly similar α and β subunits are responsible for the enzymatic reaction [1]. The amino acid sequence of the β subunit, which forms the largest part of the catalytic center, is conserved to the greatest extent, being 70% homologous in widely different species. The next best conserved subunit is α, which shows 50% homology [2]. The basic multi-subunit structure of the enzyme shows only minor differences when *E. coli*, chloroplasts, or mitochondria, obtained from yeasts and mammals, are compared [3].

In contrast to these structural data, our information about regulation of the biosynthesis and assembly of the multi-subunit enyzme is less detailed. It is well known that the synthesis of the whole complex is regulated at the level of transcription by the availability of fermentable glucose in yeast [4]. This mechanism of regulation is common to all proteins of the inner mitochondrial membrane [5]. The enzyme was postulated to be a key organizer of the self-assembly process of the inner mitochondrial membrane because, in mutant strains in which F_1 ATP synthase is completely absent, the quantities of other enzymes of the respiratory chain, such as cytochrome *c* oxidase, are severely reduced as well [6,7].

In this study, we have used yeast strains carrying point mutations of the β subunit in order to study the regulation of its expression and the synchronization with expression of other mitochondrial proteins. In these strains, the subunit is assembled into a functional complex, which, however, is extremely unstable in vitro [8]. We have asked how this instability affects the expression of the β subunit in vivo, both on the mRNA and the protein level, but also the expression of the α subunit and that of other mitochondrial proteins.

2. MATERIALS AND METHODS

2.1. *Yeast strains*

The genotypes of the *Saccharomyces cerevisiae* strains used have been described in detail elsewhere [8]. YJJ63 is the wild type strain; in DMY111cH3, the chromosomal *ATP2* gene coding for the β subunit of the F_1 ATPase was inactivated and reintroduced into the strain in a YCp50 single copy plasmid. In DMY111c21 and DMY111c14, the codon for Arg^{328}, which was shown to be important for stability [8], was replaced by lysine and alanine codons, respectively, by site-directed mutagenesis. For convenience, these strains will be called wild-type, Arg-Arg, Arg-Lys, and Arg-Ala strains, respectively.

2.2. *Growth media*

Yeast cells were grown at room temperature in liquid medium containing 1% yeast extract, 2% gelatin hydrolysate, and either 2% glucose or 3% glycerol. The rate of growth was measured by the absorbance at 600 nm of the culture grown on non-fermentable glycerol. For measurement of cytochrome *c* oxidase activity, and of the abundance of mRNAs and protein subunits of F_1 ATP synthase, cells were grown in glucose and harvested in the late exponential growth phase, when the switch from fermentative to aerobic growth occurs and mitochon-

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drial biogenesis is maximally induced. Because these parameters might fluctuate due to different culture conditons, e.g. small differences in cell density at the pre-chosen time of sampling, 3–6 cultures were grown and analyzed individually for each experiment in order to allow statistical analysis of the data.

2 3. *Assay of cytochrome c oxidase activity*

Enzyme activity was measured in fresh whole-cell extracts after digestion of the cell wall with Zymolase (ICN Inc.) by a spectrophotometric method [9]. The protein content of these samples and of samples used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was determined by the method of Lowry et al [10].

2.4. *Protein electrophoresis and immunoblotting*

Protein from purified mitochondria was separated by 12% SDS-PAGE [11] and either silver stained [12] or transferred to nitrocellulose filters by electro-blotting. The blots were incubated overnight with a polyclonal antiserum against the yeast F_1 ATP synthase in a 1 . 300 dilution [8]. After 1 h of incubation with goat anti-rabbit secondary antibody (Cooper Biomedicals), peroxidase staining was performed [13].

2.5 *cDNA and oligonucleotide probes*

The cDNAs for the α and β subunits inserted into pSP65 and pT7 plasmid vectors were generously donated by Dr Lewine, The University of Florida, and Dr. Douglas, The University of Texas, respectively. The isolated inserts were labeled by random priming [14]. A synthetic oligonucleotide corresponding to the anti-sense strand of the β cDNA (position Val^{194} to Ile^{200} according to Takeda et al. [15] as well as oligo-dT (18mer) were end-labeled with T4 kinase [14].

2.6. *RNA blotting and hybridization*

RNA was extracted by the acid guanidinium isothyocyanate method [16]. Samples were either applied in four serial dilutions (2, 1, 0.5, and 0.25 μg) to Nytran filters with a slot-blot apparatus (Schleicher & Schuell) or separated on formaldehyde-agarose gels and transferred to nitrocellulose. The filters were then consecutively hybridized to the oligo-dT probe, the α and β cDNAs, and, in some cases, to the anti-sense β oligonucleotide under standard conditions [14]. After hybridization and washing, the blots were exposed and the autoradiograms of the slot blots were scanned with a densitometer. Two or three data points in the linear range of the function densitometric values vs. μg blotted RNA were used to express mRNA levels. In order to reuse the filters, after each autoradiography the probe was removed by boiling in 0.01 × SSC, 0 01% SDS [14].

3. RESULTS

Table I shows that both strains carrying point mutations grew significantly more slowly than did the wild-type or the Arg-Arg strain. The effect of the conservative Arg-to-Lys exchange was less severe than that of the drastic Arg-to-Ala exchange. Despite these differences in growth rates, the cytochrome *c* oxidase activity was similar in all strains.

Table I

Generation time and cytochrome *c* oxidase activity in four yeast strains

	wild-type	Arg-Arg	Arg-Lys	Arg-Ala
Generation time (h)	8.23 ± 0.10	7.70 ± 0.15	9.45* ± 0.33	23.88* ± 0.26
Cytochrome *c* oxidase (nmol·mg^{-1}·min^{-1})	0.041 ± 0.006	0.053 ± 0.009	0.044 ± 0.005	0.060 ± 0.005

The data are given as means ± S.E.M. with $n = 5$ for generation time and $n = 3$ for cytochrome *c* oxidase. *Significantly different from both wild-type and Arg-Arg strain ($P < 0.01$), as determined by Student's *t*-test.

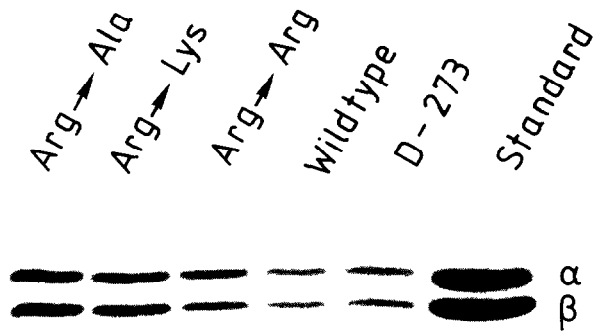

Fig. 1. Analysis of the abundance of α and β subunits of the F_1 ATP synthase by SDS-PAGE and immunoblotting in five strains of *Saccharomyces cerevisiae*; D-273 is a second wild-type strain. Samples were run together with a partially purified F_1 ATP synthase standard. The blot was probed with a polyclonal F_1 ATP synthase antiserum. The positions of α and β subunits are marked.

To determine the relative amounts of the α and β subunits of F_1 ATP synthase present in the different strains, mitochondria were isolated and mitochondrial protein was subjected to SDS-PAGE analysis together with a partially purified F_1 ATP synthase used as a standard; mitochondrial protein from another wild-type strain, D-273, was also loaded as a second control. The immunoblot of the gel (Fig. 1) showed that in both strains carrying point mutations, not only the abundance of the mutated β subunit, but also the content of the wild-type a subunit was markedly increased when compared to the Arg-Arg and to the wild-type strains. Silver staining of gels run in parallel showed the same result for these two subunits and also confirmed that equal amounts of protein had been loaded, as judged by the intensity of other, unidentified protein bands (data not shown).

Similar to protein levels, the abundance of both mRNAs encoding the α and β subunits was increased substantially in the Arg_Ala and Arg-Lys strains when compared to the Arg-Arg and wild-type strains (Table II). Small increases in the levels of both mRNAs were also noted in the Arg-Arg strain when compared to the wild-type strain. Hybridization to oligo(dT) confirmed that the same amount of poly(A)$^+$-RNA had been blotted in all strains (Table II), indicating that the increases were specific for these two mRNAs and were not due to a general increase in the amounts of poly(A)$^+$-messenger RNAs.

Northern analysis (Fig. 2) showed that, in the wild-type strain, both α and β cDNAs hybridized to one single transcript of the expected molecular weight [4,17]. However, in all other strains, which carry the β gene on

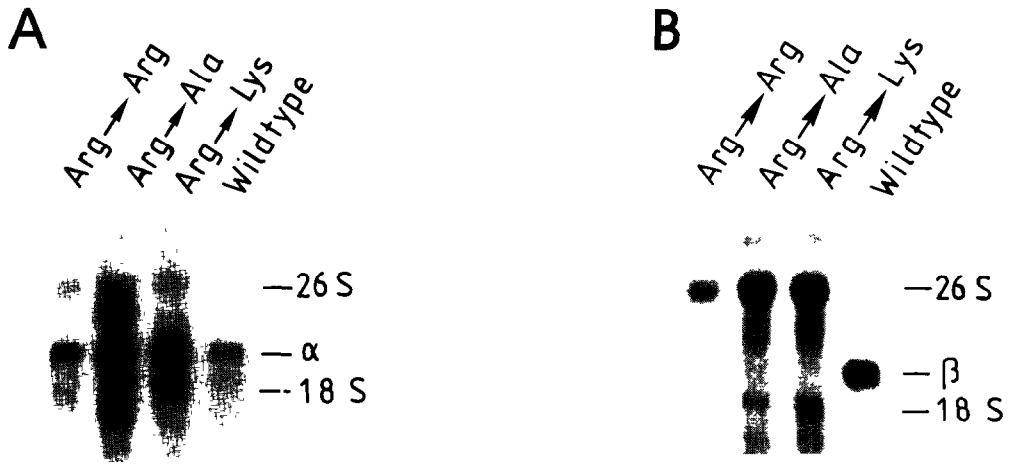

Fig. 2. Northern analysis of mRNAs coding for the α (A) and β (B) subunits of the F_1 ATP synthase in four strains of *Saccharomyces cerevisiae*. The positions of the cognate mRNAs in the wild-type strain are marked. The positions of 26 S and 18 S ribosomal RNAs are also indicated.

a plasmid, the results were different. The β cDNA hybridized to one major and three minor RNA species differing in molecular weight from the wild-type strain. Initially, we suspected that some of these species might be transcripts derived from the antisense strand of the plasmid, which contains a tetracycline resistance gene. If the transcription termination signal of this gene were leaky, transcripts extending into the *ATP2* gene would be synthetized, and these would hybridize to a double-stranded β cDNA. Thus, slot blots were probed with a 20mer oligonucleotide specific for the antisense strand within the *ATP2* gene, but they completely failed to hybridize (data not shown).

Thus, we hypothetize that all four transcripts detected in these strains are β mRNA species, derived both from the chromosomal *ATP2* gene, which still bears a functioning promoter [8], as well as from the β gene located on the plasmid.

In addition to the cognate α mRNA, two new transcripts appeared exclusively in the Arg-Ala strain; this will be discussed in detail later.

Table II

Relative amounts of poly(A)$^+$ RNA and of mRNAs encoding the α and β subunits of F_1 ATP synthase in four yeast strains, measured by slot blot analysis

	Poly(A)$^+$	Alpha	Beta
Wild-type	1.70 ± 0.20	3.50 ± 0.58	4.51 ± 0.31
Arg-Arg	1.80 ± 0.11	5.48 ± 0.14*	5.26 ± 0.12
Arg-Ala	1.56 ± 0.15	9.00 ± 0.16**	8.47 ± 0.15**
Arg-Lys	1.65 ± 0.15	7.29 ± 0 16**	7.50 ± 0.14**

The data are given as densitometric units/μg blotted RNA and are means ± S.E.M. (n = 6). Significance was analyzed by Student's t-test. *Significantly different from wild-type strain, $P < 0.01$; **Significantly different from both wild-type and Arg-Arg strains, $P << 0.01$.

4. DISCUSSION

How cells regulate their mitochondrial content and how the synthesis of all of the necessary proteins is coordinated during mitochondrial biogenesis is still largely unknown (for review, see [18]). Yeast cells offer a convenient model for the study of this question, because molecular-genetic approaches can be used and because the biogenesis of the whole organelle is regulated by the availability of fermentable glucose and oxygen.

In order to study coordination, several laboratories obtained mutant yeast strains in which either the α or the β subunit of the F_1 ATP synthase was completely absent. In most cases, this resulted in a drastic decrease of the other, non-affected subunit as well as of other proteins such as cytochrome *c* oxidase or NADH-cytochrome *c*-reductase [19–21]. However, in one nuclear *pet* mutant strain with severely reduced levels of the α subunit, the abundance of β and γ subunits was normal [17], and in one *ATP2* null mutant strain in which the β gene was disrupted, expression of the α subunit seemed to be essentially unaltered [15].

We have used yeast strains carrying well characterized point mutations of the β gene, which are less severe than the deletion mutants discussed above. In our strains, a functional F_1 ATP synthase is assembled, but the enzyme is unstable when assayed in vitro [8]. Because the β subunit is the most highly conserved subunit among the species, could it be the leading signal for regulation of the synthesis of the holoenzyme? Our results show that the destabilization of the complex obviously also impairs F_1 ATP synthase activity in vivo, leading to significantly slower growth rates of these strains when depending on oxidative phosphorylation, i.e. growth on a non-fermentable carbon source. At the same time, both the mitochondrial content of the mu-

tated β subunit, and that of the wild-type α subunit were increased. Despite these changes, levels of other, non-identified mitochondrial proteins, and specifically cytochrome *c* oxidase, were unaffected.

The increase in both ATP synthase subunits was accompanied by an increase in levels of mRNAs coding for these two proteins, indicating that regulation occurs at a pre-translational level. Such an increase can be caused either by an increased rate of transcription or by stabilization of the mRNA. Our Northern analysis provides some clues concerning these possibilities. In the Arg-Arg, Arg-Ala, and Arg-Lys strains, the β cDNA probe hybridized to four bands, which are probably transcripts derived from both the *ATP2* gene located on the plasmid, as well as from the inactivated *ATP2* gene located on the chromosome, which is interrupted by the *LEU2* gene [8]. Although only transcripts derived from the plasmid are translated into a functional protein, the levels of all transcripts are upregulated equally in the strains carrying the destabilizing mutations. Because regulation of mRNA degradation, and hence mRNA stability, is closely linked to the process of translation [22], we hypothetize that the increased levels of all transcripts are due to regulation of the activity of the cognate promoters on both the chromosome and the plasmid, and thus due to regulation of transcription. In the case of α mRNA, Northern analysis provided even more support for transcriptional regulation. In the Arg-Ala strain, where the β subunit is destabilized most severely, two new mRNAs are transcribed from the wild-type chromosomal *ATP1* gene. The differences in length compared to the wild-type message are too large to be explainable by differences in the length of the poly(A) tails, which generally confer differential stabilities to mRNAs [22]. Thus, we hypothetize that the use of previously undetected transcription initiation or termination sites in the *ATP1* gene is responsible for the two new transcripts. Mapping these different transcripts with oligonucleotides specific for certain regions of the two genes or S1 nuclease analysis will clarify their identity, but these experiments are beyond the aim of the present work.

We conclude that a specific signal communicates the actual activity of the F_1 ATP synthase to the promoters of the extramitochochondrial genes encoding both α and β subunits of the enzyme. In contrast to the *ATP2* mutant strain previously described [15], in which no β subunit was assembled at all, in our mutants both genes are activated, apparently as part of a mechanism designed to compensate the poor performance of the enzyme. Because no other mitochondrial proteins are affected, especially cytochrome *c* oxidase, this signal is obviously different from the one that induces a general upregulation of mitochondrial proteins, e.g. when the switch from fermentative to oxidative growth occurs. Thus, these yeast strains may be valuable tools for elucidating the pathway communicating the actual energetic output of the mitochondria to nuclear genes.

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