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Translational control of endogenous and recoded nuclear genes in yeast mitochondria: regulation and membrane targeting

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Abstract. Mitochondrial gene expression in yeast, *Saccharomyces cerevisiae*, depends on translational activation of individual mRNAs by distinct proteins encoded in the nucleus. These nuclearly coded mRNA-specific translational activators are bound to the inner membrane and function to mediate the interaction between mRNAs and mitochondrial ribosomes. This complex system, found to date only in organelles, appears to be an adaptation for targeting the synthesis of mitochondrially coded integral membrane proteins to the membrane. In addition, mRNA-specific translational activation is a rate-limiting step used to modulate expression of at least one mitochondrial gene in response to environmental conditions. Direct study of mitochondrial gene regulation and the targeting of mitochondrially coded proteins in vivo will now be possible using synthetic genes inserted into mtDNA that encode soluble reporter/passenger proteins.
Key words. *Saccharomyces cerevisiae*; mitochondria; mRNA-specific translational activation; synthetic genes; gene regulation.

Introduction

The chief function of gene expression within mitochondria is to supply a handful of hydrophobic protein subunits for membrane-bound respiratory complexes. One can infer from this fact that mitochondrial genetic systems are likely to be highly specialized for the production and assembly of integral membrane proteins. Are the mechanisms for targeting mitochondrial gene products to the inner membrane similar to those used by their presumed eubacterial ancestors? This question remains unanswered, but studies in yeast (*Saccharomyces cerevisiae*) suggest that membrane-bound mRNA-specific translational activators in these organelles may be an adaptation for protein targeting that has not (yet) been identified in bacterial or eukaryotic nucleo-cytoplasmic systems. Furthermore, these activators appear to play a role in modulating the levels of organellar gene expression.

The mechanisms employed in the regulation of mitochondrial genes, and the assembly of their products into functional complexes, have been difficult to study directly and remain poorly understood. Mitochondria must be descendants of procaryotic ancestors [1], but their gene expression systems [2–5] bear little clear resemblance to those of bacteria. For example, while yeast mitochondria contain a transcriptional specificity factor that resembles bacterial *sigma* subunits [6], the mitochondrial catalytic transcriptase of yeast [7], and apparently of most other eukaryotes [8, 9], is a single polypeptide resembling the RNA polymerases of phages T3 and T7. Furthermore, mitochondrial translation initiation does not utilize the bacterial mechanism of Shine-Dalgarno pairing between the mRNA and small rRNA [5], and only about half of the identified yeast mitochondrial ribosomal proteins have detectable homology with bacterial counterparts [10, 11] (see also the review by T. L. Mason in this issue of Experientia). Finally, yeast mitochondria probably do not contain a system highly similar to the eubacterial *sec* machinery for inserting their gene products into the inner membrane, and exporting some polar domains through it, since there are no genes coding for clear homologues of the known *sec* proteins in the virtually complete yeast genomic sequence.

Yeast mitochondrial translation depends on mRNA-specific translational activators encoded in the nucleus

A major tool for analyzing mitochondrial gene expression has been the study of yeast nuclear mutants that block it. These studies were pioneered by Gottfried Schatz and colleagues [12–15], whose work laid the foundation for demonstrating the importance of post-transcriptional steps in controlling mitochondrial genes. They identified nonrespiratory nuclear mutations in two genes that specifically blocked the production of cytochrome *c* oxidase by preventing accumulation of single mitochondrially coded subunits: the *pet494* mutant lacked coxIII and the *pet111* (originally *petE11*) mutant lacked coxII. This kind of specificity was surprising.

Mutants in a number of other nuclear genes causing similar phenotypes have been identified over the years,

affecting expression of several yeast mitochondrial genes [16]. None of these nuclear mutations affect mitochondrial transcription, but instead block various posttranscriptional steps in the organellar gene expression pathway, including translation: mutations in *pet494* and *pet111* block translation of the *COX3* and *COX2* mRNAs, respectively [17–20]. To date, the translation of five of the seven major yeast mitochondrially coded membrane proteins has been shown to depend on specific nuclear genes [4, 5, 21]. The best studied cases are translation of the *COX2*, *COX3* and *COB* mRNAs. (The eighth major yeast mitochondrial translation product is a hydrophilic ribosomal protein.) Interestingly, translation of at least some chloroplast mRNAs also depends on the activity of specific nuclear genes [22].

mRNA-specific translational activator proteins are associated with the inner mitochondrial membrane

Translation of the *COX3* mRNA depends on the proteins specified by three nuclear genes, *PET494* and two others discovered subsequently, *PET54* and *PET122* [23–26]. (*PET54* is also required for splicing an optional intron in the *COX1*-pre-mRNA [27].) These proteins were detected immunologically exclusively in mitochondria, although Pet494p and Pet122p were detectable only in strains that overproduced them [18, 24, 28]. Analysis of submitochondrial fractions from wild-type cells revealed that roughly half of the Pet54p was bound to the inner membrane while the rest was soluble in the matrix [28]. The membrane bound Pet54p was extractable with alkaline carbonate, indicating a peripheral association. While this behavior is typical for yeast mitochondrial ribosomal proteins, Pet54p could not be detected in ribosomes. Both overproduced Pet122p and overproduced Pet494p were strongly membrane bound, substantially resisting extraction with alkaline carbonate.

While it has not yet been possible to purify a complex containing the three *COX3*-mRNA-specific translational activator proteins, it is clear that they interact with each other. The best evidence for physical interactions comes from studies using the yeast two-hybrid system, which detects protein-protein interactions by virtue of their ability to bring two halves of a transcriptional activator together in the yeast nucleus [29]. Pairwise interactions were detected between Pet54p and Pet122p, as well as between Pet54p and Pet494p, but not between Pet122p and Pet494p [30]. However, the ability of all three proteins to interact simultaneously was demonstrated by bridging the interaction between Pet122p and Pet494p with Pet54p in a trimeric complex (N. G. Brown, J. B. Silverman and T. D. Fox, unpublished observation). In addition, a functional interaction in mitochondrial gene expression was demonstrated by allele-specific suppression of a *pet54* missense mutation by a missense substitution in *PET122* [30].

Translation of the *COB* mRNA, encoding apo-cytochrome *b*, depends on two nuclear genes, *CBS1* and *CBS2* [31, 32]. The proteins encoded by these genes are both located in mitochondria and are membrane-bound [33, 34]. Cbs1p is an integral membrane protein while Cbs2p is peripherally associated and may also be associated with the small subunit of mitochondrial ribosomes. While no interactions have been detected between Cbs1p and Cbs2p it is nevertheless likely that they are components of a *COB*-mRNA-specific translational activator complex.

PET111 is the only nuclear gene known to be required specifically for *COX2* translation. Its protein product is located in mitochondria [35] and, when overproduced to allow detection, behaves like an integral membrane protein (C. A. Strick and T. D. Fox, unpublished observation).

mRNA-specific translational activators recognize sites in the 5′-untranslated leaders of their target mRNAs and interact functionally with the mitochondrial ribosomal small subunit

The specificity of translational activation for mitochondrial mRNAs is determined by their 5′-untranslated leaders (5′-UTLs). This was clearly shown by examining in vivo translation of chimeric transcripts with the 5′-UTLs of the *COX2*, *COX3* and *COB* mRNAs fused to each other's coding sequences and 3′-trailers [20, 32, 36]. In each case, translation of the chimeric mRNA depended on the translational activator specified by the 5′-UTL.

There has been limited success to date in defining target sequences within the 5′-UTLs. The *COX3* and *COB* 5′-UTLs are 613 and 954 bases long, respectively, and highly rich in A + U. The results of deletion analyses have been complex, presumably because these leaders have extensive folded structures. Nevertheless, the phenotypes of *COX3* 5′-UTL deletions and their revertants indicate that the activation site of that mRNA lies in a 151 base region between −480 and −330, relative to the translation start [37, 38]. Deletion analysis of the *COB* mRNA 5′-UTL indicates that bases −954 to −898, and/or −170 to −1 contain a target for translational activation [39]. The *COX2* mRNA 5′-UTL is only 54 bases long, the shortest of the major mitochondrial messages. Thus the prospects for defining an activator target appear best here. Indeed, Clark-Walker and coworkers have identified a short conserved sequence in the *COX2* 5′-UTLs of several budding yeasts [40, 41]. Mutational analysis of the *S. cerevisiae COX2* 5′-UTL in this lab has defined a 31 base region required for translation, that includes this conserved sequence in a stem structure (H. M. Dunstan and T. D. Fox, unpublished observation).

Analysis of genetic interactions shows clearly that the *COX2*- and *COX3*-specific activators have intimate functional interactions with their mRNA targets, most probably reflecting direct contact. In both cases mutations in the 5′-UTLs, generated in vitro and inserted into mtDNA in place of wild-type sequences by transformation and homologous recombination, could be suppressed allele-specifically by missense substitutions in translational activator proteins and overproduction of wild-type activator proteins. Certain mitochondrial mutations affecting the *COX3* 5′-UTL were suppressed by alterations of Pet122p and overproduction of Pet494p [37, 38]. A mutation affecting the *COX2* 5′-UTL was suppressed by a missense substitution in Pet111p and by overproduction of wild-type Pet111p [42]. Consistent with the idea that these genetic interactions reflect physical interactions, all three subunits of the *COX3*-specific activator complex can be cross-linked to RNA in vitro by UV radiation, although the reaction is not specific for the *COX3* 5′-UTL (C. A. Butler, G. Wiesenberger and T. D. Fox, unpublished observation).

The translational activation pathway must at some point involve general components of the mitochondrial protein synthetic machinery. In at least the case of *COX3*-specific activation, this component appears to be the ribosomal small subunit. Mutations that truncate the carboxy-terminus of the activator subunit Pet122p are nonfunctional but are suppressed by nuclear mutations in the genes coding any one of three mitochondrial ribosomal small subunit proteins [43–46]. These suppressors do not restore function to *pet122* deletions or a *pet122* missense mutation, indicating that they work by specifically restoring the interaction of the ribosome with truncated Pet122p. Although all three of these ribosomal proteins are required generally for mitochondrial protein synthesis, they are not homologous to known ribosomal proteins from other systems. Thus, they appear to belong to the group of mitochondrial ribosomal proteins that carry out functions unique to this organellar system [47]. Recent evidence indicates that other small subunit ribosomal proteins of this class may work in conjunction with mRNA-specific activators to recognize targets in mRNA 5′-UTLs (N. S. Green-Willms, M. C. Costanzo and T. D. Fox, unpublished observation).

Translation initiation at the surface of the inner membrane?

It appears that yeast mitochondrial ribosomes cannot simply initiate translation by interacting, unassisted, with mRNAs in the matrix. Instead, productive association of ribosomes with mRNAs seems to require mediation by mRNA-specific translational activators that recognize targets in the mRNA 5′-UTLs and interact with the ribosomal small subunit to promote translation. Since the translational activators studied to date are associated with the mitochondrial inner membrane, it appears that translation can only begin after an mRNA has been localized to the membrane surface. Thus this system could provide a method of targeting translation of mitochondrial gene products to their sites of insertion into the membrane, an end achieved using different means via the SRP system in higher eukaryotic cytoplasms. Very little is known about the mechanisms employed to insert mitochondrial gene products into the inner membrane and, in some cases, export polar domains through it. Available evidence from studies of in organello translation suggests that insertion occurs cotranslationally [48], although posttranslational insertion is possible [49].

For comparison, it is interesting to consider the one major yeast mitochondrial translation product, Var1p, that is not inserted into the inner membrane. Var1p is a hydrophilic protein of the yeast mitochondrial ribosomal small subunit [50, 51]. No nuclear mutations blocking expression of the *VAR1* gene have been isolated. If there are translational activators specific for the *VAR1* mRNA, then mutations in their genes would have gone undetected in screens for nuclear mutants, since blocking Var1p synthesis would destroy mitochondrial protein synthesis entirely, leading to instability of *rho*$^+$ mtDNA [52]. However, a gene encoding Var1p in the universal genetic code has recently been synthesized [53]. Expression of this gene, *VAR1*^{u}, in the nucleus provides a cytoplasmically synthesized protein that is imported into mitochondria where it functions in mitochondrial ribosomes. Using strains containing *VAR1*^{u} to provide this essential ribosomal protein in trans, it will now be possible to examine genetically *VAR1* mRNA translation in mitochondria: are there *VAR1* mRNA-specific activators and if so are they membrane bound?

Low-level, modulated expression of a translational activator subunit

If translational activation is a rate-limiting step in mitochondrial gene expression, then expression of at least some activator complex subunits should be very low and might be modulated in response to environmental conditions that alter the levels of respiratory chain enzymes. Expression of the nuclear gene *PET494* has been carefully studied, monitoring the levels of β-galactosidase in cells containing a *pet494::lacZ* gene fusion chromosomally integrated at the *PET494* locus [54]. *PET494* expression is indeed modulated over a wide range depending upon growth conditions. Cells grown anaerobically on glucose medium express the *pet494::lacZ* fusion at one fifth the level of cells grown aerobically on glucose, while cells grown aerobically on

ethanol have six times the expression level of aerobic glucose-grown cells.

The absolute level of *PET494* expression is very low: the *pet494::lacZ* gene produced a β-galactosidase specific activity of 0.076 units/mg in extracts of aerobic glucose-grown diploid cells (expressed as nanomoles ONPG hydrolyzed per minute) [54]. This corresponds to roughly ten β-galactosidase polypeptides per diploid cell. (The estimate is based on a specific activity of 300,000 units/mg for pure β-galactosidase [55] and a total protein content of eight picograms per diploid yeast cell [56].) This is probably an overestimate of the number of Pet494p molecules per cell since β-galactosidase is very stable in yeast, accumulating to high levels when expressed by a strong promoter [57], while Pet494p appears to be relatively unstable [28]. Thus, the maximum possible number of Pet494p molecules per cell varies roughly between 2 and 60, depending upon growth conditions, and the true numbers are probably considerably lower. For comparison, one can use published data [58] in a similar calculation to estimate that there must be at least 10,000 sites per cell where cytoplasmically synthesized precursor proteins can be imported into mitochondria.

It appears, therefore, that there are a very limited number of sites on the surface of the mitochondrial inner membrane where translation of the *COX3* mRNA can initiate. Indeed, there are fewer such sites than there are copies of the *COX3* gene in mtDNA, which is present in roughly 100 copies per diploid yeast cell [59]. Sites for *COX2* mRNA translation are probably similarly limited since the expression level of the nuclear gene *PET111*, encoding its translational activator, is comparable to that of *PET494* (C. A. Strick and T. D. Fox, unpublished observation). Nothing is known about levels of the *COX1* mRNA-specific translational activators.

Why are there distinct translational activators for the three mitochondrially coded subunits of cytochrome *c* oxidase? Perhaps there is no selective advantage to this complexity. However, it is tempting to speculate that there may be a higher order organization of translational activators at a discrete number of sites on the inner membrane where synthesis and assembly of cytochrome *c* oxidase occurs. Distinct translational activators could allow synthesis and membrane insertion of the three subunits in a defined topological orientation that is advantageous for enzyme assembly.

Expression of a recoded nuclear gene within yeast mitochondria: an organellar reporter-gene passenger-protein system

Direct studies of mitochondrial gene regulation, as well as of targeting and assembly of mitochondrial gene products, requires the ability to express reporter genes encoding soluble proteins within the organelle. Standard reporter genes, such as *lacZ*, will not work because the genetic code in which they are written is different from that used in yeast mitochondria [60]. To circumvent this problem, a synthetic gene has been created that specifies, using the yeast mitochondrial genetic code, the soluble matrix enzyme acetylornithine aminotransferase, which is normally coded by the nuclear gene *ARG8* [61]. When inserted into mtDNA in place of the *COX3* structural gene, this synthetic gene, $ARG8^m$, fully complements the Arg^- growth phenotype of a nuclear *arg8* deletion, directs the production of an immunologically detectable soluble protein, and results in enzymatic activity that can be quantitatively assayed in crude cellular extracts. Expression of $ARG8^m$ at the *COX3* locus exhibits glucose repression and is dependent on the presence of all three subunits of the *COX3* mRNA-specific translational activator complex.

Quantitative assays of $Arg8^m$p levels in strains with a variety of nuclear genotypes indicate that expression of the nuclear gene *PET494* is indeed limiting for *COX3* expression [61]. For example, diploids heterozygous for a *pet494* deletion express the *cox3::*$ARG8^m$ gene at half the level of homozygous wild-type diploids. In contrast, heterozygosity for a *pet54* deletion has no effect on $Arg8^m$p levels. This is not surprising, since *PET54* is known to be expressed roughly ten-fold more highly than *PET494* [62]. A heterozygous *pet122* deletion reduces $ARG8^m$ expression by 38%, consistent with the observation that the level of *PET122* expression is comparable to that of *PET494* (B. A. Barlow and T. D. Fox, unpublished observation). Since expression of the nuclear gene *PET494* is regulated in response to environmental conditions [54] and is limiting for expression of the mitochondrial *COX3* gene, it appears that modulation of the mitochondrial gene is achieved at the level of translation via control of the nuclear gene.

In addition to providing an important new tool for the study of mitochondrial gene regulation, organellar expression of synthetic genes will allow direct in vivo approaches to other questions of cell biological interest. For example, $Arg8^m$p can be synthesized within mitochondria fused to domains of endogenous mitochondrial gene products and used as a passenger protein to study the process of membrane insertion and export to the intermembrane space (S. He and T. D. Fox, unpublished observation). In addition, mitochondrial expression of a recoded gene specifying the green fluorescent protein [63] allows visualization of the organelle in living cells based on the activity of its endogenous genetic system (J. S. Cohen and T. D. Fox, unpublished observation).

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