Determinants for binding of a 40 kDa protein to the leaders of yeast mitochondrial mRNAs

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ABSTRACT

An abundant yeast mitochondrial 40 kDa protein (p40) binds with high specificity to the 5'-untranslated region of cytochrome c oxidase subunit II (COX2) mRNA. Using mobility shift and competition assays, we show here that purified p40 complexes with the leaders of all eight mitochondrial mRNAs of Saccharomyces cerevisiae. The location of the protein binding site on the different leaders is not conserved with respect to the AUG start codon. In vitro RNA footprint and deletion experiments have been used to define the p40-binding site on the leaders of COX1 and ATP9 mRNAs. Nucleotides at, and near, a single stranded region are protected or exposed for DEPC modification by binding of p40 to these leaders. Removal of this region from the COX1 messenger shows that it is essential for the protein-RNA interaction. While no obvious sequence similarity can be detected between the single stranded regions in different leaders, a nearby helical segment is conserved. A consensus model for p40-RNA interactions is presented and the possible biological function of p40 is discussed.

INTRODUCTION

The 5' untranslated regions of mitochondrial mRNAs in the yeast Saccharomyces cerevisiae are exceptional in several respects. Most leaders are extremely long and A+U rich, and are not capped at their 5'-ends. The start codon is seldom the first AUG and is not surrounded by obvious discriminatory sequences [1]. These features seem to exclude a translation initiation mechanism either by direct ribosome-mRNA recognition as in prokaryotes [2], or by a scanning model for eukaryotic cytoplasmic translation initiation [3,4]. As a consequence, recognition of the leaders by mitochondrial ribosomes and translation initiation in yeast mitochondria are, at present, poorly understood processes. Genetic studies have shown that expression of a number of mitochondrial genes is specifically controlled at the level of translation by positive regulatory proteins encoded by nuclear genes [5,6]. Although translation of at least five out of the eight mitochondrial mRNAs requires the presence of these specific activators, their mode of action is largely unknown [7-16]. Rearrangements of mitochondrial DNA, leading to fusion of all or part of the 5'-untranslated region of one mRNA to the coding region of another, have been shown to suppress mutations in several nuclear genes that code for specific activators, localizing the site of action of these proteins to the leaders of mitochondrial mRNAs [15-17]. Some activators seem to associate with the mitochondrial small ribosomal subunit, others have been shown to interact with the inner membrane of mitochondria [18,19]. For the translation of COX3 and COB mRNA, this has led to the proposal of a model in which the activators function to target the mRNA and ribosome to the membrane where cytochrome c oxidase subunit III and cytochrome b are synthesized near their final destination [5,19].

The 5'-untranslated regions of numerous prokaryotic and eukaryotic mRNAs have been shown to be involved in the regulation of protein synthesis. While primary structure in the leaders has long been recognized as an important determinant for efficient translation initiation, higher order structures contribute significantly to the efficiency and fidelity of protein synthesis, by masking the ribosome binding site or start codon, or impeding the scanning ribosome [2,20,21]. Secondary structures are also thought to be involved in the internal initiation of translation on the leaders of picornaviral mRNAs [22]. The vast majority of leader binding regulatory proteins inhibit translation by stabilization of secondary structures. Translational repression of the synthesis of some E.coli ribosomal proteins [23,24], several bacteriophage encoded proteins [2] and the production of ferritin in eukaryotes [25] have been studied extensively. Apart from the mitochondrial trans-acting factors, examples of positive translational regulation by specific leader binding proteins are surprisingly scarce [26,27].

Previously we identified a protein with an apparent molecular weight of 40 kDa (p40) that interacts with the 5' untranslated region of the yeast mitochondrial COX2 gene [28]. The significance for binding of a single stranded area near the AUG-start codon was revealed by site directed mutagenesis and DEPC-footprinting. Furthermore, Bal31 deletion experiments indicated the importance of secondary structure for the formation of the correct binding site [28]. The protein was demonstrated not to be any of several

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genetically identified mRNA specific activators, and is abundant in yeast mitochondria (~25,000 copies per cell [29]).

In this study, we present evidence for the interaction of p40 with the 5' untranslated regions of mRNAs of all protein coding genes in mitochondria. In a number of leaders, the binding site is localized in different positions with respect to the start codon, but always adopts a similar secondary structure. A consensus model for p40 binding to the different leaders of mitochondrial mRNAs is presented. The possible role of p40 in mitochondria is discussed.

MATERIALS AND METHODS

Plasmid constructions and strains

All plasmids were constructed by standard techniques [30] in the transcription vectors pEP30 or pEP40 [31], harbouring the T7 and SP6 promoters on either side of the polynucler of pUC18. 5' deletions of the COXI leader were made by digestion of pKL41, containing a 2 kb Sau3A fragment spanning the entire COXI leader from S.cerevisiae KL14-4A mitochondrial DNA, with EcoRI, subsequent Bal31 exonuclease treatment, digestion with BamHI and blunt ligation into the Smal site of pEP40. The extent of the deletion of the resultant subclones COXIΔ28, Δ38, Δ13, Δ35, Δ31 and Δ43 was checked by sequence analysis. COX2 subclones have been described previously [28]. Subclones of pSP65XAI (kindly provided by Dr. T.D. Fox), containing the COX3 leader from the XbaI site at -610 until the Accl site at +20, were constructed by digestion with MboII or Dral plus HindIII, with subsequent blunt ligation in the Smal site of pEP40, yielding plasmids pCOX3MA and pCOX3DA. Constructs of the COB leader were generated by digestion of pBS/2-2 (gift of Dr. C. Dieckmann), encompassing a 1 kb COB leader fragment, with BamHI, Dral and EcoRI and subcloning of the fragments in the BamHI/EcoRI, BamHI/HinIII, Smal or Smal/EcoRI sites of pEP30, resulting in plasmids pCOBBE, pCOBBD, pCOBDD and pCOBDE. Plasmid pCOBBHE was made by digestion of pCOBDE with HindII, and back ligation. Leaders of ATP6 and ATP8 were obtained by cloning of a 2.5 kb EcoRI fragment (RR7), encompassing both leaders from S.cerevisiae KL14-4A, in the EcoRI site of pEP30 (pAP8-6). This plasmid was further digested with XbaI or MspI, and fragments were subcloned in the XbaI and Accl site of pEP30, yielding plasmids pATP6XE, pATP6M and pATP8M. The leaders of ATP9 and VARI were amplified by PCR on mitochondrial DNA of S.cerevisiae D273-10B, with primers complementary to the transcription start (5'-AATAATATATAGTTTATCTA-3') and the 3' end of the coding sequence of the ATP9 gene (5'-TTATAAACCGAGATTATAATAA-3'), or complementary to the VARI 5'-processing site (5'-ATAATGTTAATATAACTGC-3') and internal in the VARI coding sequence (5'-CCTAATTCCGTTGAAACACC-3'). Subsequent to amplification, fragments were treated with Klenow DNA polymerase, 5' phosphorylated and then cloned in the Smal site of pEP30, resulting in plasmids pATP9 and pVARI. All subclones were created in E.coli strains HB101 or JM101 and routinely checked with restriction enzymes and sequence analysis of the junctions.

In vitro transcription

Templates for run-off in vitro transcription were produced by digestion of subclones with suitable restriction endonucleases (schematically represented in Figure 3). After purification of digested DNA, run-off transcripts were made by incubation of 1 µg of template DNA for one hour at 37°C in the presence (for direct binding studies) or absence (for competition assays) of [α-32P]UTP, with T7 (pCOX1Δ28, pCOX1Δ31, pCOX1Δ35, pCOX2Δ21, pCOX2Δ29, pCOX2Δ33, pCOX3MA, pCOX3DA, pCOBBD, pATP8-6, pATP8M) or SP6 RNA polymerase (pCOXIΔ13, pCOXIΔ38, pCOXIΔ43, pCOX2Δ29, pSP65XAI, pCOBBE, pCOBDE, pCOBDD, pATP6XE, pATP6M, pATP9, pVAR1) according to standard procedures [32]. Unincorporated nucleotides were removed by purification over a 1 ml Sephadex-G50 column. Yields and quality of transcripts were routinely checked on agarose or polyacrylamide gels.

Analysis of RNA protein interactions

Analysis of p40 binding to the mitochondrial leader RNAs was essentially according to [28]. In effect approximately 2 ng of [α-32P] UTP-labeled RNA (50-150 cps) was mixed with 50-200 ng of purified p40 and 0.5-1.0 µg of either total purified E.coli or calf liver RNA (Sigma) to minimize non-specific p40-RNA interactions in a final volume of 30 µl binding buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 3 mM MgCl2, 50 mM NaCl, 5% glycerol). For competition experiments p40 was first incubated with a 25-100 fMolar excess of cold leader RNA for 15 min. at 30°C before addition of the radioactive labeled probe. Protein-RNA contacts were established in 15 min. at 30°C, after which complexes were resolved by electrophoresis through native polyacrylamide gels.

DEPC-footprinting analysis was essentially performed according to [28]. Approximately 500 cps (~20 ng) of 5'-end labeled COXlΔ13*DraI and ATP9*AsciI RNA (gel purified) was mixed with, 0.5-1.0 µg of purified p40 (or with an equivalent volume of protein storage solution) and 1 µg of calf liver RNA in 50 µl 50 mM sodium cacodylate (pH7.5), and incubated for 15 min. at 30°C (these conditions are sufficient to complex all COXI or ATP9 RNA, as assayed by gel retardation). 2 µl of DEPC (Sigma) was added to the incubation mixture and after thorough mixing the incubation was continued for 15 min. at 30°C. Analysis of modified RNA was essentially according to [33]. RNA was purified by phenolization and ethanol precipitation, and chain scission was induced by incubation in 20 µl freshly prepared 1 M aniline (Merck) in 0.3 M sodium acetate (final pH 4.5) at 60°C for 20 min. Two n-butanol precipitations were performed to remove the aniline, after which RNA pellets were carefully rinsed with ethanol, dried and resuspended in layer mix (0.03% bromphenol blue, 0.03% xylene cyanol, 25 mM EDTA in formamide). After boiling for 2 min., care was taken to layer equal amounts of radioactive material onto a 12% polyacrylamide-7 M urea gel. After fixation in 10% acetic acid, gels were dried and autoradiographed.

RNase T1 fractionation analysis

2000 cps (~80 ng) of [α-32P] UTP-labeled RNA transcribed from the appropriate templates (see figure legends) was mixed in a final volume of 50 µl binding buffer containing 2 µg of calf liver RNA, 1.5 µg of purified p40, and incubated for 15 min. at 30°C. Subsequently, the mixture was treated with 20 U of RNase T1 (Boehringer) for 15 min. at 30°C. 5 µl of the reaction mixture was phenolized and precipitated (total digest fraction), 30°C. Analysis of modified RNA was essentially according to [33]. RNA was purified by phenolization and ethanol precipitation, and chain scission was induced by incubation in 20 µl freshly prepared 1 M aniline (Merck) in 0.3 M sodium acetate (final pH 4.5) at 60°C for 20 min. Two n-butanol precipitations were performed to remove the aniline, after which RNA pellets were carefully rinsed with ethanol, dried and resuspended in layer mix (0.03% bromphenol blue, 0.03% xylene cyanol, 25 mM EDTA in formamide). After boiling for 2 min., care was taken to layer equal amounts of radioactive material onto a 12% polyacrylamide-7 M urea gel. After fixation in 10% acetic acid, gels were dried and autoradiographed.
was directly loaded onto a native 4% polyacrylamide gel in TBE and run for two hours at 30 mA at 4°C. Gels were directly autoradiographed and retarded material was eluted for 16 hours at room temperature in elution buffer. Eluted material was purified, resuspended in layer mixture, boiled for 2 min. and separated on 12% polyacrylamide—7 M urea gels.

Truncations by ribonucleases
2600 cpv (≈ 100 ng) [α-32P]UTP-labeled COX1Δ35*DraI RNA was digested in 20 μl binding buffer with 50 U of RNase T1 for 15 min. at 37°C. RNA was purified and separated on a 12% polyacrylamide—7 M urea gel, and directly autoradiographed. The appropriate RNase T1 fragment (COX1Δ35*T1) was eluted and purified by phenol extraction and ethanol precipitation. RNase CL3 digestion was done in the same manner, but ≈ 50 ng of COX1Δ35*T1 was digested with 0.05 U RNase CL3 (Boehringer) in 4 μl 8 M urea, 20 mM Tris-HCl (pH 8.0) for 15 min. at 50°C, and purified (yielding COX1Δ35*T1*CL3). The ribonucleosome is under these conditions C-specific.

RESULTS
p40 binds to the leaders of all mitochondrial mRNAs
Previously we demonstrated that full length leader RNAs of the COX1, COX3 and COB genes compete efficiently for binding of p40 to the leader of the COX2 gene. The antisense transcripts of the same constructs were unable to compete for this interaction, suggesting that p40 binds specifically to the leaders of these genes. Since yeast mitochondrial DNA harbours only seven major protein coding genes, beside COX2, we asked whether all 5′-untranslated regions of mitochondrial genes have the same effect. Mitochondrial DNA fragments accommodating the full length leaders of the ATP6, ATP8, ATP9 and VAR1 genes were cloned in the transcription vectors pEP30 as described in Materials and methods. In vitro transcripts of these constructs were assayed for competition of the COX2 leader-p40 interaction. As shown in Figure 1, an excess of sense transcripts from these constructs competes efficiently for binding of p40 to the leader of COX2 (lanes 6—9). Competition by these leaders was as efficient as competition by COX2 or COB leaders (compare to lanes 3—5). Since this protein-RNA interaction is not competed by transcripts lacking leader sequences, even when present in high excess, it is likely that all leaders of mitochondrial mRNAs contain one or more specific binding site(s) for p40.

Positioning of the p40 binding site within the leaders of mitochondrial mRNAs
Except for the leader of the COX2 gene, the leaders of mitochondrial genes are extremely large (>500 nt) and therefore not suitable for direct protein-RNA interaction studies by band-shift assays, as performed with the COX2 leader (Figure 1, lane 2). To investigate the binding of p40 to the leaders directly, they had to be truncated to more suitable sizes (<200 nt). Shorter run-off transcripts of several leaders were produced by digestion of the DNA with suitable restriction endonucleases. DNA fragments encoding the leaders were shortened at the 5′-ends by Bal3I exonuclease, or digested with restriction endonucleases, and subcloned in the transcription vectors pEP30 and pEP40 (see Materials and methods). This approach yielded a set of subclones containing relatively small fragments of all mitochondrial leaders (see Figure 3). Transcripts produced from these fragments were tested for their ability to interact with purified p40 by band-shift analysis. A large excess (>250 fold) of total E. coli or calf liver RNA, to compete for nonspecific binding, was always included in the binding reactions. Figure 2 shows a result of such an analysis. Only the smallest transcripts, of subclones of the leaders of seven mitochondrial mRNAs, that were still able to interact with p40 are indicated. Direct p40 binding to the VAR1 leader is not examined yet. At higher protein concentrations the RNAs sometimes complexed with p40 in two bands (see Figure 2). This might be due to binding of more than one p40 molecule to the leader, or to an alternative conformation of the RNA. The migration of both COX3Δ3*AcoRI (lane 3) and ATP8ΔM*Ndel RNA (lane 7) as two separate bands on this native gel is also likely to be a conformational effect since all RNAs used in binding experiments migrated as single bands on denaturing polyacrylamide gels. The capacity of all RNAs to interact with p40 is represented schematically in Figure 3. Since transcripts lacking leader sequences were not able to interact with p40 [28],
the binding of this protein to the leaders of mitochondrial mRNAs must be considered specific. Transcripts of larger subclones, containing all sequences required for binding, sometimes exhibited no binding activity (see Figure 3, COX1). Computation-aided folding, with the RNA-FOLD program [34] predicted, however, a different secondary structure for these RNAs than for the full length leader transcripts or the truncated RNAs that were still able to bind to p40 (data not shown). For instance, COX1 constructs COX1A38*BamHI and COX1A28*MspI, which do not exhibit p40 binding activity (see Figure 3), are predicted to fold entirely differently from COX1A28*EcoRI or COX1A13*DraI RNAs, which do show p40 binding. This indicates that secondary structure in the RNAs is an important parameter for binding of p40.

The RNA fragments of the different leaders that were still able to interact with p40, varied surprisingly in their position with respect to the AUG start codons of the different mRNAs (Figure 4). The binding site ranges from the 5'-end of the ATP9 leader, the middle of the COX1 leader to the 3'-end of the COX3 leader. The regions in the mitochondrial leaders sufficient for binding of p40 (hatched boxes in Figure 4) do not show a significant sequence match to the single stranded 5'-UAAA- GUUUAA-3' sequence motif shown to be essential for p40 binding to the COX2 leader [28]. All leaders compete, however, for binding to the COX2 leader (see Figure 1 and [28]), so they all probably encompass a similar binding site for p40. This again indicates that higher order structures are important in forming the binding site for p40.

Demarcation of the p40 binding sites on the leaders of COX1 and COX3

To further define the binding site of p40 on the leaders of mitochondrial mRNAs, we designed a technique that is based on the G-deficient character of the mitochondrial leaders. If the binding site contains G-residues, as with the COX2 leader, we expect to disturb the interaction with p40 by digestion with RNase T1. However, if the binding site does not contain any G-residues, it should be possible to isolate an RNase T1 fragment containing the binding site in the form of an RNA-protein complex. The protein-bound RNase T1 fragment can be specifically retained by isolation from a retardation gel or by direct filtration through nitrocellulose and further analysed.

We performed this experiment on a transcript that contained the entire COX1 leader (COX1Δ28*EcoRI), a shorter form of this (COX1Δ13*DraI), the entire COX3 leader (SP65XAI*HindIII) and a truncated form (COX3DA*EcoRI), that were all
shown to bind p40 (see Figure 3). In contrast to protein binding to the leader of COX2, complexes of the leaders of COXI or COX3 with p40 were shown not to dissociate upon digestion with RNase T1, as judged by the presence of a retarded RNase T1-fragment in gel mobility shift assays (results not shown). Only a 78 nt RNA fragment is specifically bound by p40 with both COXI transcripts (Figure 5, lanes 2 and 6). The isolation of the same RNase T1 fragment from the smaller RNA molecule (COXI A13*DraI) as from the full length COXI leader indicates that a bona fide p40 binding site is present on COXI A13*DraI, as from the full length COXI leader indicates that a bona fide p40 binding site is present on COXI A13*DraI. The COXI leader therefore only contains one efficient p40 binding site, located within this 78 nt RNase T1 fragment, that is situated between positions −385 and −307 relative to the initiation codon.

Figure 4. Localization of the p40 binding site on the leaders of all mitochondrial mRNAs. Only the in vivo 5'-untranslated regions, as far as the AUG initiation codon, are indicated. RNA fragments sufficient for p40 binding are hatched. Arrows indicate in vivo transcription starts, circles indicate putative processing sites.

Figure 5. RNase T1 fractionation analysis of the leaders of COXI and COX3. Total RNase T1 digests of COXI A28*EcoRI (lane 1), SP65XAI*Hinfl (lane 3), COXI A13*DraI (lane 5) and COX3*EcoRI (lane 7), and RNase T1 fractions collecting with p40 were isolated by nitrocellulose filtration (lanes 2 and 4), or elution from retardation gels (lanes 6 and 8), and separated on a 12% polyacrylamide/7M urea gel. Sizes in nucleotides of the RNase T1 fragments are indicated.

The p40 binding site on the COXI leader maps to a single stranded region

To determine more precisely the binding site for p40 within the 78nt RNase T1 fragment of COXI leader RNA, studies on transcripts that overlap with this fragment were carried out. Predicted secondary structure folding of the COXI A13*DraI RNA (from −424 until −288), that accommodates the complete 78 nt RNase T1 fragment, and was shown to interact with p40 (Figure 3), revealed that this molecule has the capacity to form two separate hairpin structures (Figure 6A). Cotranscribed polylinker sequences fold into a separate domain and are not depicted in this figure. The existence of both hairpins was confirmed by nuclease protection experiments, although it is probable that the base of each helix folds into one large single stranded loop since these nucleotides were more exposed to RNase A than nucleotides in the helices (data not shown). To localize the p40 binding site to either the 5'- or the 3'-half of the molecule, we produced shorter transcripts of COXI A13 by digestion of the DNA template with AseI (COXI A13*AseI) and by digestion of Bal31 deletion clone COXI A35 with DraI (COXI A35*DraI). These molecules accommodate helix 1 and of COXI A13*DraI respectively (Figure 6A), with conservation of wild type secondary structures predicted by computer modelling. Gel mobility shift experiments with both molecules and p40 shows that with the 5'-arm (COXI A13*AseI) only a minute amount of complex was observed, compared to the 3'-arm (COXI A35*DraI, compare lanes 2 and 3 to 5 and 6 of Figure 6B), so the main p40 binding site is located in the 3'-arm of COXI A13*DraI (helix 2). We investigated the COXI A35*DraI molecule further in two steps, making use of base-specific RNases. First, the 3'-17 nt were removed by RNase T1, leaving a molecule of 42 nt (COXI A35*T1) that consists entirely of bases belonging to the 78nt RNase T1 fragment isolated from the wild type leader (Figure 5, lane 2).
molecule was purified on a 12% polyacrylamide gel (7M urea) and is still predicted to form helix 2 and a 5' single stranded region (Figure 6A). As can be seen in Figure 6B this deletion has no effect on the affinity of the molecule for p40 (lane 8). In the second step we also removed the 5' 11 nt unpaired stretch of COX1Δ35*T1 with the C-specific RNase CL3 (COX1Δ35*T1*CL3). Removal of these bases completely abolishes the ability of the molecule to bind p40 (Figure 6B, lane 10). Since the only difference between COX1Δ35*T1 and COX1Δ35*T1*CL3 is the absence of the predicted single stranded stretch of nucleotides of the former molecule, the difference in p40 binding properties must be ascribed to this stretch. Therefore, we conclude that this single stranded region is an essential determinant in the formation of the recognition site for p40 in the wild type leader of COX1 mRNA.

p40 binding exposes and protects nucleotides against DEPC modification in the leaders of COX1 and ATP9

In order to identify the nucleotides directly involved in binding of p40, we performed diethylpyrocarbonate (DEPC) footprinting experiments. The reliability of such an experiment is heavily dependent on the size of the RNA molecule tested. Therefore we chose two small transcripts, that still retained the capacity to interact with p40, COX1Δ13*DraI (136 nt) and ATP9*Asel (92 nt, see Figure 3). Predicted secondary structures of both molecules are depicted in Figure 7B. As for COX1Δ13*DraI, the structure of ATP9*Asel was confirmed by nuclease protection experiments (data not shown). The computer predicted structure of this molecule was similar to those of larger ATP9 leader transcripts shown to interact with p40 and, therefore, probably folds as the wild type configuration of this part of the ATP9 leader. Purified RNA molecules were 5'-end labeled and, after incubation with purified p40, modified with DEPC (carboxyethylation at the N7-position of purines). The modified ribonucleotides were cleaved with aniline and separated on a 12% polyacrylamide gel (7M urea). Comparison of the modified nucleotides of COX1Δ13*DraI and ATP9*Asel RNAs in the absence or presence of p40 yields both protection and exposure of nucleotides (Figure 7A). Protected bases in the COX1Δ13*DraI RNA surround the single stranded region shown to be essential for p40 binding (see previous section). With the ATP9*Asel molecule, protection predominates in the 3' arm, near or at the loop (Figure 7A and B). The most likely explanation for this observation is that the single stranded region in the COX1Δ13*DraI molecule and the loop in the ATP9*Asel molecule are directly involved in binding of p40 and the nucleotides are thereby protected from DEPC modification by the protein. Exposed nucleotides in the COX1Δ13*DraI RNA are mainly present at the loop of the 3' hairpin, 3' of the protected nucleotides. Exposure in the ATP9*Asel molecule is oriented 5' of the protected area, on the 5' arm of the molecule (Figure 7A and B). Surprisingly, the exposure of nucleotides is, in both molecules, much clearer than the protection. Exposure of nucleotides probably originates from structural changes in the RNA molecules and could, therefore, be functionally significant. It should be noted that the exposed areas in both COX1 as in ATP9 have a potential to base pair with a region around their respective start codons (Figure 7C). Whether this ability is significant, or coincidental, remains to be established.

DISCUSSION

In order to obtain a definition of the p40 binding site, we have compared primary and secondary structure motifs in the leaders of COX1, COX2 and ATP9 mRNAs. By DEPC footprinting, site-directed mutagenesis and deletion analysis we have established that in the leaders of COX2 [28], COX1 and ATP9 (this study) a single stranded region appears to be required for p40 binding, but no obvious sequence similarity exists in this region of the leaders. Since p40 binds to the leaders with high specificity, an unpaired non-conserved sequence cannot be the only determinant for this interaction. Closer examination of the
three leaders reveals an helical region with conserved sequence in the immediate vicinity of the single stranded region (Figure 8). This suggests that p40 recognizes an helical region with conserved sequence 5'-UUUAUA-3' in combination with a stretch of unpaired nucleotides that is situated at the immediate 5' side of this sequence. The COX1A13*AseI molecule, which has residual binding activity (Figure 6B, lanes 2–3), contains a 'degenerate consensus' (G-U pair instead of A-U, Figure 8), that may be responsible for the marginal binding activity of the molecule.

The secondary structures proposed have been confirmed by nuclease protection experiments on the constructs COX1A13*DraI and ATP9*AseI. Computer modelling of large constructs of the COX1 and ATP9 leader, that have retained p40 binding activity, predicts similar local structures (data not shown). These have yet to be confirmed experimentally.

Some of the sequence similarities may be coincidental: the A+U richness of the leaders of mitochondrial mRNAs results in an almost one in two chance that nucleotides in similar positions in two RNAs will have the same base. However, the essential proximity of a single stranded region renders the binding site more specific. Studies on autoregulatory ribosomal proteins of E.coli revealed that often their RNA binding sites are short helical segments with unusual bulge and loop features [23]. Since the A form RNA helix has a deep, narrow major groove, which may exclude protein binding, helix sequences appear important only at base pairs adjacent to bulges or loops, where the helix is probably distorted from its regular conformation [23]. The consensus presented here for the p40 binding site shows something similar. The essential single stranded region is likely to have a weakening effect on the stability of the helical conformation, rendering the nucleotides more accessible to protein contacts.

In both prokaryotes and eukaryotes, primary and secondary structure features within the 5' leaders of mRNAs are often involved in regulation of gene expression. Secondary structures normally have an inhibitory effect on translation initiation, either
by shielding the ribosome binding site and/or the start codon in prokaryotes (reviewed in [2]), or inhibition of scanning by the eukaryotic ribosome (reviewed in [20]). Regulatory proteins usually interact with specific RNA sequences located in the 5' untranslated region and thereby stabilize secondary structure and repress translation. Activators of translation have also been identified, but are less common. Nuclear encoded factors that activate translation of specific mitochondrial mRNAs have been found in S.cerevisiae and are thought to interact with specific sites in the 5' leaders of the mRNAs [5]. The protein identified as p40 (28; this study) is capable of interacting with the leaders of all mitochondrial mRNAs (Figure 3). The steady state concentration of mitochondrial mRNAs, in derepressed conditions, is approximately 100 to 500 copies per transcript per cell [35]. Since the concentration of p40 is at least 25,000 copies per cell [29], the protein has the capacity to complex all mRNA molecules present.

The abundance and general character of p40 suggests to us it is ill-suited for fine regulation of protein synthesis. The apparent lack of conservation of the location of the p40 binding site on the different leaders (Figure 4) does not, however, exclude a direct or indirect function in the translation of the messages. For example, the L10-L7/L12 ribosomal protein complex of E.coli represses translation of rplJ mRNA by binding to a site that is more than 100 bases upstream of the affected ribosome binding site [36]. In analogy to this, virtually nothing is known about the secondary and tertiary structure of mitochondrial leaders, so the distance in bases between the p40 binding site and the initiation codon may not reflect their span in the folded RNA.

We previously suggested that p40 could activate translation in some way by interacting with mitochondrial mRNAs [28]. In two instances, however, mRNAs lacking putative p40 binding sites have been shown to be translated in yeast mitochondria. The 5' two-third of the 5' untranslated region of COX3 mRNA (as far as position -172) fused to the COB structural gene, lacks the 79nt RNase T1 fragment (-76 to +3) demonstrated to bind to p40 (Figure 5, lanes 4 and 8), but is still capable of directing translation of the COB gene [17]. This indicates that the p40 binding site at the COX3 leader identified by us (Figure 4) is not essential for translation initiation. However, p40 might stimulate COX3 mRNA translation by binding to the second site isolated by RNase T1 fractionation experiments (Figure 5, lane 4, 166 nt. fragment). If this is true, the function of the binding site nearer to the initiation codon is not clear.

Chimeric mRNAs containing the 5'-third of the COB leader attached to the 3'-half of the ATP9 leader can direct translation on the COB or ATP9 structural genes [37,38]. Both chimeric RNAs are stable and competent in translation initiation in the absence of the binding site for p40 in both the ATP9 leader (located at the 5' end), and in the COB leader (located at the 3' end). This appears to indicate that p40 is not essential for stability or translation of mitochondrial transcripts. However, as for the COX3 leader, more p40 binding sites could be present on the leaders of either COB or ATP9 or both, and stimulate translation of the chimeric mRNAs. In addition, the secondary structure of the fusion transcripts are likely to deviate considerably from the wild type folding of either of their constituents. The possibility that alternative p40 binding sites might be formed in these transcripts cannot be formally ruled out.

An intriguing possibility is that the function of the p40-leader interaction could be the stabilization of certain structures in the leaders, that may have a negative influence on the translatability of the mRNAs. We have noticed a marked increase in the stability of both the COXI13*Drax and ATP9*Asel molecules under the influence of p40, assayed by nuclease protection (data not shown). In addition, the interaction of p40 with these molecules also resulted in the exposure to DEPC of an area that has the capacity to base pair with the respective initiation codons (Figure 7C). A structural change in the leaders, imposed by p40, possibly renders this area more accessible to base pair contacts. Closer inspection of the DEPC footprint on the leader of COX2 (including the initiation codon) published earlier [28], reveals protection by p40 of nucleotides in the vicinity of the start codon. The base pair interaction may have already occurred here, shielding the start codon for ribosome recognition. If this base pairing is indeed a common phenomenon in all leaders, the initiation codons of all mRNAs could be shielded by a secondary or tertiary interaction imposed by p40, leading to an inhibition of protein synthesis.

Premature synthesis of the mitochondrially encoded hydrophobic proteins in the mitochondrial matrix could result in misfolding and degradation of the translation products. Association of p40 (which is located in the mitochondrial matrix, unpublished results) with all mRNAs could prevent premature protein synthesis in the matrix. This block in translation might only be reversible by interaction of this complex with the mRNA-specific activators, that are thought to act on the leaders of mitochondrial mRNAs [16,17]. Several of these trans acting factors are largely associated with the mitochondrial inner membrane [5,19] and two of them (PET122 and CBS2) interact with the small ribosomal subunit [18,19]. These factors have, therefore, the capacity to associate mRNA and ribosomes at the inner membrane of mitochondria, where the translation products can assemble into respiratory chain complexes. Translation of chimeric mRNAs lacking the p40 binding site would, in this model, only lead to a less economic protein synthesis, not to a block in translation. Some mRNAs might be prematurely translated in the matrix, because of the lack of translation inhibition, but others might still reach the specific translational activators at the inner membrane. Consequentially, the trans-activators would, in this case, still be required to target the mRNA and ribosome to the membrane. In this model, the presence of p40 ensures that the assembly of an initiation complex and translation of mitochondrial mRNAs only occur near the final destination of the protein, i.e. at the inner membrane.

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