3'-Untranslated regions of oxidative phosphorylation mRNAs function *in vivo* as enhancers of translation

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Recent findings have indicated that the 3'-untranslated region (3'-UTR) of the mRNA encoding the β -catalytic subunit of the mitochondrial H⁺-ATP synthase has an *in vitro* translation-enhancing activity (TEA) [Izquierdo and Cuezva, Mol. Cell. Biol. (1997) 17, 5255–5268; Izquierdo and Cuezva, Biochem. J. (2000) 346, 849–855]. In the present work, we have expressed chimaeric plasmids that encode mRNA variants of green fluorescent protein in normal rat kidney and liver clone 9 cells to determine whether the 3'-UTRs of nuclear-encoded mRNAs involved in the biogenesis of mitochondria have an intrinsic TEA. TEA is found in the 3'-UTR of the mRNAs encoding the α - and β -subunits of the rat H⁺-ATP synthase complex, as well

as in subunit IV of cytochrome *c* oxidase. No TEA is present in the 3'-UTR of the somatic mRNA encoding rat mitochondrial transcription factor A. Interestingly, the TEA of the 3'-UTR of mRNAs of oxidative phosphorylation is different, depending upon the cell type analysed. These data provide the first *in vivo* evidence of a novel cell-specific mechanism for the control of the translation of mRNAs required in mitochondrial function.

Key words: cytochrome *c* oxidase, green fluorescent protein, H⁺-ATP synthase, mitochondria, mitochondrial transcription factor A.

INTRODUCTION

The biogenesis of mitochondria is a complex cellular process that requires a concerted expression of two genomes that are physically separated in the cell. However, the vast majority of mitochondrial proteins are encoded in the nuclear genome. The analysis of the mechanisms controlling the expression of nuclear genes required for the biogenesis of mitochondria during development of the liver [1–3], in brown adipose tissue [4] and in cancer cells [5] has provided paradigms in which the control of gene expression is also exerted at the level of translation. In this regard, in both fetal rat liver and hepatoma cells, the transcript encoding the β -catalytic subunit of the mitochondrial H⁺-ATP synthase is accumulated in a translationally repressed ribonucleoprotein particle [6,7] as a result of the interaction of the 3'-untranslated region (3'-UTR) of the transcript with cellular proteins, 3'-UTR β -F1 ATPase mRNA binding proteins (3'- β FBPs), which interfere with normal mRNA translation [5,8]. Interestingly, it appears that the control of β -mRNA translation is exerted by the 3'-UTR of the mRNA [5,8]. In fact, when the 3'-UTR of β -mRNA was placed in the inter-cistronic region of dicistronic constructs, a test for internal ribosome-entry sequences (IRESs) [9], it promoted the efficient translation of the second cistron to a level comparable with that of the most active IRES present in picornavirus [10]. This finding strongly suggested an 'IRES-like' functional activity for the 3'-UTR of β -mRNA. In the same *in vitro* study, it was found that the 3'-UTRs of other mRNAs of oxidative phosphorylation were either less active or devoid of such activity [10].

In this work, we have undertaken a study aimed at analysing the putative translation-enhancing activity (TEA) of the 3'-UTRs of essential nuclear-encoded components of both the

bioenergetic and replication/transcription machinery of the organelle in cells in culture. To meet this aim, we have studied the expression of the green fluorescent protein (GFP) derived from mRNA chimaeras containing the 3'-UTRs of these transcripts. The results reveal that the 3'-UTRs of mRNAs involved in oxidative phosphorylation function as cell-type-specific enhancers of mRNA translation, whereas the 3'-UTR of the somatic mitochondrial transcription factor A (Tfam) mRNA does not. These findings suggest that, in certain cell types, the control of translation of the set of transcripts involved in the bioenergetic function of the organelle might be an additional mechanism of regulation of gene expression for adapting the mitochondrial function of the cell to changes in energy requirements.

EXPERIMENTAL

Cell cultures and DNA transfection assays

Normal rat kidney (NRK) and rat liver clone 9 (C9) cells were grown in Dulbecco's Modified Eagle's medium (Gibco Life Technologies, Paisley, Scotland, U.K.), supplemented with a mixture containing 400 μ M of each of the following amino acids: Ala, Asn, Asp, Glu, Pro (Merck, Darmstadt, Germany) and 1 mM Gln (Merck), plus 5% (NRK) or 10% (C9) (v/v) fetal-calf serum (Biological Industries, Kibbutz Bet Haemek, Israel), 100 units/ml penicillin and 0.1 mg/ml streptomycin (Sigma, St. Louis, MO, U.S.A.) in an atmosphere of air/CO₂ (93:7). For transfection experiments, cells were used at 60–80% confluence. Transfection was performed using 1 μ l of FUGENE 6 (Boehringer Mannheim, Mannheim, Germany) and 0.5 μ g of DNA/1 $\times$ 10⁵ cells in 1 ml of culture medium, according to the manufacturer's instructions.

Abbreviations used: 3'-UTR, 3'-untranslated region; 3'- β FBPs, 3'-UTR β -F1 ATPase mRNA binding proteins; IRES, internal ribosome-entry sequence; TEA, translation-enhancing activity; GFP, green fluorescent protein; Tfam, mitochondrial transcription factor A; NRK, normal rat kidney; α - and β -F1 ATPase, subunits α and β of the mitochondrial H⁺-ATP synthase; COXIV, subunit IV of cytochrome *c* oxidase; SV40, simian virus 40; GFP*, modified GFP; a.u., arbitrary units; mtDNA, mitochondrial DNA; CMV, cytomegalovirus.

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Table 1 Oligonucleotides used in this study

The bold-type letters in the sequence of the sense oligonucleotides indicate the stop codons.

Oligonucleotide	Orientation	Sequence	3'-UTRs
A	Sense	5'-GCG CGG CTC GAG TAA AAG CCC TGC CAC TGT C-3'	α -F1 ATPase
B	Antisense	5'-GCG CGG AAG CTT CAA TAT GGA ACT TTT ATT CAT G-3'	α -F1 ATPase
C	Sense	5'-GCG CGG CTC GAG GTG AGG GGC CCT TCA GCC-3'	β -F1 ATPase
D	Antisense	5'-GCG CGG AAG CTT AGG GTA TAT ACT TTA TTG-3'	β -F1 ATPase
E	Sense	5'-GCG CGG CTC GAG GTG AGA GCC TGC T-3'	COXIV
F	Antisense	5'-GCG CGG AAG CTT CCA CGT AAA CTG GTC ATT TA-3'	COXIV
G	Sense	5'-GCG CGG CTC GAG TAA AGA TTG AAG ACA GAG TTG TCA-3'	Tfam
H	Antisense	5'-GCG CGG ACG CGT GGA AAA TTA GAG TTT TTA TTC TAT G-3'	Tfam

Plasmid construction

The original pEGFP-C2 plasmid (Clontech, Palo Alto, CA, USA) was modified (see the Results section) by eliminating the *XhoI*–*XbaI* fragment of the polylinker from the vector. The resulting plasmid was named CDL-GFP-SV40 (plasmid SV40). The cDNAs, corresponding to the 3'-UTRs of the nuclear transcripts encoding the relevant rat mitochondrial proteins [α - and β -subunits of the F1-ATPase complex (α - and β -F1 ATPase respectively), subunit IV of cytochrome *c* oxidase (COXIV) and Tfam] were obtained by PCR using synthetic oligonucleotides (Isogen, Maarsen, The Netherlands) (Table 1), and plasmids described previously [10] or cDNA (A. Rantanen and N. G. Larsson, unpublished work) as templates. The Tfam 3'-UTR cDNA was cloned between the *XhoI* and *MluI* sites of the pEGFP-C2 plasmid. The other cDNAs obtained from the PCRs were cloned between the *XhoI* and *HindIII* sites of the polylinker of the same plasmid. The constructs were digested further with *HindIII* and *MluI* enzymes in order to remove the 3'-UTR and polyadenylation signal of simian virus 40 (SV40) from the original pEGFP-C2 plasmid. The resulting constructs were named as follows: CDL-GFP- α -3'-UTR (plasmid α), CDL-GFP- β -3'-UTR (plasmid β), CDL-GFP-COX-3'-UTR (plasmid COX) and CDL-GFP-Tfam-3'-UTR (plasmid Tfam), for the plasmids expressing chimaeric mRNAs of GFP with the 3'-UTRs of α -F1-ATPase, β -F1-ATPase, COXIV and Tfam respectively. The sequence of all constructs was verified by automated DNA sequencing (Servicio de Secuenciación, Universidad Autónoma de Madrid, Madrid, Spain).

Fluorescence microscopy

After transfection (24 h), the cells were analysed by fluorescence microscopy using a Leica DMIRB microscope equipped with a GFP excitation filter (Leica, Heerbrugg, Switzerland; BP 470/40). Digital image acquisition and processing were performed with a Leica DC100 camera and the Leica QWin software package respectively. At least ten different cellular fields were examined in each plate at 10 $\times$ or 20 $\times$ magnification. Image acquisition within the same cell type studied was performed under constant conditions in every field examined and for each of the transfected plasmids. Fluorescent images were quantified by two different approaches, as defined by the QWin software package: interactive (to quantify the number of cells/field expressing detectable levels of GFP as well as the relative fluorescence intensity/cell) and total-field fluorescence.

DNA and RNA manipulation

DNA extraction and Southern blotting experiments were performed as described previously [5,11]. In brief, total DNA was

extracted from the cells after digestion with RNase and Proteinase K [5]. Of the DNA, 5–10 μ g was digested overnight at 37 °C with *BglII* (Boehringer Mannheim), fractionated on 0.8% (w/v) agarose gels and blotted on to GeneScreen membranes. Hybridization was carried out in the presence of radiolabelled probes (5 $\times$ 10⁸ c.p.m./ μ g DNA) using (1–2) $\times$ 10⁶ c.p.m./ml, by standard procedures [5,11]. RNA was prepared by the method described in [12]. RNA fractionation by gel electrophoresis, Northern blotting and hybridization were performed by standard methods described previously [2,5]. Membranes were exposed to AGFA RP2 films for various lengths of time.

Electrophoresis of proteins and Western blotting

Cells were recovered from the plates by trypsin treatment, washed twice with PBS and boiled in loading buffer. Cellular proteins were fractionated by SDS/PAGE (12% gels), and then were transferred on to PVDF membranes (Millipore, Bedford, MA, U.S.A.). Western blotting of the membranes was performed essentially as described in [2]. The primary monoclonal antibodies used were an anti-GFP (1:1000 dilution) (Clontech) and anti- α -tubulin (1:1000 dilution) (Sigma). Peroxidase-conjugated anti-mouse IgGs (Nordic Immunology, Tilburg, The Netherlands) were used as secondary antibodies. The blots were revealed using the ECL[®] reagent (Amersham Pharmacia Biotech, Little Chalfont, U.K.), according to the manufacturer's instructions.

RESULTS

Design of the GFP-reporter plasmids

Preliminary experiments, designed to investigate the TEA of the 3'-UTR of β -mRNA in cells in culture, disclosed an anomalous behaviour, most likely cytotoxic [13], of the GFP derived from the original pEGFP-C2 plasmid (C. M. Di Liegro and J. M. Cuezva, results not shown). The cells transfected with the pEGFP-C2 plasmid revealed a preferential accumulation of GFP in the nucleus, with a subsequent loss of cellular morphology and final cellular detachment from the plate. Therefore we decided to design the GFP constructs in such a way that: (i) they are less toxic to the cells; (ii) all the plasmids express the same GFP; and (iii) all the plasmids have a unique polyadenylation signal for the processing of the transcript, which is provided by the cloned 3'-UTR fragment. The approach followed is summarized in the Experimental section, and is outlined in Figure 1(a). In brief, this consisted of the removal of part of the polylinker (*XhoI*–*XbaI* fragment) of the pEGFP-C2 plasmid. This deletion eliminated the last 20 residues of the commercial GFP fusion, an amino acid sequence that is encoded by the polylinker and that contains a stretch of basic residues (Arg–Arg–Tyr–Arg) that mimics a nuclear localization signal [14,15].

Figure 1 Plasmids used in transient transfection experiments

(a) Schematic representation of the plasmids is presented. The CMV promoter (PCMV), GFP coding region and the different 3'-UTRs (boxes with different shadings) are indicated. pEGFP-C2 is the commercially available plasmid. 'SV40' is a 60-bp-deleted version (the *XhoI*-*XbaI* fragment) of the pEGFP-C2 plasmid that expresses a GFP protein (GFP*) that lacks the last 20 amino acids encoded by the polylinker of the pEGFP-C2 plasmid. The α , β , COX and Tfam constructs bear the 3'-UTRs of the mRNAs encoding the rat α - and β -subunits of the H⁺-ATP synthase, COXIV and Tfam respectively. The expected size (nt) of the GFP mRNAs obtained from each construct is shown on the right. (b) Representative Southern blots of total DNA (10 μ g) extracted from NRK and C9 cells 24 h after transfection with the constructs indicated above the slot. DNA was digested with *Bgl*II enzyme. The blots were hybridized to a 0.7 kb *Eco*RI-*Hind*III 12 S rRNA probe and to a 0.8 kb *Eco*47III-*Bgl*II GFP probe, as described in the Experimental section.

The resulting plasmid derived from this basic construct expresses a modified GFP (GFP*) that is not preferentially accumulated in the nuclei of mammalian cells, and is apparently devoid of the undesired cytotoxic effects when compared with the one expressed from the pEGFP-C2 plasmid (C. M. Di Liegro and J. M. Cuezva, results not shown). In addition, all the plasmids express the same GFP* protein and generate a unique RNA species of the predicted size (see below). Furthermore, elimination of the *XhoI*-*XbaI* fragment of the pEGFP-C2 plasmid and substitution of the SV40 polyadenylation signal for that contained in the different 3'-UTRs does not affect the efficiency of transfection of the resulting plasmids in any of the cell lines analysed, as revealed by the hybridization signal of the incorporated GFP* cDNA relative to that of the mitochondrial 12 S rRNA gene, the latter used as a standard for normalization of cellular abundance (Figure 1b).

TEA of the 3'-UTRs of transcripts of oxidative phosphorylation in NRK cells

The expression level of GFP* was determined 24 h after transfection of the cells with the different plasmids by monitoring both the GFP* fluorescence (Figure 2A) and the relative cellular content of the protein (Figure 2C). Figure 2(A) illustrates a field of confluent NRK cells from a representative experiment. It is evident that the number of cells expressing detectable levels of GFP* from the Tfam plasmid is lower (33 ± 3 cells/field) than the number of those expressing the SV40, α , β and COX plasmids (49 ± 5 cells/field for the β plasmid). Likewise, the fluorescence intensity was lower in cells transfected with the Tfam plasmid [98 ± 3 arbitrary units (a.u.)/pixel] than in those transfected with any of the other plasmids (150 ± 6 a.u./pixel for the β plasmid). The total-field fluorescence of the expressed GFP* in NRK cells is shown in Figure 2(A). It is worth mentioning that the SV40 3'-UTR has been previously shown to possess TEA [bibr,16].

The determinants of mRNA stability usually reside within the 3'-UTR of the transcripts [17,18]. Therefore it was possible that

differences in GFP* expression between the various constructs could arise from differences in the steady-state cellular mRNA levels. Thus we determined the GFP* mRNA levels in the transfected cells (Figure 2B). The results illustrated that the expressed GFP* mRNA derived from each plasmid has its predicted size, as judged from the cloning strategy followed (see also Figure 1a). In addition, the results showed that, with the exception of the mRNA derived from the SV40 plasmid (Figure 2B), the relative cellular content of the GFP* mRNAs containing the 3'-UTRs of nuclear-encoded mitochondrial mRNAs was not significantly different. The former reveals a significant 50% increase ($P < 0.01$) when compared with cells transfected with the β plasmid (Figure 2B).

The expression level of GFP* was analysed further by immunological procedures (Figure 2C). The results obtained by this approach revealed that NRK cells transfected with the Tfam plasmid had a 10–13-fold lower cellular amount of GFP* relative to cells transfected with the SV40, α , β or COX plasmids. The large differences observed between the fluorescence and immunological procedures are most likely to result from the more accurate estimation of GFP* expression by the latter method. In this case, the whole cellular amount of GFP* is determined, whereas fluorescence microscopy only provides an estimate of GFP* expression that depends upon the threshold amount of GFP required in a cell to provide a detectable fluorescence value. Calculation of the relative translational activity of each transcript, as the ratio of immunodetectable GFP*/GFP* mRNA levels normalized to the less active 3'-UTR of the Tfam mRNA (arbitrarily set to the value of 1), revealed that, in NRK cells, the activity of COX, β , α and SV40 transcripts was significantly higher than that of the Tfam transcript (Figure 2D). Furthermore, it was observed that the activity of the 3'-UTR of the COX transcript was higher ($P < 0.01$) than that of the SV40 enhancer [16]. These results indicate that, in NRK cells, the 3'-UTRs of nuclear-encoded mRNAs of oxidative phosphorylation have an intrinsic enhancer activity in translation that is not

Figure 2 TEA of the 3'-UTRs of oxidative phosphorylation mRNAs in NRK cells

(A) After DNA transfection (24 h), NRK cells were observed by fluorescence microscopy. At least ten different fields were photographed at $10\times$ magnification, with an exposure of 1.2 s. A field from a representative experiment is shown. The histogram to the right shows the total-field fluorescence. The results shown are the means $\pm$ S.E.M. (B) Northern blot analysis of total RNA extracted from NRK cells. After transfection (24 h), cellular RNA was isolated and processed (20 μ g). The blots were hybridized to the GFP and mitochondrial 12 S rRNA DNA probes. A 7 h-exposed blot from a representative experiment is shown. The histogram below shows the relative cellular amount of each GFP* mRNA normalized to the 12 S signal. The results shown are the means $\pm$ S.E.M. for five independent experiments. ns, non-significant; *, $P < 0.01$ when compared with β -transfected by Student's *t* test. (C) Western blots of whole cellular extracts prepared from NRK cells. After transfection (24 h), the cells [(1–2) $\times 10^5$] were collected and resuspended in loading buffer. Cellular proteins were fractionated by SDS/PAGE. After transfer, the membranes were incubated with GFP (gfp) and α -tubulin (tub) monoclonal antibodies. Blots from a representative experiment are shown. The histogram below shows the relative cellular amount of GFP* normalized to the α -tubulin content. The results shown are the means $\pm$ S.E.M. for six independent experiments. ns, non-significant; *, $P < 0.0025$ and **, $P < 0.0005$ when compared with COX transfected by Student's *t* test. (D) The histogram shows the relative translational activity of each GFP* mRNA (GFP* protein/GFP* mRNA ratio) normalized to the less active one derived from the Tfam plasmid that is arbitrarily set to the value of 1. *, $P < 0.025$; **, $P < 0.01$ and ***, $P < 0.0005$ when compared with Tfam transfected; ●, $P < 0.01$ when compared with SV40 transfected, by Student's *t* test.

present in the 3'-UTR of the mRNA encoding the somatic transcription factor of mitochondrial DNA (mtDNA).

TEA of the 3'-UTRs of oxidative phosphorylation transcripts in rat liver C9 cells

Transfection efficiencies of C9 cells were comparable with those obtained in NRK cells, as assessed by Southern blotting of the

GFP plasmids (Figure 1b). However, it is evident that the number of cells expressing detectable levels of GFP* was much lower in C9 (Figure 3A) than in NRK (Figure 2A) cells. This difference could result from a variation in the cytomegalovirus (CMV) promoter activity of the plasmid and/or in the turnover of the mRNAs in each cell type. In fact, the relative cellular abundance of GFP* mRNAs was much less in C9 (Figure 3B) than in NRK (Figure 2B) cells. In C9 cells transfected with the

Figure 3 TEA of the 3'-UTRs of oxidative phosphorylation mRNAs in C9 cells

(A) After DNA transfection (24 h) the C9 cells were observed by fluorescence microscopy. At least ten different fields were photographed at $10\times$ magnification with an exposure of 2 s. A field from a representative experiment is shown. The histogram to the right shows the total-field fluorescence. The results shown are the means $\pm$ S.E.M. (B) Northern blot analysis of total RNA extracted from C9 cells. After transfection (24 h), cellular RNA was isolated and processed (20 μ g). The blots were hybridized to the GFP (24–36 h of exposure) and mitochondrial 12 S rRNA (7 h exposure) DNA probes. Blots from a representative experiment are shown. The histogram below shows the relative cellular amount of each GFP* mRNA normalized to the 12 S signal. The results shown are the means $\pm$ S.E.M. for three independent experiments. (C) Western blots of whole cellular extracts prepared from C9 cells. After transfection (24 h) the cells (2×10^5) were collected and resuspended in loading buffer. Cellular proteins were fractionated by SDS/PAGE. After transfer, the membranes were incubated with GFP (gfp) and α -tubulin (tub) monoclonal antibodies. Blots from a representative experiment are shown. The histogram below shows the relative cellular amount of GFP* normalized to the α -tubulin content. The results shown are the means $\pm$ S.E.M. for seven independent experiments. *, $P < 0.05$; **, $P < 0.025$ and ***, $P < 0.0005$ when compared with β -transfected by Student's *t* test. (D) The histogram shows the relative translational activity of each GFP* mRNA (GFP* protein/GFP* mRNA ratio) normalized to the less active one derived from the Tfam plasmid that is arbitrarily set to the value of 1. *, $P < 0.01$ and **, $P < 0.005$ when compared with Tfam transfected; ●, $P < 0.025$ when compared with β transfected, by Student's *t* test.

β plasmid, both the number of GFP*-positive cells and the fluorescence intensity of them (25 ± 2 cells/field and 141 ± 7 a.u./pixel respectively) were significantly higher than in cells transfected with the Tfam plasmid (12 ± 2 cells/field and 104 ± 8 a.u./pixel respectively) (Figure 3A). The total-field fluorescence of the expressed GFP* in C9 cells was also significantly higher in cells transfected with the β -plasmid than in cells transfected with the Tfam plasmid (Figure 3A). Cells transfected with the SV40,

α and COX plasmids demonstrated fluorescence properties that were intermediate between these two extremes (Figure 3A).

In C9 cells, the GFP* mRNA derived from each plasmid had its predicted size (Figure 3B; also see Figure 1a). In this cell line, no significant differences were observed in the steady-state content of the mRNAs derived from the different plasmids (Figure 3B). Immunological determination of the expressed GFP* protein in C9 cells revealed that the relative cellular

abundance varied in the following order of plasmids: $\beta > \text{SV40} = \alpha > \text{COX} \gg \text{Tfam}$ (Figure 3C). The calculation of the relative translational activity of each construct (GFP* protein/GFP* mRNA ratio) when normalized to the less active 3'-UTR provided by the Tfam plasmid (arbitrarily set to the value of 1), revealed that, in C9 cells, the activity of the 3'-UTRs of β , α , SV40 and COX transcripts were significantly higher than that of the Tfam transcript (Figure 3D). These findings indicate that in C9 cells too the 3'-UTRs of oxidative phosphorylation mRNAs have an intrinsic TEA that is not present in the mRNA encoding the somatic transcription factor of mtDNA. However, in this cell line the 3'-UTR of the COX transcript was not the most active one; in fact, its activity on translation was significantly lower ($P < 0.025$) than that of the β transcript (Figure 3D).

DISCUSSION

Recently, we provided evidence *in vitro* that the 3'-UTR of the mRNA encoding the β -F1 ATPase is a non-coding regulatory sequence element required for mRNA translation [8,10]. Interestingly, this element is able to promote the internal initiation of mRNA translation [10], functionally resembling the IRES [10,19,20], albeit placed at the opposite end of the transcript. In the same study, we reported that the 3'-UTR of the α -subunit mRNA, a counterpart protein of the mitochondrial H^+ -ATP synthase, was devoid of such activity, whereas the 3'-UTR of the mRNA encoding COXIV was less active than that of β -mRNA on dicistronic assays [10]. In the present investigation, we have analysed the putative TEA of the 3'-UTRs of these mRNAs in cells in culture by monitoring the expression level of GFP* derived from chimaeric plasmids containing such sequence elements. We have included in the analysis the 3'-UTRs of an enhancer sequence described previously, the 3'-UTR of SV40 [16], and that of the mRNA encoding the somatic form of Tfam [21]. In contrast with the findings *in vitro* [10], the results in the present study indicate that all the tested 3'-UTRs of nuclear-encoded mRNAs involved in the bioenergetic function of the organelle (α , β and COXIV mRNAs) have an intrinsic TEA *in vivo* that is at least equal to that of the SV40 enhancer [16]. However, no TEA is present in the 3'-UTR of the Tfam mRNA. In addition, the results demonstrate that the relative activity on translation of the 3'-UTRs depends on the cell type studied. Although the results obtained in cells in culture need not necessarily be extrapolated to the physiological situation of the cells, it is reasonable to infer that the activity of the 3'-UTRs on translation might also operate in the control of the expression of these mRNAs in kidney and liver cells.

The expression of the mitochondrial genes, especially those that belong to the same protein complex (such as the α - and β -subunits of the H^+ -ATP synthase), is expected to be exerted in concert for the appropriate assembly and function of the mitochondrial activity in which they are involved. Thus the findings *in vivo* solved the apparent paradox resulting from the *in vitro* studies [10], revealing that the 3'-UTRs of the α - and β -mRNAs essentially have the same activity on translation. In addition, and within the translationally active 3'-UTRs (SV40, α , β and COXIV), it is observed that their activity on translation depends upon the cell type studied. These findings suggest that the control of translation of the mRNAs bearing these sequence determinants depends on the availability/activity of cell-type-specific factors. In fact, riboprobes derived from these sequences provided specific RNA-protein complexes for each of them, and the profile of the RNA-protein complexes formed varied among different cell lines ([10], and results not shown). Other findings also argue in favour of a tissue-specific regulation of the

expression of oxidative phosphorylation genes at both the transcriptional [22,23] and post-transcriptional [8,24–26] levels.

The observation that the 3'-UTRs of SV40, α , β and COXIV mRNAs have no obvious sequence determinants in common suggests that their activity on mRNA translation is exerted by the existence of a common 'scaffold' used by all them. In any case, the 3'-UTRs active in mRNA translation should recognize and interact with components of the translational machinery of the cell [8], for promoting the required communication between the 5'- and 3'-ends of the mRNAs for the functioning of an IRES-like activity placed at the 3'-end of the transcripts [10].

Recent findings indicated that the altered biogenesis of mitochondria in rat hepatomas results from a reversion in tumour cells to the mechanism that controls the translation of β -mRNA in fetal liver [5]. This mechanism of translational repression [5,8] is expected to be conserved for the expression of counterpart subunits of the same oligomeric complex and, perhaps, for other mitochondrial proteins involved in the bioenergetic function of mitochondria. Repression of β -mRNA translation is exerted by a specific set of 3'- β FBPs that are expressed in a developmental [8], tissue-specific [8] and pathologically regulated manner [5]. However, both the α - and COXIV-3'-UTRs provided different patterns of RNA-protein complexes when UV-cross-linked to proteins of reticulocyte lysates [10] and tissue extracts (results not shown). The observation that the 3'-UTRs of transcripts involved in oxidative phosphorylation possess TEA suggests that an upstream factor, presumably involved in the recognition of the determinants that define the TEA of each transcript, might cause the expected concerted repression of the translation of these mRNAs in carcinogenesis.

Finally, two biological programmes control the activity of mitochondria within the hepatocyte during development, which have been respectively defined as mitochondrial proliferation and differentiation (for a review, see [3]). The differentiation of mitochondria in rat liver promotes the rapid acquisition of a functional energy-transducing organelle [27], and is mainly controlled at the level of translation [1,2,8]. In contrast, mitochondrial proliferation during liver development, which represents an increase in the complement of mitochondria within the cell, i.e. the accumulation of mtDNA relative to the nuclear genome [11], requires most of the developmental period of the mammal [2,28]. The finding that the 3'-UTRs of oxidative phosphorylation mRNAs possess TEA, whereas that of the somatic Tfam mRNA does not, suggest further that mechanisms that control mRNA translation might define the rate of the bioenergetic differentiation of mitochondria after birth. In contrast, the concerted regulation of gene transcription [29] might define the rate of accretion of mtDNA during organelle proliferation.

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REFERENCES

- 1 Luis, A. M., Izquierdo, J. M., Ostronoff, L. K., Salinas, M., Santarén, J. F. and Cuezva, J. M. (1993) Translational regulation of mitochondrial differentiation in neonatal rat liver. Specific increase in the translational efficiency of the nuclear-encoded mitochondrial β -F1-ATPase mRNA. *J. Biol. Chem.* **268**, 1868–1875

- 2 Izquierdo, J. M., Ricart, J., Ostronoff, L. K., Egea, G. and Cuezva, J. M. (1995) Changing patterns of transcriptional and post-transcriptional control of β -F1-ATPase gene expression during mitochondrial biogenesis in liver. *J. Biol. Chem.* **270**, 10342–10350
- 3 Cuezva, J. M., Ostronoff, L. K., Ricart, J., López de Heredia, M., Di Liegro, C. M. and Izquierdo, J. M. (1997) Mitochondrial biogenesis in the liver during development and oncogenesis. *J. Bioenerg. Biomembr.* **29**, 365–377
- 4 Tvrdik, P., Kuzela, S. and Houstek, J. (1992) Low translational efficiency of the F1-ATPase β -subunit mRNA largely accounts for the decreased ATPase content in brown adipose tissue mitochondria. *FEBS Lett.* **313**, 23–26
- 5 López de Heredia, M., Izquierdo, J. M. and Cuezva, J. M. (2000) A conserved mechanism for controlling the translation of β -F1-ATPase mRNA between the fetal liver and cancer cells. *J. Biol. Chem.* **275**, 7430–7437
- 6 Egea, G., Izquierdo, J. M., Ricart, J., San Martín, C. and Cuezva, J. M. (1997) mRNA encoding the β -subunit of the mitochondrial F1-ATPase complex is a localized mRNA in rat hepatocytes. *Biochem. J.* **322**, 557–565
- 7 Ricart, J., Egea, G., Izquierdo, J. M., San Martín, C. and Cuezva, J. M. (1997) Subcellular structure containing mRNA for β subunit of mitochondrial H^+ -ATP synthase in rat hepatocytes is translationally active. *Biochem. J.* **324**, 635–643
- 8 Izquierdo, J. M. and Cuezva, J. M. (1997) Control of the translational efficiency of β -F1-ATPase mRNA depends on the regulation of a protein that binds the 3' untranslated region of the mRNA. *Mol. Cell. Biol.* **17**, 5255–5268
- 9 Jackson, R. J. and Kaminski, A. (1995) Internal initiation of translation in eukaryotes: the picornavirus paradigm and beyond. *RNA* **1**, 985–1000
- 10 Izquierdo, J. M. and Cuezva, J. M. (2000) Internal ribosome entry site functional activity of the 3'-untranslated region of the mRNA for the β -subunit of mitochondrial H^+ -ATP synthase. *Biochem. J.* **346**, 849–855
- 11 Ostronoff, L. K., Izquierdo, J. M., Enríquez, J. A., Montoya, J. and Cuezva, J. M. (1996) Transient activation of mitochondrial translation regulates the expression of the mitochondrial genome during mammalian mitochondrial differentiation. *Biochem. J.* **316**, 183–191
- 12 Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159
- 13 Liu, H. S., Jan, M. S., Chou, C. K., Chen, P. H. and Ke, N. J. (1999) Is green fluorescent protein toxic to the living cells? *Biochem. Biophys. Res. Commun.* **260**, 712–717
- 14 Garcia-Bustos, J., Heitman, J. and Hall, M. N. (1991) Nuclear protein localization. *Biochim. Biophys. Acta* **1071**, 83–101
- 15 Dingwall, C. and Laskey, R. A. (1991) Nuclear targeting sequence. *Trends Biochem. Sci.* **16**, 478–481
- 16 Wang, E., Ma, W. J., Aghajanian, C. and Spriggs, D. R. (1997) Posttranscriptional regulation of protein expression in human epithelial carcinoma cells by adenine-uridine-rich elements in the 3'-untranslated region of tumor necrosis factor- α messenger RNA. *Cancer Res.* **57**, 5426–5433
- 17 Yeilding, N. M., Rehman, M. T. and Lee, W. M. (1996) Identification of sequences in c-myc mRNA that regulate its steady-state levels. *Mol. Cell. Biol.* **16**, 3511–3522
- 18 Wickens, M., Anderson, P. and Jackson, R. J. (1997) Life and death in the cytoplasm: messages from the 3' end. *Curr. Opin. Genet. Dev.* **7**, 220–232
- 19 Pelletier, J. and Sonenberg, N. (1988) Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. *Nature (London)* **334**, 320–325
- 20 Johannes, G. and Sarnow, P. (1998) Cap-independent polysomal association of natural mRNAs encoding c-myc, BIP, and eIF4G conferred by internal ribosome entry sites. *RNA* **4**, 1500–1513
- 21 Larsson, N. G., Garman, J. D., Oldfors, A., Barsh, G. S. and Clayton, D. A. (1996) A single mouse gene encodes the mitochondrial transcription factor A and a testis-specific nuclear HMG-box protein. *Nat. Genet.* **13**, 296–302
- 22 Li, K., Hodge, J. A. and Wallace, D. C. (1990) OXBOX, a positive transcriptional element of the heart-skeletal muscle ADP/ATP translocator gene. *J. Biol. Chem.* **265**, 20585–20588
- 23 Barazzoni, R., Short, K. R. and Nair, K. S. (2000) Effects of aging on mitochondrial DNA copy number and cytochrome *c* oxidase gene expression in rat skeletal muscle, liver, and heart. *J. Biol. Chem.* **275**, 3343–3347
- 24 Izquierdo, J. M. and Cuezva, J. M. (1993) Evidence of post-transcriptional regulation in mammalian mitochondrial biogenesis. *Biochem. Biophys. Res. Commun.* **196**, 55–60
- 25 Preiss, T. and Lightowler, R. N. (1993) Post-transcriptional regulation of tissue-specific isoforms. A bovine cytosolic RNA-binding protein, COLBP, associates with messenger RNA encoding the liver-form isopeptides of cytochrome *c* oxidase. *J. Biol. Chem.* **268**, 10659–10667
- 26 Connor, M. K., Takahashi, M. and Hood, D. A. (1996) Tissue-specific stability of nuclear- and mitochondrially encoded mRNAs. *Arch. Biochem. Biophys.* **333**, 103–108
- 27 Valcarce, C., Navarrete, R. M., Encabo, P., Loeches, E., Satrustegui, J. and Cuezva, J. M. (1988) Postnatal development of rat liver mitochondrial functions. The roles of protein synthesis and of adenine nucleotides. *J. Biol. Chem.* **263**, 7767–7775
- 28 Izquierdo, J. M., Jiménez, E. and Cuezva, J. M. (1995) Hypothyroidism affects the expression of the β -F1-ATPase gene and limits mitochondrial proliferation in rat liver at all stages of development. *Eur. J. Biochem.* **232**, 344–350
- 29 Scarpulla, R. C. (1997) Nuclear control of respiratory chain expression in mammalian cells. *J. Bioenerg. Biomembr.* **29**, 109–119

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