Internal-ribosome-entry-site functional activity of the 3′-untranslated region of the mRNA for the β subunit of mitochondrial H$^+$-ATP synthase

José M. IZQUIERDO and José M. CUEZVA[1]

Departamento de Biología Molecular, Centro de Biología Molecular 'Severo Ochoa' (C.S.I.C. - U.A.M.), Universidad Autónoma de Madrid, 28049 Madrid, Spain

Translation *in vitro* of the mammalian nucleus-encoded mRNA for the β subunit of mitochondrial H$^+$-ATP synthase (β-mRNA) of oxidative phosphorylation is promoted by a 150 nt translational enhancer sequence in the 3′-untranslated region (3′ UTR). Titration of the eukaryotic initiation factor eIF4E with cap analogue revealed that translation of capped β-mRNA was pseudo-cap independent. The 3′ UTR of β-mRNA stimulates the translation of heterologous uncapped mRNA species, both when the 3′ UTR is placed at the 3′ end and at the 5′ end of the transcripts. The 3′ UTRs of the α subunit of mitochondrial H$^+$-ATP synthase (α-F1-ATPase) and subunit IV of cytochrome *c* oxidase (COX IV) mRNA species, other nucleus-encoded transcripts of oxidative phosphorylation, do not have the same activity in translation as the 3′ UTR of β-mRNA. On dicistronic RNA species, the 3′ UTR of β-mRNA, and to a smaller extent that of COX IV mRNA, is able to promote the translation of the second cistron to a level comparable to the activity of internal ribosome entry sites (IRESs) described in picornavirus mRNA species. These results indicate that the 3′ UTRs of certain mRNA species of oxidative phosphorylation have IRES-like functional activity. Riboprobes of the active 3′ UTRs on dicistronic assays formed specific RNA–protein complexes when cross-linked by UV to proteins of the lysate, suggesting that cytoplasmic translation of the mRNA species bearing an active 3′ UTR is assisted by specific RNA–protein interactions.

Key words: mitochondria, mRNA-binding proteins, oxidative phosphorylation, translation, translational enhancers.

INTRODUCTION

The regulation of the expression of the mammalian β-catalytic subunit of the H$^+$-ATP synthase (β-F1-ATPase), an essential protein required for the biogenesis of mitochondria [1] and in programmed cell death [2], has been studied in different tissues and during the development of the liver [1,3–7]. The expression of the gene for β-F1-ATPase, both in the liver [7,8] and in brown adipose tissue [9], pointed out that the regulation of the stability and translational efficiency of the mRNA are primary sites for the spatial and temporal expression of the protein [10]. In this context, we have provided evidence indicating that translation *in vitro* of the mRNA for β-F1-ATPase (β-mRNA) required a full-length 3′ UTR [11]. This highly conserved 150 nt AU-rich sequence element, when placed at the 3′ end of a reporter, promoted an increase in the translational efficiency of the chimaeric construct [11], closely resembling the translational enhancers described in certain mRNA species of plant viruses [12–14]. Developmental and tissue-specific translation of β-mRNA was further correlated with the expression of a regulatory protein that inhibits β-mRNA translation by binding the 3′ UTR of the transcript [11].

The control of eukaryotic mRNA translation is primarily exerted at the initiation level and is mediated by a complex set of protein–protein and protein–RNA interactions between specific sequence elements within the mRNA molecule (*cis*-acting elements) and *trans*-acting components that define the site of ribosome binding for the initiation of translation [15,16]. The recruitment of the 40 S ribosomal subunits to most mRNA species near the capped 5′ end is mediated by the eukaryotic initiation factor 4E (eIF4E) component of the eIF4F cap-binding protein complex [17–19], an interaction that is greatly facilitated by the poly(A) tail of the mRNA [20–22]. Alternatively, 40 S subunits can be recruited to certain viral [23,24] and cellular [25,26] mRNA species at internal ribosome entry sites (IRESs), a set of *cis*-acting elements that are located hundreds of nucleotides downstream of the capped 5′ end and generally placed upstream of the open reading frame. IRES-mediated translation initiation is both cap- and end-independent. In addition, the 3′-end-located translational enhancers found in viral RNA species [12–14] promote translation initiation of the mRNA by a cap-independent mechanism, i.e. functionally resembling the activity of IRESs, albeit placed at the opposite end of the mRNA.

The existence of a translational enhancer in the 3′ non-coding region of β-mRNA [11] led us to characterize its functional role in translation initiation. Here we provide evidence that the translation-enhancing activity of the 3′ UTR of β-mRNA functionally resembles an IRES. Interestingly, the 3′ UTRs of other nucleus-encoded transcripts of oxidative phosphorylation lack the same activity of the 3′ UTR of β-mRNA.

EXPERIMENTAL

Cloning technologies

Fetal rat liver RNA (10–20 μg) and a 30-mer anti-sense oligonucleotide (1 μg) (oligo G, 5′-GCGAGCTCCGCGAAGCTTT-

Abbreviations used: ARF, bovine ADP ribosylation factor; CAT, chloramphenicol acetyltransferase; COX IV, subunit IV of cytochrome *c* oxidase; eIF, eukaryotic initiation factor; EMCV, encephalomyocarditis virus; α- and β-F1-ATPase, subunits α and β of mitochondrial H$^+$-ATP synthase respectively; FMDV, foot-and-mouth disease virus; IRES, internal ribosome entry site; LUC, luciferase; β-mRNA, mRNA for β-F1-ATPase; pβ, precursor protein of the β-subunit of the F1-ATPase; UTR, untranslated region.

[1] To whom correspondence should be addressed (e-mail jmcuezva@cbm.uam.es).

TTTTTTTTTT-3′) [11], complementary to the poly(A) tail of the mRNA species, were used as template and primer of the reverse transcriptase (Promega) respectively. The fragments of the cDNA species, corresponding to the 3′ UTRs of the mRNA species for the α-subunit of the mitochondrial F1-ATPase complex (α-F1-ATPase) and for subunit IV of cytochrome *c* oxidase (COX IV) from rat liver, were generated by PCR with sense oligonucleotides (oligo L, 5′-CGCGCCTCGAGCACCAGAGCCTCTTGGG-CA-3′; oligo M, 5′-GCGCGGCTCGAGGTGAGAGCCTGC-TGCTGT-3′ for α-F1-ATPase [27] and COX IV [28] cDNA species respectively).

Construction of the plasmids and of the dicistronic vectors

Plasmids pJMI-β-F1, pJMI3′UTR-β-F1 and pARF (in which ARF stands for bovine ADP ribosylation factor) have been previously described in detail [11]. To obtain the recombinant plasmids containing the 3′ UTR of α-F1-ATPase and COX IV mRNA species, the amplified cDNA species were cloned between the *Xho*I and *Hin*dIII sites of the pBSSK plasmid (Stratagene). These plasmids were named pJR3′UTR-α-F1 and pJMI3′UTR-COX IV.

The dicistronic and monocistronic vectors used in this study were made from the dicistronic plasmid pBIC (wt-plasmid) kindly provided by Dr E. Martínez-Salas [29]. The wt-dicistronic plasmid contains, as the first cistron, the chloramphenicol acetyltransferase (CAT) cDNA under the control of the T7 phage promoter. The second cistron is the luciferase (LUC) cDNA. Translation of the LUC reporter is under the control of the IRES of foot-and-mouth disease virus (FMDV). This plasmid was modified to generate two additional restriction sites at the 5′ and 3′ ends of the FMDV IRES, the *Apa*I site (at the 5′ end) and the *Hin*dIII site (at the 3′ end). For this purpose, two oligonucleotides were synthesized, each containing a unique *Sac*I (underlined) (sense oligo A, 5′-GCGCGGGAGCTC**GGGCC-C**AGCAGGTTTCCCCAACTGACACAA-3′; anti-sense oligo B, 5′-GCGCGGGAGCTC**AAGCTT**AGGGTCATTAATTGT-AAAGGAAAGG-3′) and *Apa*I (oligo A, in bold) or *Hin*dIII (oligo B, in bold) restriction sites. After PCR with these oligos, using the wt-dicistronic plasmid as template, the amplified fragments were digested with *Sac*I and further subcloned in the wt-dicistronic plasmid. Clones with the sense (wt-ApHi) and anti-sense orientation (wt-HiAp) of the FMDV IRES were selected. The inserts containing the 3′ UTRs of β-F1-ATPase, α-F1-ATPase and COX IV mRNA species were excised from their corresponding plasmids by digestion with *Apa*I and *Hin*dIII and ligated with the wt-ApHi dicistronic plasmid. This set of plasmids were named Dicis-3′βUTR, Dicis-3′αUTR and Dicis-3′COX UTR respectively. The cDNA corresponding to the 3′ UTR of β-F1-ATPase mRNA was also subcloned into the wt-HiAp dicistronic plasmid and the resulting construct was named InvDis-3′βUTR.

To generate the monocistronic plasmids of the LUC series, the dicistronic plasmids were digested with *Apa*I and *Hpa*I to release the cDNA species containing the IRES of FMDV and the 3′ UTRs of oxidative phosphorylation transcripts (β-F1, α-F1 and COX IV) upstream of the LUC reporter. The monocistronic LUC cDNA lacking a 5′ non-coding region was obtained by digestion of the InvDis-3′βUTR plasmid with the same enzymes. The purified inserts were subcloned between the *Apa*I and *Sma*I sites of the pBSSK plasmid (Stratagene) with the correct orientation downstream of the T7 promoter. This set of plasmids were named MoLu-FMDV IRES, MoLu-3′βUTR, MoLu-3′αUTR, MoLu-3′COX UTR and MoLu respectively.

Transcription reactions

Transcription reactions were performed with phage T7 RNA polymerase (10 units) and linearized plasmid DNA (1 μg) as template with the mCAP RNA Capping kit (Stratagene) as described previously [11]. The pJMI-β-F1 plasmid was digested with *Hin*dIII or *Apa*I, to generate the full-length and 3′β-UTR-deleted mRNA species of β-F1-ATPase respectively. The pARF plasmid was digested with *Apa*I to generate the ARF mRNA. The wt-dicistronic and Dicis-plasmids were linearized with *Hin*dIII to generate the set of CAT monocistronic mRNA species. The wt-dicistronic, InvDis and Dicis plasmids were linearized with *Hpa*I to generate the set of dicistronic mRNA species. The MoLu plasmids were linearized with *Bam*HI to generate the set of LUC monocistronic mRNA species. As indicated, both capped and uncapped versions of the transcripts were synthesized. Capped transcripts were synthesized with 5′7mGpppG5′cap analogue in accordance with the suppliers' recommendations. The uncapped transcripts were prepared essentially as described above, without adding cap structures, in the presence of additional rGTP. The encephalomyocarditis virus (EMCV) RNA was obtained from Amersham (Little Chalfont, Bucks., U.K.).

Translation assays and analysis of translation products

Monocistronic and dicistronic RNA species were translated at 30 °C, in the presence of 20 μCi of L-[^{35}S]methionine (more than 1000 Ci/mM) and 1 unit/μl of RNasin (Boehringer), using the Amersham and Promega nuclease-treated rabbit reticulocyte lysates respectively. The translation products were analysed by electrophoresis on 12% (w/v) polyacrylamide gels containing 0.1% SDS and further processed for fluorography as described previously [11]. To monitor cap-independent translation, 5′7mGpppG5′ (Stratagene) (0–1 mM) was added to the translation assay *in vitro* to compete for the cap-binding proteins required for cap-dependent translation.

UV cross-linking assays

UV cross-linking assays were performed with the modifications previously described in detail [11]. In brief, 2 μl of rabbit reticulocyte lysates was incubated with radiolabelled probes [(1–5) × 10^5 c.p.m.] at 30 °C for 10 min. The reaction mixtures were exposed to UV (254 nm) (Stratalinker 1800; Stratagene) for 6 min on ice, before the addition of 20 units of RNase T1 (Boehringer). The RNA–protein complexes were resolved by SDS/PAGE [12% (w/v) gel]. For competition studies, an excess of unlabelled RNA was added 10 min before the addition of the radiolabelled RNA [11]. After electrophoresis, the gels were dried and exposed to X-ray films. For the generation of RNA riboprobes, the plasmids pJMI3′UTR-β-F1, pJR3′UTR-α-F1 and pJMI3′UTR-COX IV were digested with *Hin*dIII. Radiolabelled RNA probes were prepared, without adding cap structures, in the presence of 0.05 mM unlabelled rUTP plus 50 μCi of [α-^{32}P]UTP (400 Ci/mmol) (Amersham).

RESULTS

Apparent lack of function for the 5′ cap on β-mRNA translation

We have shown previously that the 3′ UTR of β-mRNA was essential for its efficient translation *in vitro* [11]. To investigate further the role of the 3′ UTR on β-mRNA translation, capped

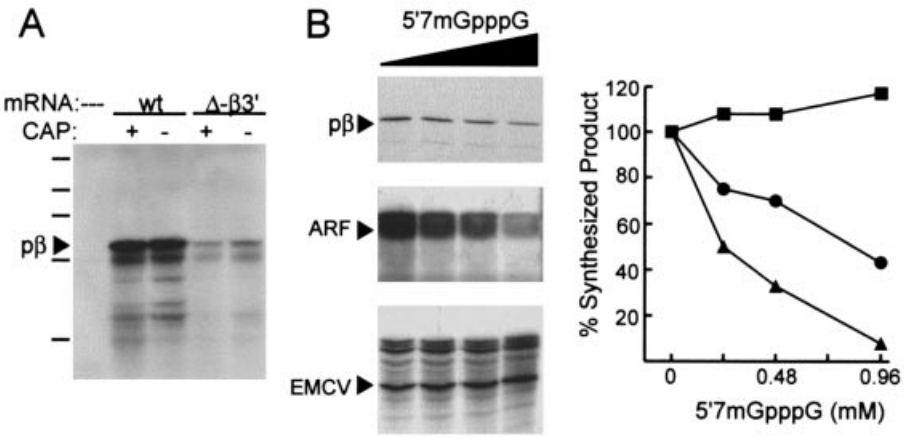

Figure 1 Pseudo cap-independent translation of β-F1-ATPase mRNA

(**A**) Capped (+) and uncapped (−) versions of the full-length (wt) and 3′ UTR deleted (Δ-β3′) β-F1-ATPase mRNA species were synthesized. 0.55 pmol of each mRNA form were translated in rabbit reticulocyte lysates at 30 °C in the presence of ^{35}S-methionine. 4 μl aliquots of the *in vitro* synthesized products were analysed by 12% SDS-PAGE. The migration of the main translation product (closed arrowhead) corresponds to pβ. The positions of molecular mass markers (from the top: 200, 97, 69, 46 and 30 kDa) are shown at the left. (**B**) Capped full-length β-F1-ATPase (1.6 pmol) (top panel) and ARF (1.6 pmol) (middle panel) mRNA species and 1.1 pmol of uncapped EMCV RNA (bottom panel) were translated *in vitro* in the presence of increasing amounts (0–1 mM) of 5′7mGpppG cap analogue. Representative fluorograms of the synthesized protein products (pβ, ARF and EMCV) are shown. The graph illustrates the relative amount of synthesized pβ (●), ARF (▲) and the 52 kDa peptide derived from the uncapped EMCV RNA (■) as a function of the concentration of cap analogue present in the assay.

and uncapped versions of the full-length and the 3′-UTR-deleted β-mRNA were prepared by transcription of the pJMIβ-F1 plasmid *in vitro* in the presence or absence of 5′7mGpppG5′ cap analogue. Figure 1(A) illustrates the synthesized protein product derived from the two versions of the β-mRNA species generated. Deletion of the 3′ UTR of the transcript almost completely abolished β-mRNA translation in both its capped and uncapped versions. In fact, the presence of the 3′ UTR downstream of the β-mRNA sequence promoted a 60-fold increase in the amount of synthesized precursor protein of the β-subunit of the F1-ATPase (pβ) in comparison with the translation of the transcript lacking the 3′ UTR. Remarkably, the translational efficiency of capped β-mRNA was found to be essentially the same as that of the uncapped mRNA version (Figure 1A).

Pseudo cap-independent translation of β-mRNA

The apparent lack of function of the 5′-cap on β-mRNA translation led us to study the translational efficiency of the capped full-length β-mRNA in conditions under which the availability of eIF4E of the lysate varied. eIF4E titration was achieved by the addition of increasing amounts (0–1 mM) of the 5′7mGpppG cap analogue [30]. The same study was performed with two additional mRNA species that represented the cap-dependent (ARF mRNA) and cap-independent (EMCV mRNA) pathways of mRNA translation (Figure 1B). As expected, the translational efficiency of the picornaviral EMCV mRNA was not affected by increasing concentrations of the cap analogue. The EMCV mRNA contains an IRES, thus allowing mRNA translation without the usual mode of scanning from the 5′ mRNA terminus [31]. The translational efficiency of capped ARF mRNA was significantly decreased (50 %) at concentrations of the cap analogue (240 μM) known to inhibit the cap-dependent pathway of mRNA translation [30]; 95 % inhibition of ARF mRNA translation was attained at concentrations of the cap analogue close to 1 mM (Figure 1B). The translational efficiency of β-mRNA was much less sensitive to the titration of eIF4E (Figure 1B). In fact, to achieve a 50 % inhibition of β-mRNA translation, a 4-fold higher concentration of the cap analogue was required in comparison with a similar translation arrest exerted on ARF mRNA (Figure 1B). Taken together, these findings suggest that translation of β-mRNA *in vitro* is less dependent on the 5′ cap structure of the mRNA. In other words, and in keeping with more recent terminology [30], β-mRNA translation seems to be a pseudo cap-independent mRNA.

Translation-enhancing features of the 3′ UTR of β-mRNA

In a previous study we found that the 3′ UTR of β-mRNA exerted a translation-enhancing activity when placed downstream of a capped reporter chimaeric mRNA [11]. We wondered whether this property of the 3′ UTR of β-mRNA was common to other 3′ UTRs of mammalian mRNA species involved in oxidative phosphorylation. To explore this possibility, we cloned and analysed the putative translation-enhancing activity of the 3′ UTRs of other nuclear genes involved in oxidative phosphorylation. Figure 2(A) shows that the 3′ UTR of β-mRNA promoted a significant (3-fold) increase in the translational efficiency of uncapped CAT mRNA when placed at the 3′ end of the reporter. In a previous study we observed that the translation-enhancing activity of the 3′ UTR of β-mRNA with the ARF reporter under capped conditions promoted a 7 ± 1-fold (mean ± S.E.M.) increase in the amount of ARF synthesized [11]. Analysis of the translation-enhancing activity of the 3′ UTRs of other nucleus-encoded mRNA species of mitochondrial proteins (COX IV and the α-subunit of the H^+-ATP synthase) revealed that the COX IV 3′ UTR promoted a 2-fold increase in the amount of CAT synthesized, whereas the 3′ UTR of α-mRNA was devoid of translation-enhancing activity (Figure 2A).

Position-independent function of the 3′ UTR of β-mRNA

The position-independent translation-enhancing activity of the 3′ UTRs was analysed in uncapped RNA species containing at the 5′ end of the LUC reporter the 3′ UTR sequences of interest.

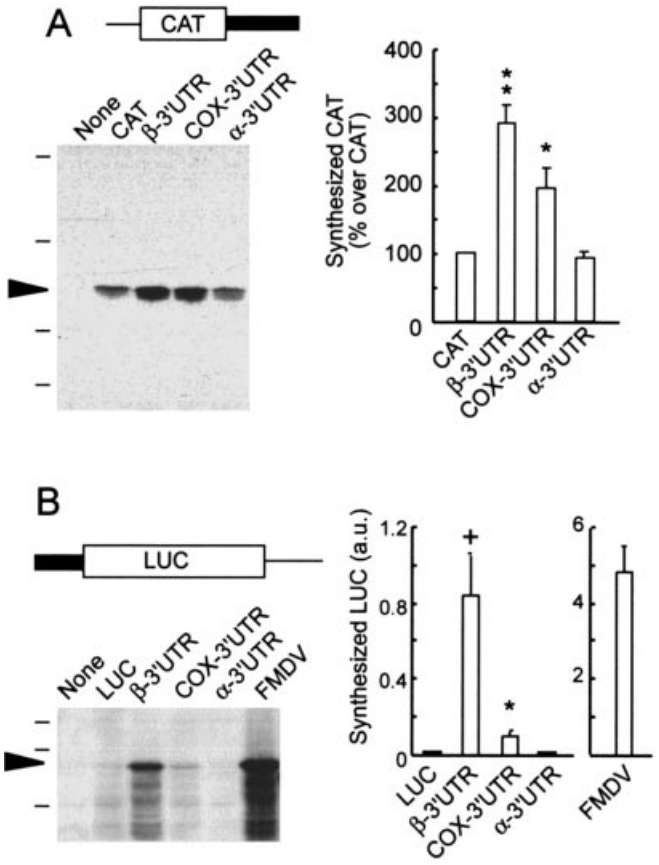

Figure 2 Position-independent translation-enhancer activity of the 3′ UTR of β-F1-ATPase mRNA

(**A**) Scheme illustrating the monocistronic CAT mRNA species containing the different 3′ UTRs (black box) downstream of the CAT cistron. The uncapped CAT mRNA species were synthesized after digestion of the wt-ApHi dicistronic (CAT), Dicis-3′βUTR (β-3′UTR), Dicis-3′COX UTR (COX-3′UTR) and Dicis-3′αUTR (α-3′UTR) plasmids as described in the Experimental section. Each mRNA [0 ng (none) or 250 ng] was translated by using reticulocyte lysates; the reaction products were fractionated on SDS/PAGE gels. An arrowhead indicates the migration of the synthesized CAT reporter. A representative fluorogram is shown. The positions of molecular mass markers (46, 30, 21 and 14 kDa, from top to bottom) are shown at the left. The histogram shows the synthesized CAT reporter derived from each chimaeric mRNA as a percentage of that synthesized in parallel assays from the CAT mRNA. The results are means ± S.E.M. for four independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ in comparison with CAT by Student's *t* test. (**B**) Scheme of the monocistronic LUC mRNA species containing different non-coding RNA sequences (black box) upstream of the LUC cistron. The uncapped LUC mRNA species were synthesized after the digestion of MoLu (LUC), MoLu-3′βUTR (β-3′UTR), MoLu-3′COX UTR (COX-3′UTR), MoLu-3′αUTR (α-3′UTR) and MoLu-FMDV-IRES (FMDV) plasmids as described in the Experimental section. Each mRNA 0 ng (none) or 500 ng] was translated and processed as described above. An arrowhead indicates the position of migration of the synthesized LUC reporter. A representative fluorogram is shown. The positions of Molecular mass markers (97, 69 and 46 kDa, from top to bottom) are shown at the left. The histogram shows the synthesized LUC reporter (arbitrary densitometric units, a.u.) derived from each chimaeric mRNA. The results are means ± S.E.M. for three independent experiments. $^{*}P < 0.025$; $+P < 0.005$ in comparison with β-3′ UTR and FMDV respectively by Student's *t* test.

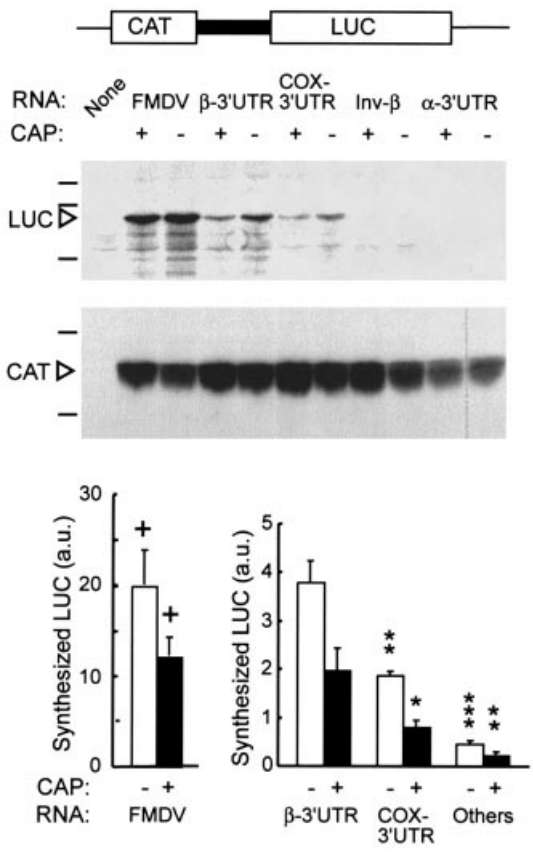

Figure 3 IRES-like translational activity of the 3′ UTRs of β-mRNA and COX IV mRNA

Schematic representation illustrating the series of dicistronic mRNA species containing, in the intercistronic region between the CAT and LUC cistrons, the following RNA sequences: the IRES of FMDV (FMDV), the 3′ UTRs of β-F1-ATPase (β-3′UTR), COX IV (COX IV-3′UTR) and α-F1-ATPase (α-3′UTR) and the anti-sense sequence of the 3′ UTR of β-F1-ATPase (Inv-β). The synthetic dicistronic mRNA species were generated from their corresponding plasmids in both their capped (+) and uncapped (−) versions. Each mRNA [0 ng (none) or 500 ng] was translated and processed as described. Arrowheads indicate the migrations of the synthesized LUC and CAT reporters. The positions of molecular mass markers (97, 69, 46, 30 and 21 kDa, from top to bottom) are shown at the left. A representative experiment is shown. To illustrate the effect of the cap structure on the translation of the first cistron, a different exposure of the fluorogram for the CAT product is presented. The histograms show the synthesized LUC reporter (arbitrary densitometric units, a.u.) derived from each chimaeric mRNA under both capped (+, black bars) and uncapped (−, white bars) conditions. The results are means ± S.E.M. for four independent experiments. In the histogram, 'others' are a compilation of the results derived from both α-3′ UTR and Inv-β because both transcripts revealed a very low synthesis of LUC. $+P < 0.01$; $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0005$ in comparison with β-3′ UTR by Student's *t* test.

In addition, and for comparative purposes, a construct containing the 5′ non-coding region of FMDV mRNA, a sequence that contains an IRES, was also analysed (Figure 2B). The results obtained illustrated that the 3′ UTR of β-mRNA was able to promote a significant yield of LUC, whereas those of COX IV and α-mRNA were not (Figure 2B). These findings reinforce the translation-enhancing activity of the 3′ UTR of β-mRNA and strongly suggest that its activity is exerted independently of its position within the mRNA sequence. It should be noted that the translation-enhancing activity of the 3′ UTR of β-mRNA is 20 % of that of one of the most active IRES described so far (Figure 2B) [32].

Functional resemblance between IRES and the 3′ UTR β-mRNA

Our previous findings suggested that the 3′ UTR of β-mRNA is able to interact with the translational machinery because, when added in *trans* to the translation assay, it inhibited the translation of β-mRNA. Furthermore, it was shown that, in competition assays, the mRNA bearing the 3′ UTR of β-mRNA was

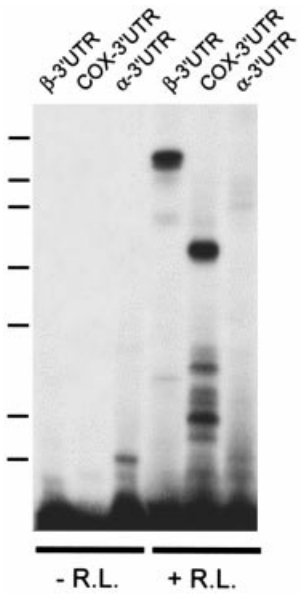

Figure 4 Specific RNA–protein complexes of reticulocyte lysates formed with 3′ UTRs of oxidative phosphorylation transcripts

The sense 3′ UTR riboprobes of β-F1-ATPase (β-3′UTR), COX IV (COX-3′UTR) and α-F1-ATPase (α-3′UTR) mRNA species were synthesized from the *Hin*dIII-digested pJMI3′UTR-β-F1, pJMI3′UTR-COX IV and pJR3′UTR- α-F1 plasmids respectively, with the use of T7 viral RNA polymerase. The corresponding ^{32}P-labelled RNA probes were incubated with none (−RL) or 2 μl of reticulocyte lysates under conditions of translation (+RL). After incubation, the samples were digested with RNase T1 and subjected to cross-linking with UV. The RNA–protein complexes were resolved by SDS/PAGE [12% (w/v) gel]. The positions of protein standards of 200, 97, 69, 46, 30, 21 and 14 kDa (from top to bottom) are indicated at the left.

translated preferentially over mRNA not containing this sequence element [11]. These findings suggested that the 3′ UTR of β-mRNA could behave very similarly to an IRES, despite its opposite positioning within the sequence of the mRNA. *Cis*-acting RNA sequence elements are functionally designated as an IRES when they can promote the translation of a second cistron in a heterologous dicistronic mRNA [33]. We therefore next analysed this property for heterologous capped and uncapped versions of dicistronic mRNA species that contained, between the first (CAT) and second (LUC) cistrons, the 3′ UTRs of oxidative phosphorylation transcripts (Figure 3). The results showed that for both the capped and uncapped versions of the dicistronic mRNA species the 3′ UTRs of β-mRNA and COX IV mRNA were able to promote the translation of the second cistron (LUC reporter) with a significant yield (Figure 3), although the β-mRNA sequence was twice as active than that of COX IV. In contrast, dicistronic RNA species containing the anti-sense sequence of the 3′ UTR of β-mRNA or that with the 3′ UTR of α-mRNA were unable to promote the translation of LUC (Figure 3). The former construct further suggested that essential RNA sequences or structures, with had an essential role in the translation-enhancing activity of the 3′ UTR of β-mRNA, had been inactivated. Remarkably, the translation of the second cistron was always more efficient when derived from the uncapped versions of the dicistronic RNA species (Figure 3), suggesting competition for the available translational machinery between the first and second cistrons. As shown previously, the translational activity of the 3′ UTR of β-mRNA was approx. 20% less active than that of the IRES of FMDV in both the capped and uncapped versions of the dicistronic RNA (Figure 3). As expected, it was noted that the translation of the first cistron in all dicistronic RNA species under uncapped condition was approximately half of that derived from the capped versions ($P < 0.025$ (Figure 3). Taken together, these results argued in favour of an IRES-like function for the 3′ UTR of β-mRNA in the control of the translation of this transcript: a functional activity that is not present in the 3′ UTR of α-mRNA but might be active in the 3′ UTR of COX IV mRNA.

Reticulocyte lysates contain specific proteins able to interact with the 3′ UTRs of β-mRNA and COX IV mRNA

UV cross-linking assays of the 3′ UTRs of oxidative phosphorylation transcripts with proteins of the reticulocyte lysate, under conditions of mRNA translation, indicated the existence of two major protein–RNA complexes (p158 and p126, migrating as a doublet) when the 3′ UTR of β-mRNA was used as riboprobe (Figure 4). Remarkably, it should be noted that reticulocyte lysates show negligible binding activity of the 3′βFBPs (p50) previously described as being involved in the translational repression of β-mRNA in the fetal liver [11]. The negligible binding activity of the 3′βFBPs observed (Figure 4) could result from either a low representation of their activity and/or because the strong binding of p158/126 prevented the binding of the 3′βFBPs to the riboprobe in the lysate. The 3′ UTR COX IV riboprobe provided a major RNA–protein complex (p50) and an additional five less-abundant complexes in the 15–25 kDa range (Figure 4). No RNA protein complexes were observed when the 3′ UTR of α-mRNA riboprobe was used (Figure 4).

DISCUSSION

The 3′ UTR of β-mRNA is a *cis*-acting sequence element that is required for the translation of the mRNA. To our knowledge, this is the first mammalian non-coding sequence element, placed at the 3′ end of the transcript, known to exert this function in translation. Translation-enhancing sequences have been mostly described at the 3′ end of viral RNA species [12–14,34] and at the 5′ end of viral [23,24] and cellular mRNA species [25,26]. The effect on translation of the 3′ UTR is observed with both capped and uncapped versions of β-mRNA. However, the presence of a cap structure in β-mRNA did not further increase the yield of synthesized pβ. Furthermore, the translation of capped β-mRNA was pseudo cap-independent. These findings suggest that the 3′ UTR is itself sufficient to replace the function of the cap structure for the recruitment of eukaryotic initiation factors to the 5′ mRNA terminus and the subsequent initiation of mRNA translation [15].

We have investigated the characteristics of the 3′ UTR of β-mRNA that determine its stimulatory activity, to understand how it might functionally replace the cap structure in the initiation of translation. The observation that the translation-enhancing activity of the 3′ UTR of β-mRNA is maintained when the 3′ UTR is placed both downstream and upstream of the reporters suggested that its function is to stimulate translation initiation, because in the latter condition it functionally replaces the cap structure. Similar observations have been reported for the translational enhancers found in the 3′ UTRs of PAV barley yellow dwarf virus [14,34,35] and satellite tobacco necrosis virus [36] RNA species. The observation in dicistronic RNA species of an increased output of translation product from the second cistron in the absence of a cap structure suggests strongly that the 3′ UTR of β-mRNA and, to a smaller extent, that of COX IV mRNA are able to promote the recruitment of the translational

machinery internally as the IRES of FMDV. In other words, that translation of the second cistron in dicistronic RNA species containing the 3′ UTR of β-mRNA and COX IV mRNA is not due to the leaking of ribosomes through the intercistronic sequences. In fact, it is worth noting that the 3′ UTR of β-mRNA, both when placed at the 5′ end of the reporter and when an intercistronic sequence, has 20% of the translation-enhancing activity of the IRES of FMDV. The IRES of this aphthovirus has been shown to drive the translation of RNA species in reticulocyte lysates in a very accurate and efficient way [32]. In this regard, the basal translation-enhancing activities of the 3′ UTRs of β-mRNA and COX IV mRNA are comparable to the IRES activity of the hepatitis A and C viruses, 10-fold more active than the IRES activity of enterovirus (poliovirus) and the JV4 strain of ECHO virus type 25 and 100-fold more active than the IRES activity of human rhinovirus [32].

The results in this work indicate that the 3′ UTRs of β-mRNA and COX IV mRNA stimulate the translational efficiency of the corresponding mRNA species by a preferential interaction of these non-coding RNA sequences with *trans*-acting factors involved in the initiation of translation. In fact, the 3′ UTRs of both β-mRNA and COX IV mRNA formed specific RNA–protein complexes with proteins from the reticulocyte lysate, whereas the 3′ UTR of α-mRNA, which lacks an IRES-like functional activity, did not. It is possible that the differences in RNA–protein complexes observed between the 3′ UTRs of β-mRNA and COX IV mRNA reveal specific RNA-binding proteins required for the recognition of each 3′ UTR by the translational machinery of the cell. Alternatively, they might illustrate the differential affinity of each 3′ UTR for different proteins of the canonical translation initiation factors.

Irrespectively of the mechanism by which the 3′ UTR of β-mRNA is able to recruit the translational machinery to the 3′ trailer of both homologous and heterologous mRNA species, it implies a communication between the 3′ and 5′ ends of the mRNA, in this way enhancing the delivery of 40 S subunits to the initiator AUG codon. This communication resembles the 3′-translation enhancers found in viral RNA species and the promotion of internal initiation of uncapped mRNA species by the poly(A) tail [37].

The question here is: What is the biological significance of 3′-translation enhancers in certain mRNA species of oxidative phosphorylation? As with most of the nucleus-encoded cellular mRNA species, the nuclear transcripts of oxidative phosphorylation in the cellular context are capped and have a poly(A) tail. It is therefore reasonable to assume that their translation under normal cellular conditions would follow the pathway of mRNA translation established by eIF4F [38–42]. In this context, the role of 3′ UTRs with translation-enhancing activity would be to facilitate the rapid recycling of ribosomes to the 5′ end and therefore to increase the reinitiation of translation, as has been suggested for 3′-translation enhancers found in viral mRNA species [12–14,34]. This is supported by two additional findings: (1) the lower sensitivity of capped β-mRNA translation to the titration of eIF4E, as revealed by the fact that in rabbit reticulocyte lysates reinitiating ribosomes are less sensitive to inhibition by cap analogues than those participating in primary initiation [43], and (2) β-mRNA in rat liver has been shown to be associated with high-density polysomes [8] and forming a cluster structure approx. 150 nm in diameter [44] that contains translational machinery [10]. Remarkably, this localization of β-mRNA is not observed for the nucleus-encoded α-mRNA [44], for which no translation-enhancing activity has been observed. These findings therefore suggest that β-mRNA might be added to the list of cellular mRNA species whose translation is less dependent on the amount of functional eIF4F, such as heat shock mRNA species [45] and IRES-containing cellular mRNA species [25,26].

Finally, a translation mechanism mediated by enhancer elements placed either at the 3′ end (this study) or at the 5′ end [25,26] of the mRNA confers on the mRNA species bearing such sequence elements a clear competitive translational advantage over other cellular mRNA species that lack such enhancers. This is specially relevant under stressful cellular conditions, such as viral infection, that result in the limitation of the availability of eIF4E [18,46] or of eIF4G [47]. The shut-down of cellular mRNA translation on viral infection can compromise the viability of the cell, and therefore viral replication and morphogenesis, if it also affects the translation of essential cellular mRNA species required in housekeeping cellular functions. In fact, recent evidence has indicated a structural and functional association between mitochondria and the morphogenetic pathway of the african swine fever virus in infected cells [48]. The identification of an enhancer element in the 3′ UTR of β-mRNA, an essential protein required in the provision of cellular ATP, is therefore not so surprising.

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