Role of mRNA stability and translation in the expression of cytochrome *c* oxidase during mouse myoblast differentiation: instability of the mRNA for the liver isoform of subunit VIa

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The role of mRNA stability and translation in mediating the expression of selected subunits of cytochrome *c* oxidase (COX) was examined during the differentiation of mouse myoblasts into myotubes in cell culture. The expression of the liver (L) and heart (H) isoforms of COX VIa, which undergo an isoform switch during muscle development, as well as of the Va subunit, which is expressed in all tissues, was analysed. The translational efficiencies of COX Va, VIa-L and VIa-H, as well as of mitochondrially encoded COX mRNAs, were inferred from their distribution in polysome gradients. These experiments suggest that the translational efficiencies of these mRNAs do not change during myoblast differentiation, although the nuclear mRNAs for COX Va, VIa-L and VIa-H are translated more efficiently than the mitochondrial mRNAs. Analysis of mRNA stability using the tetracycline-repressible promoter system and/or actino-

mycin D indicates that COX VIa-L mRNA decays with a half-life of ~5–6 h in both myoblasts and myotubes, whereas COX VIa-H and Va mRNAs decay with half-lives of >15 h in myotubes. This relative instability of COX VIa-L mRNA serves to limit the accumulation of COX VIa-L mRNA in these myogenic cells, as compared with mRNAs for other COX subunits. Deletion/replacement mapping experiments suggest that the COX VIa-L 3' untranslated region contains a destabilization element. Analysis of the rate of poly(A) tail shortening on COX VIa-L and stable α -globin mRNAs suggests that the overall rate of poly(A) shortening *per se* is not rate limiting for the degradation of COX VIa-L mRNA.

Key words: COX, mitochondria, muscle, myotubes, polysomes.

INTRODUCTION

Cytochrome *c* oxidase (COX) is the terminal component of the electron transport chain and consists of 13 subunits, three of which are encoded by the mitochondrial genome. Three of the nuclear-encoded subunits, VIa, VIIa and VIII, have isoforms that are expressed in a tissue-specific manner (for reviews, see [1,2]). During muscle development of most mammals there is a switch from the liver (L) or non-muscle isoform to the heart (H) or striated muscle isoform of these subunits [3–8]. Isoform switching of metabolic enzymes such as COX is widespread during muscle development [9–12] and imparts striated muscle with the ability to meet the high and variable energy demands of muscle contraction [13,14]. The importance of the COX VIa isoform switch is especially well documented. The COX VIa-H isoform, but not the COX VIa-L isoform, contains an ADP-binding site, and binding of ADP to this site increases the efficiency of respiration [13,14]. Therefore both the increased expression of the COX VIa-H isoform and the reduced or limited expression of the COX VIa-L subunit are essential for COX to be regulated by nucleotides in striated muscle. This switch also occurs during the differentiation of myoblasts into myotubes in cell culture, facilitating analysis of the mechanisms regulating the COX VIa isoform switch [5,6]. We and others have shown that reciprocal changes in the levels of COX VIa-L and VIa-H mRNAs are associated with the isoform switch.

The present study addresses the role of mRNA translation and stability in mediating the expression of mRNAs for COX VIa isoforms during the differentiation of mouse myoblasts into

myotubes. The stability and translation of COX Va mRNA, the level of which does not change during myoblast differentiation [5], was also assessed in order to determine whether any of the mechanisms mediating expression of VIa isoforms are also used to control expression of COX mRNAs lacking isoforms. The role of translational regulation in controlling the expression of COX subunits and in isoform switching is not well understood. In bovine heart tissue, mRNA for COX subunit VIII-L is detected by RNA gel blot analysis, but a proportional amount of the VIII-L protein is not detected by Western blot analysis [15]. This suggests either that COX VIII-L mRNA is not translated efficiently or that the VIII-L protein is synthesized but rapidly turns over. It is also possible that differential translation of nuclear compared with mitochondrial COX subunits plays a role in regulating the equimolar accumulation of COX nuclear and mitochondrial subunits. Mitochondrial COX mRNAs are present in a 3–10-fold higher concentration than nuclear COX mRNAs [5,8], yet equimolar ratios of the subunits assemble into the COX holoenzyme. This suggests either that mitochondrial subunits are synthesized in excess and then are degraded, or that the mitochondrial COX mRNAs are not translated as efficiently as the nuclear COX mRNAs. To address these questions we have measured the translational efficiencies of COX VIa-L, VIa-H and Va mRNAs, as well as of mRNAs for mitochondrial COX subunits I, II and III, in myoblasts and myotubes.

The steady-state level of an mRNA is a function of both its transcription or synthesis and its degradation. The present experiments have focused on the role of mRNA stability in regulating COX VIa isoform switching during myoblast differentiation,

Abbreviation used: ARE, AU-rich element; COX, cytochrome *c* oxidase; COX VIa-L and VIa-H, liver and heart isoforms respectively of COX subunit VIa; RNP, ribonucleoprotein particle; mRNP, RNP containing mRNA; tTA, tetracycline-controlled transactivator; UTR, untranslated region.

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because previous experiments have suggested that the activation of COX VIa-H expression after myoblasts differentiate into myotubes is controlled at the level of transcription by elements that mediate the expression of other muscle-specific genes [16]. Although much less is known concerning the mechanisms that control mRNA stability, it is becoming evident that mRNA stability is an important aspect of gene regulation in eukaryotes (for a review, see [17]). Many rapidly degraded mRNAs, such as that of c-Fos, contain an AU-rich element (ARE) in their 3' untranslated region (UTR) that stimulates shortening of the poly(A) tail and subsequent degradation of the mRNA ([18]; for reviews, see [19,20]). Sequence elements that stabilize mRNA have also been identified, for example in the 3' UTR of α -globin mRNA [21]. However, the role of mRNA stability in mediating the accumulation of COX VIa-H and VIa-L mRNAs during the differentiation of myoblasts is unknown.

Our results show that the relative instability of COX VIa-L mRNA as compared with the mRNAs for other COX subunits plays an important role in limiting the expression of COX VIa-L mRNA in developing muscle. Furthermore, the instability of COX VIa-L mRNA is controlled by a transplantable destabilizing element that is located in the 3' UTR of COX mRNA. This element does not induce rapid poly(A) tail shortening. In fact, the rate of shortening of the poly(A) tail on COX VIa-L mRNA is similar to that on the considerably more stable α -globin mRNA. Our experiments also show that regulation at the level of translation is not important for the expression of COX subunits during myoblast differentiation.

EXPERIMENTAL

Cell culture

The MM14DZ myoblast cell line was cultured in Ham's F12 medium containing 15% (v/v) horse serum and crude fibroblast growth factor, as described previously [22,23]. The culture medium was changed 24 h after plating of cells. In our culture conditions, myoblasts divide rapidly for 2–3 days with a generation time of ~15 h. They then withdraw permanently from the cell cycle and fuse to form multinucleated myotubes. By the fourth day virtually all the nuclei are contained in multinucleated myotubes, which then increase in size.

Mouse L cells were grown in Eagle's minimum essential medium containing 5% (v/v) newborn calf serum.

RNA isolation

Whole-cell RNA was isolated using Proteinase K, phenol/chloroform and LiCl [24,25] or Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH, U.S.A.). In experiments that involved the transient expression of transfected genes, RNA preparations were also digested with DNase I to remove residual DNA.

Plasmid construction

The entire mouse COX VIa-L cDNA sequence (GenBank accession no. U08440), previously cloned into pBluescript SK(–) (Stratagene, La Jolla, CA, U.S.A.), was amplified using PCR techniques. As this VIa-L cDNA sequence starts just after the AUG codon, the nucleotides TCCAAGAT, corresponding to rat VIa-L AUG, were included in the 5' oligonucleotide used to amplify the mouse COX VIa-L cDNA. The entire rabbit α -globin cDNA (accession nos. V00875 and J00658) was amplified by reverse transcriptase-PCR starting with RNA isolated from rabbit reticulocytes. The tet-COX and tet-GLO plasmids were generated by cloning the COX VIa-L and α -globin sequences

respectively into the unique *SacII* (blunt-ended with T4 DNA polymerase) and *HpaI* sites of the vector pUHD 10-3 [26], which contains the tetracycline-repressible promoter.

The tet-COX-GLO and tet-GLO-COX chimaeric plasmids were generated by ligating chimaeric cDNAs into pUHD 10-3, as described above. The appropriate 5' and 3' fragments of the α -globin and COX VIa-L mRNAs were amplified by PCR and then ligated. The tet-GLO-COX chimaera contains the first 461 nucleotides of α -globin, including the first two nucleotides of the 3' UTR, linked to the last 194 nucleotides of COX VIa-L mRNA, which includes the last three nucleotides of the coding region and the entire 3' UTR. The tet-COX-GLO chimaera contains 334 nucleotides of COX VIa-L mRNA, including the entire coding region and four nucleotides of the 3' UTR, linked to the last 87 nucleotides of the α -globin mRNA, which includes the entire 3' UTR and the last two nucleotides of the coding region. The plasmid BBB-COX was constructed by cloning the last 194 nucleotides of COX VIa-L mRNA into the unique *BglIII* site located in the 3' UTR of the β -globin gene in the vector BBB [18].

Cell transfection

Myoblasts and myotubes were transfected using the calcium phosphate technique as described previously [23]. Myoblasts were transformed with the ptet-tak [27] and pSV2-neo [28] plasmids to generate lines expressing the tetracycline-controlled transactivator (tTA). Colonies were selected for growth in the presence of G418, as described previously [22]. To generate cell lines stably expressing COX VIa-L from the tetracycline-repressible promoter, the tTA cell lines were transfected with tet-COX and pSV2-hph (American Type Culture Collection #37647), followed by selection with 200 μ g/ml hygromycin. Alternatively, myoblasts were transfected simultaneously with tet-COX, ptet-tak and SV2-neo to generate cell lines expressing both tTA and the tet-COX gene. Cell lines were tested for their ability to support tetracycline-regulated expression from the tetracycline-repressible promoter by transiently transfecting these lines with pUHC 13.3 [25], which contains the luciferase gene located downstream of the tetracycline-repressible promoter. Only lines that expressed luciferase at high levels and whose luciferase expression could be reduced by at least 85–90% by addition of 0.5 μ g/ml tetracycline for 20 h were used in these studies. In transient transfection experiments, 100 mm-diam dishes were typically transfected with 5 μ g of tet-COX or tet- α -globin and 3 μ g of the ptet-tak plasmids, as described previously [23], and two or more plates were pooled for each time point to reduce or eliminate variability due to plate-to-plate differences in transfection efficiency.

Actinomycin D experiments

Actinomycin D was added to myotube cultures to a final concentration of 1 μ g/ μ l, and RNA was isolated at various times thereafter. Whole-cell RNA obtained at various times of the chase was fractionated on denaturing agarose gels [24]. The levels of specific mRNAs were assessed by RNA gel-blot techniques using nick-translated cDNA hybridization probes [24]. The relative amounts of specific mRNAs were determined by densitometry of autoradiograms and normalized to the amount of rRNA loaded on the corresponding lane, as indicated by ethidium bromide staining. The normalized levels of specific mRNAs at various times of the chase were expressed as a fraction of the normalized level of that specific mRNA at 0 h of the chase and plotted (see Figure 1). These decay plots actually represent the decay of specific mRNAs as compared with that of rRNA. Since rRNA is stable in MM14DZ myoblasts and

myotubes [29], analysis of the decay curves yields half-lives of mRNAs directly.

Tetracycline chase and RNase protection experiments

Tetracycline was added to a final concentration of 0.5–1.0 $\mu\text{g}/\text{ml}$ to specifically block transcription of genes cloned downstream of the tetracycline promoter. The levels of the mRNAs derived from the transfected genes and the endogenous COX VIa-L gene were measured by RNase protection using 15000–30000 c.p.m. of probe per sample. Hybridization and RNase digestion were performed as described previously [25], except that 2.2 $\mu\text{g}/\text{ml}$ RNase A and 44 units/ml T1 RNase were used. Alternatively, for analysis of COX VIa-L mRNA expressed from stably incorporated tet-COX genes, cells were harvested using Direct Protect lysis buffer (Ambion Inc, Austin, TX, U.S.A.). Equal fractions of the lysate from each time point (usually 5–10 μl) were then hybridized at 55 °C in a 30 μl hybridization reaction containing 5 μg of tRNA, 200 mM NaCl, 20 mM Pipes (pH 6.8), 0.5 mM EDTA and 17% formamide, and digested with RNase as described above. Radioactivity was detected using a Molecular Dynamics Storm PhosphorImager.

The RNA probes were synthesized using T7 polymerase from templates generated by PCR of the tet-COX or tet-GLO plasmids [25] using 3' primers that contained T7 promoter sequences. The RNA probe used to detect mRNAs having COX VIa-L sequences at their 5' ends contained 87 nucleotides of upstream non-transcribed sequences, ~68 nucleotides of transcribed vector sequences and 250 nucleotides of COX VIa-L sequence. tet-COX mRNA protects 318 nucleotides of the probe, whereas the endogenous COX VIa-L mRNA protects 250 nucleotides of the probe. Two different probes were used to detect mRNAs containing tet-GLO sequences at their 5' ends, resulting in a protected species of either 215 or 303 nucleotides.

Data analysis

For each time point, the signal from the mRNA derived from the transfected gene was normalized to the signal from the endogenous COX VIa-L mRNA. For myotubes the normalized values at various times of the tetracycline chase are expressed as a fraction of the normalized value at 0 h of the chase and plotted. No additional manipulation of the myotube data is required, because the level of the endogenous COX VIa-L mRNA does not change during the course of the chase of myotubes, either on a per cell basis or on a per plate basis, as the nuclei of myotubes do not divide. In contrast, plotting the normalized values from myoblasts does not yield the half-life directly, because the amount of endogenous COX VIa-L mRNA per plate increases logarithmically, since tetracycline does not inhibit cell division or expression of endogenous COX VIa-L mRNA. The normalized value for each time point in myoblasts was multiplied by $e^{(t)\ln 2/t_D}$, where t is the time of the chase and t_D is the myoblast generation time (15 h), so that the resulting plot reflects only the decay of mRNA rather than a combination of mRNA decay and dilution due to cell division.

Serum-inducible promoter experiments

Myotube cultures were transfected with vector BBB-COX or BBB [18]. Two or more plates were pooled to reduce variability due to plate-to-plate variation in transfection efficiency. In some experiments the cultures were also transfected with p_{tet}-tak and tet-GLO. The expression of the tet-GLO mRNA serves as an internal control for transfection efficiency and RNA recovery. After transfection, cultures were placed into medium containing

19 parts fresh Ham's F12 and 1 part conditioned medium, so that the concentration of horse serum was 0.75% (v/v). Medium was conditioned by incubation with muscle cell cultures for 4 days. After 28–36 h the medium was changed to fresh medium containing 15% (v/v) horse serum to initiate the transcriptional pulse. RNA was isolated at various times thereafter, and the amount of BBB-COX or BBB mRNA was determined by RNase protection using a 313-nucleotide probe, of which 135 nucleotides are protected by correctly initiated β -globin transcripts. The amount of BBB or BBB-COX mRNA was normalized to the amount of endogenous COX VIa-L or tet-GLO measured as described above and plotted.

Poly(A) measurements

Samples of 5 μg of RNA isolated at various time points of the chase were fractionated on 4% (w/v) polyacrylamide gels containing 7 M urea. A 5 μg aliquot of RNA from the $t = 0$ time point was incubated with RNase H and oligo(dT), as described by the supplier (United States Biochemical, Cleveland, OH, U.S.A.), to remove the poly(A) tail. The RNase H-treated sample was fractionated with the other samples to identify the size of deadenylated mRNAs. After electrophoresis the RNA was electroblotted on to Nytran Plus membranes (Schleicher & Schuell, Keene, NH, U.S.A.), cross-linked to the membrane and hybridized to RNA probes as described by the manufacturer.

Polysome analysis

Cell extracts were fractionated on sucrose gradients, and the relative level of COX mRNA measured in each fraction was determined using RNA gel-blot analysis as described previously [23,24]. The distribution of mitochondrial COX mRNAs in polysome gradients was determined similarly, except that the harvesting buffer contained 50 $\mu\text{g}/\text{ml}$ chloramphenicol to inhibit elongation, and the gradients were centrifuged for 3.5 h instead of 2 h to separate the smaller mitochondrial ribosomes and polysomes. Pilot experiments indicated that >90% of mitochondrial polysomes are solubilized by our standard polysome extraction procedures. Nick-translated cDNA probes were used to detect the nuclear-encoded COX mRNAs [5]. Probes derived from nucleotides 6717–7220, 9047–10084 and 366–1860 of the mitochondrial genome (accession no. J01420) were used to detect COX I and II mRNAs, COX III mRNA and mitochondrial rRNAs respectively.

RESULTS

Analysis of mRNA stability using actinomycin D

The stabilities of the COX VIa-L, VIa-H and Va mRNAs were measured during the differentiation of myoblasts into myotubes initially using actinomycin D. Unlike the COX VIa-L and VIa-H mRNAs, the level of COX Va mRNA does not change during myoblast differentiation [5]. Myotube cultures were treated with an amount of actinomycin D shown in pilot experiments to inhibit the incorporation of [³H]uridine into RNA by >95% in a 30 min pulse. Under these conditions, myotubes maintained normal morphology for at least 20 h. The amounts of COX VIa-L, VIa-H and Va mRNAs remaining at various times after the addition of actinomycin D were measured using RNA gel-blot techniques and normalized to the amount of RNA loaded on the corresponding lane. Figure 1 shows that there was little loss of COX VIa-H or Va mRNAs during the actinomycin D chase, suggesting that these mRNAs have half-lives of >15–20 h in myotubes. In contrast, COX VIa-L mRNA decayed with a half-

Figure 1 Actinomycin D chase of MM14DZ myotubes

Cultures of myotubes were incubated with 1 μ g/ml actinomycin D to inhibit transcription. RNA was isolated at the indicated times thereafter, fractionated on agarose gels, blotted to charged nylon membranes and hybridized to probes to COX V1a-L, V1a-H and Va mRNAs. The gel was stained with ethidium bromide before blotting to monitor loading of the RNA. The hybridization signal was normalized to the amount of RNA loaded on to each lane. Data from two independent experiments are plotted in (A), as described in the Experimental section, and the autoradiograms and ethidium bromide-stained gel from one experiment are shown in (B).

life of approx. 5–6 h during the actinomycin D chase. The relative instability of COX V1a-L mRNA would serve to limit the accumulation of this mRNA in myotubes as compared with that of the other COX mRNAs.

Analysis of mRNA stability using the tetracycline-repressible promoter system

Although actinomycin D is used widely to measure mRNA stability, it blocks the transcription of all genes, not just the gene

of interest, and can be toxic to cells. In particular, myoblasts stop dividing and detach from the plate after approx. 10 h of actinomycin D treatment, making actinomycin D unsuitable for stability measurements in myoblasts. The tetracycline-repressible promoter system [26] was therefore used to measure the stability of COX V1a-L mRNA in myoblasts and to verify the results obtained with actinomycin D in myotubes. Myoblasts divide and differentiate normally in the presence of tetracycline. Furthermore, tetracycline only blocks transcription of genes located downstream of the tetracycline-repressible promoter, and does not have a general effect on RNA metabolism.

Myoblasts were stably transformed with the tet-COX plasmid, which contains COX V1a-L mRNA sequences downstream of the tetracycline-repressible promoter and the tTA, to facilitate analysis of the stability of COX V1a-L mRNA. Tetracycline binds to tTA, causing it to dissociate from the tetracycline-repressible promoter, thereby inhibiting transcription. Tetracycline was added to these cultures of myoblasts and myotubes, and the amount of tet-COX mRNA remaining at various times thereafter was determined using RNase protection techniques. The endogenous COX V1a-L mRNA, the level of which should not decrease during a tetracycline chase, was also detected by this probe, and serves as an internal control for recovery. Although tetracycline is effective in blocking transcription (see the Experimental section), it is possible that transcription is not completely inhibited in the $\sim$ 1–2 h of the tetracycline chase. This would cause an overestimation of mRNA half-life. Figure 2 shows that tet-COX mRNA decayed with a half-life of approx. 5–6 h in both myoblasts and myotubes. Similar results were obtained when the stability of tet-COX mRNA was measured after transient transfection of cells with tTA and tet-COX, and after transient transfection of cells stably expressing tTA with tet-COX (results not shown). Furthermore, the stability of the tet-COX mRNA was also measured as a function of its level of expression, which was manipulated by altering the concentration of tetracycline in the medium. These experiments showed that tet-COX mRNA has the same stability regardless of whether it is expressed at about 10 times or one-third the level of endogenous COX V1a-L mRNA (results not shown).

To determine if COX V1a-L mRNA is also unstable in other cells, the stability of COX V1a-L was measured in mouse L cells. Figure 2 shows that tet-COX mRNA decayed with a half-life of approx. 12 h in dividing mouse L cells, which is significantly longer than the half-life of 5–6 h for tet-COX mRNA in both myoblasts and myotubes. This result suggests that COX V1a-L mRNA has different half-lives in different cell types.

Another control experiment was performed to examine the possibility that the instability of COX V1a-L mRNA derived from the tet-COX gene was due to the CMV (cytomegalovirus) sequences located at its 5' end, rather than to some inherent property of COX V1a-L mRNA. To this end, the rabbit α -globin mRNA was cloned downstream of the tetracycline-repressible promoter, and the stability of the derived mRNA (tet-GLO) was measured in myotubes. Figure 3 shows that the tet-GLO mRNA had a half-life of at least 15 h in myotubes, which is considerably greater than the half-life of 5–6 h for COX V1a-L mRNA. This indicates that the instability of COX V1a-L mRNA is due to an inherent property of this mRNA, rather than being a property of the tetracycline-repressible promoter system.

The 3' UTR of COX V1a-L mRNA contains an instability element

To identify the sequences that confer tissue-specific instability on COX V1a-L mRNA, chimaeras were constructed between the tet-COX and tet-GLO genes (Figure 3A). In the tet-COX-GLO

Figure 2 Tetracycline chase of myoblasts, myotubes and mouse L cells expressing the tet-COX mRNA

Cultures containing both the tTA and the tet-COX gene stably incorporated into the genome were incubated with 1 μ g/ml tetracycline to block transcription of the transfected tet-COX gene. The amounts of tet-COX mRNA and of the endogenous COX VIa-L mRNA were measured using RNase protection techniques. The signal from the tet-COX mRNA was normalized to that from the endogenous COX VIa-L mRNA and plotted as described in the Experimental section. The data in (A) are derived from two independent experiments. The autoradiograms from one experiment are shown in (B). The signal derived from the tet-COX mRNA and the endogenous COX VIa-L mRNA (endo. COX) are indicated.

plasmid, the COX VIa-L 3' UTR is replaced by the tet-GLO 3' UTR. In the tet-GLO-COX plasmid, the 3' UTR of tet-GLO is replaced by the 3' UTR of tet-COX. These genes were transfected together into myotubes, and the relative and absolute stabilities of the derived mRNAs were determined in a tetracycline chase experiment. Figure 3 shows that the tet-COX-GLO mRNA was stable, having a half-life similar to that of tet-GLO mRNA, whereas the tet-GLO-COX mRNA was unstable, similar to the tet-COX mRNA. The simplest interpretation of this result is that the 3' UTR of COX VIa-L mRNA contains an instability element.

A different transcriptional chase approach was also utilized to investigate the role of the COX VIa-L 3' UTR in mediating the instability of this mRNA in myotubes. In this experiment the 3' UTR of COX VIa-L mRNA was cloned into the 3' UTR of the rabbit β -globin gene located downstream of the serum-inducible human *c-fos* promoter in the BBB plasmid [18]. Cells grown in reduced serum were treated with medium containing high levels of serum to activate the *c-fos* promoter for a short period of time, thus creating a transcriptional pulse. The stability of the β -globin mRNA containing or lacking the COX VIa-L 3' UTR was then

inferred by measuring the loss of β -globin mRNA after the transcription pulse. This system has been used extensively to identify and characterize AREs, based on their ability to destabilize the β -globin mRNA when cloned into this same site in the β -globin 3' UTR [18,19]. Both the β -globin mRNA (BBB) and the β -globin mRNA containing the COX VIa-L 3' UTR (BBB-COX) accumulated for 5–8 h after initiation of the transcriptional pulse. Figure 4 shows that the level of BBB mRNA increased slightly after this time, suggesting, as expected, that it is stable. In contrast, the BBB-COX mRNA decayed with a half-life of approx. 8–10 h. These experiments also suggest that the 3' UTR of COX VIa-L mRNA contains a destabilization element.

Role of poly(A) shortening in mediating the instability of COX VIa-L mRNA

To determine if rapid deadenylation is associated with the degradation of COX VIa-L mRNA, the rates of poly(A) tail shortening for tet-COX (half-life $\sim$ 5–6 h) and tet-GLO (half-life $\sim$ 15 h) mRNAs were measured in myotubes. Cultures expressing tet-COX or tet-GLO were incubated with tetracycline, and the

Figure 3 Tetracycline chase of myotubes expressing tet-GLO, tet-COX-GLO and tet-GLO-COX mRNAs

The structures of these genes cloned downstream of the tetracycline-repressible promoter are indicated in (A). Myotubes were transfected with the tet-GLO gene or simultaneously with the tet-COX-GLO and tet-GLO-COX genes, and the tTA gene. The tetracycline chase was initiated 24 h after transfection by the addition of tetracycline at 1 μ g/ml, and RNA was isolated at various times thereafter. The amounts of mRNAs derived from the transfected genes and of endogenous COX VIa-L mRNA were measured using RNase protection. The signals from the tet-GLO, tet-COX-GLO and tet-GLO-COX mRNAs were normalized to that from the endogenous COX VIa-L mRNA and plotted as described in the Experimental section. The data in (B) are derived from two independent experiments, and the autoradiograms from one experiment are shown in (C). The signal from the endogenous COX mRNA (endo. COX) is also indicated.

poly(A) tail lengths of these mRNAs were measured at various times of the chase using Northern analyses and high-resolution acrylamide-gel electrophoresis. Deadenylated tet-COX or tet-GLO mRNAs were generated by oligo(dT) and RNase H treatment and included on each gel. Figure 5 shows that, at 0 h of the chase, the size of the poly(A) tail for both mRNAs was heterogeneous, with a median length of approx. 100 nucleotides. This represents the steady-state distribution of poly(A) tail length. The median poly(A) tail length of both tet-COX and tet-GLO mRNAs had decreased to approx. 55 nucleotides by 8–12 h of the chase. The poly(A) tail length is still heterogeneous at the end of the chase, with poly(A) tails ranging from 20 to 120 nucleotides. A similar pattern of poly(A) tail shortening was displayed by the tet-COX-GLO and tet-GLO-COX mRNAs (results not shown). Within the resolution of this analysis, the rate and extent of

poly(A) tail shortening is the same for both the tet-COX and tet-GLO mRNAs.

Translational efficiencies of COX mRNAs

The translational efficiencies of the mRNAs for the nuclear-encoded COX subunits VIa-H, VIa-L and Va in myoblasts and myotubes were inferred from their distribution in polysome gradients. The more ribosomes translating an mRNA the more efficiently it is translated, assuming there are no differences in the elongation rate. These experiments were undertaken to determine if the expression of these mRNAs is regulated at the level of translation. Myoblast and myotube cell extracts were fractionated on sucrose gradients to separate polysomes from non-polysomal ribonucleoprotein particles (RNPs), such as ribosomal

Figure 4 Stability of rabbit β -globin mRNA containing the COX VIa-L 3' UTR

Myotubes were transfected with the rabbit β -globin gene cloned downstream of the human *c-fos* promoter (BBB), or the rabbit β -globin gene containing the COX VIa-L 3' UTR cloned downstream of the human *c-fos* promoter (BBB-COX). The transcriptional pulse was initiated at $t = 0$, and RNA was isolated at various times thereafter. The levels of rabbit β -globin mRNA and of endogenous COX mRNA (endo. COX) were measured using RNase protection. The signal from rabbit β -globin mRNA was normalized to that from the endogenous COX VIa-L mRNA. The data plotted in (A) are derived from two independent experiments, and the autoradiograms from one experiment are shown in (B). FM, fresh medium.

subunits (Figure 6). RNA was isolated from fractions of the gradient and analysed using RNA gel blots. No data are shown for COX VIa-H in myoblasts, because this mRNA is not expressed at detectable levels in these cells. More than 90% of COX VIa-H, VIa-L and Va mRNAs co-sedimented with polysomes containing three and five ribosomes in both myoblasts and myotubes (Figure 6). The sedimentation of these mRNAs in the polysome region of the gradient was disrupted by incubation of the extract with EDTA, implying that these mRNAs are associated with ribosomes rather than with large mRNPs (RNPs containing mRNA) (results not shown). These results suggest that the translational efficiencies of these nuclear-encoded COX mRNAs are similar in myoblasts and myotubes,

Figure 5 Poly(A) tail shortening of tet-COX and tet-GLO mRNAs during a tetracycline chase

Myotubes were transfected with tet-COX or tet-GLO along with the gene for the tTA. Cultures were incubated with 1 μ g/ml tetracycline 24 h after transfection. RNA was isolated at the indicated times thereafter and fractionated on a 4% acrylamide/urea gel, electroblotted to charged nylon membranes, and hybridized to a probe complementary to the transcribed sequences of the tetracycline-repressible promoter. Deadenylated tet-GLO and tet-COX mRNAs were generated using oligo(dT) and RNase H, and the predicted sizes of the deadenylated tet-GLO and tet-COX mRNAs are indicated. A *Hae*III digest of ϕ X 174 was also included on the gel as size markers, and the migration of the relevant species is indicated.

Figure 6 Distribution of nuclear-encoded COX VIa-H, VIa-L and Va mRNAs in polysome gradients

Myoblast (day 2) and myotube (day 6) extracts were fractionated on polysome gradients. RNA was isolated from fractions of the gradient, and the relative amounts of specific mRNAs in the fractions were determined by RNA gel-blot analysis using hybridization probes to COX VIa-H, VIa-L and Va mRNAs. The absorbance profile of a typical sucrose gradient is shown, with the polysome region of the gradient indicated.

and that translational regulation is not important in regulating the expression of nuclear COX mRNAs in this system.

The translational efficiencies of the mitochondrially encoded COX subunits I, II and III were also inferred from their distribution in polysome gradients. These gradients were centrifuged for longer to separate the smaller mitochondrial ribosomal subunits and polysomes. Analysis of the distribution of the

Figure 7 Distribution of mitochondrially encoded COX I, II and III mRNAs in polysome gradients

Myoblast (day 2) and myotube (day 6) extracts were fractionated on high-resolution sucrose gradients. RNA was isolated from fractions of the gradients, and the relative amounts of COX I, II and III mRNAs in the fractions were determined by RNA gel-blot analysis. The distribution of cytoplasmic (cyto) and mitochondrial (mito) rRNAs in a typical gradient, in this case a myotube gradient, is also shown. The mitochondrial rRNAs were detected by RNA gel-blot methods, whereas the cytoplasmic rRNAs were detected by ethidium bromide staining of the RNA gel before transfer to paper.

mitochondrial rRNAs in the gradient (Figure 7) indicated that the small mitochondrial rRNA subunit sedimented in fraction 3, the large subunit in fraction 4, and 58 S mitochondrial monosomes in fraction 5 (the first fraction that contains equimolar amounts of large and small subunit mRNAs). The large 60 S cytoplasmic ribosomal subunit also sedimented primarily in fraction 5, and to a lesser extent in fraction 6. Thus mitochondrial mRNAs translated by one 58 S mitochondrial ribosome should be located in fractions 5–6, and mRNAs translated by between two and four ribosomes in fractions 6–7, of the polysome gradient. Treatment of the cell extracts with EDTA to chelate magnesium caused mRNAs that sedimented with mitochondrial monosomes or polysomes (fractions 5–7) to shift to fractions 2–3 of the gradient (results not shown). The magnesium-dependence of the sedimentation of these mitochondrial mRNAs in fractions 5–7 of the gradient suggests that they are associated with ribosomes rather than with large mRNPs.

The distribution of the mitochondrially encoded COX mRNAs in the polysome gradients was the same in myoblasts and myotubes, suggesting that the translational efficiencies of the mitochondrial mRNAs, like those of the nuclear COX mRNAs, do not change during myoblast differentiation. However, the polysome size and the fraction of mRNA associated with polysomes was different for each of the mitochondrial mRNAs. A greater proportion of the smaller COX III mRNA sedimented in the subpolysomal region of the gradient and was presumably not actively translated.

To determine if the nuclear and mitochondrial COX mRNAs were translated with similar efficiencies, the packing densities of ribosomes on the mitochondrial and nuclear COX mRNAs were estimated from the sizes of the coding region and the number of ribosomes translating each mRNA (Figures 6 and 7). It was estimated that the cytoplasmic ribosomes were spaced 60–100 nucleotides apart, whereas the mitochondrial ribosomes were spaced 250–750 nucleotides apart. Elongation rates for mitochondrial ribosomes are similar to or slower than those for cytoplasmic ribosomes [30,31]. Therefore the greater spacing of the mitochondrial ribosome is not due to an increased elongation

rate, but rather suggests that mitochondrial mRNAs are translated 3–10-fold less efficiently than cytoplasmic mRNAs.

DISCUSSION

Striated muscle cells must limit the accumulation of the COX VIa-L subunit as compared with the COX VIa-H subunit and the COX subunits that do not have isoforms. Our experiments show that the instability of COX VIa-L mRNA plays a major role in limiting the expression of COX VIa-L in developing muscle as compared with other COX mRNAs. The COX VIa-L mRNA is relatively unstable as compared with the COX Va and VIa-H mRNAs, having a half-life of approx. 5–6 h as compared with > 15–20 h for the other COX mRNAs. The instability of COX VIa-L mRNA is tissue-specific, at least to some extent, as COX VIa-L has a significantly greater stability (half-life ~ 12 h) in mouse L cells. These results raise the possibility that tissue-specific mRNA instability is a general mechanism limiting the expression of non-muscle isoforms of metabolic enzymes in striated muscle.

The stability of COX VIa-L mRNA does not change during the differentiation of myoblasts into myotubes in cell culture, although the level of COX VIa-L mRNA does decrease [5]. This result indicates that changes in the stability of COX VIa-L mRNA do not control the decreased expression of this mRNA during myoblast differentiation. Rather, changes in transcription, or possibly the efficiency of mRNA processing, must mediate the decreased expression of VIa-L mRNA during myoblast differentiation. The expression of COX VIa-L mRNA is, therefore, tightly controlled in differentiating muscle cells by mechanisms acting at different levels of gene expression.

Previous experiments also point to a general role for mRNA stability in mediating the steady-state levels of COX mRNAs. COX IV, Va, Vb, VIIa-L, VIIb and VIII are reasonably stable in liver Hep G2 cells, having half-lives between 10 and 25 h. Their half-lives increase by 4–12-fold when cells are treated with the mitochondrial synthesis inhibitor thiamphenicol [32]. Furthermore, actinomycin D chase experiments in whole animals indicate that COX VIc is more stable in skeletal muscle than in the liver or heart [33].

Two approaches were used to identify the sequence element(s) in COX VIa-L mRNA that are responsible for its relative instability in myotubes. In one approach, chimaeras were constructed between COX VIa-L and α -globin genes and cloned downstream of the tetracycline-repressible promoter. The α -globin 3' UTR was replaced by the COX 3' UTR, and vice versa. In the second approach the COX VIa-L 3' UTR was inserted into the β -globin 3' UTR, a system that has been used extensively to identify destabilizing sequences [18]. In both cases mRNAs having the COX 3' UTR were destabilized. The simplest interpretation of the data is that the 3' UTR of COX VIa-L mRNA contains a tissue-specific destabilization element. Comparison of the 3' UTR of COX VIa-L mRNA with other known destabilizing and stabilizing elements indicates that this region does not contain an ARE and is not obviously similar to other known determinants of mRNA stability.

This analysis does not eliminate the possibility that COX VIa-L mRNA contains an additional destabilizing element in its coding region, similar to c-Fos mRNA [2]. For this possibility to be consistent with the observation that the tet-COX-GLO chimaera (having the 3' UTR of COX replaced with the α -globin 3' UTR) is stable (Figure 3), one would have to postulate that the α -globin 3' UTR contains a stabilizing element that is dominant to a destabilizing element in the coding region of COX VIa-L mRNA. This is not an unreasonable possibility, in that some

globin 3' UTRs contain stabilization elements [21]. Further experimentation is required to determine if the coding region of COX VIa-L mRNA contains a destabilization element. Nevertheless, taken together, the data presented here strongly suggest that COX VIa-L mRNA contains a destabilization element in its 3' UTR.

An RNA binding activity, identified as glutamate dehydrogenase [34], binds to the 3' UTR of mRNAs for non-muscle isoforms of bovine VIIa and VIII and human VIIa COX subunits [35,36]. A similar factor also binds to the 3' UTR of bovine COX VIa-L mRNA [6]. This RNA binding activity is present at high concentrations in tissues that express these non-muscle isoforms, such as liver, but present at low levels in adult muscle tissues that do not express high levels of non-muscle isoforms. It was suggested that this RNA binding activity is necessary for the expression of non-muscle isoforms, possibly by protecting these mRNAs from nucleolytic degradation. The role, if any, of this RNA binding activity in controlling the stability of COX VIa-L mRNA is unknown. The situation is complicated by the observation that COX VIa-L and VIIa-L mRNAs are not coordinately regulated during myoblast differentiation. Unlike COX VIa-L mRNA, the amount of COX VIIa-L mRNA does not decrease during myoblast differentiation [11]. It is unlikely, therefore, that glutamate dehydrogenase regulates both mouse COX VIa-L and VIIa-L mRNAs in developing muscle. Further experimentation is required to identify and characterize the factors that mediate the instability of COX VIa-L mRNA during muscle differentiation and to clarify the role of glutamate dehydrogenase in this process.

The rate of shortening of the poly(A) tail may not be rate limiting for the degradation of COX VIa-L mRNA. The poly(A) tail on some ARE-containing mRNAs, such as *c-Fos*, shortens synchronously. *c-Fos* mRNA is stable until the poly(A) tail shortens to a critical length of 30–60 nucleotides, and then is degraded rapidly, generating biphasic decay kinetics [18,19,37]. The poly(A) tail on other ARE-containing mRNAs, such as granulocyte/macrophage colony-stimulating factor, is degraded asynchronously, generating mRNAs that are totally deadenylated. mRNA decay then follows deadenylation. In this case the older mRNAs also turn over more rapidly than newly synthesized mRNA. The poly(A) tail on these mRNAs shortens with age, which increases the probability of complete deadenylation and subsequent degradation [19,37]. In contrast with these ARE-containing mRNAs, the decay of COX VIa-L mRNA approximates first-order kinetics over the entire period of the chase (Figures 1 and 2). This result indicates that decay of COX VIa-L mRNA is stochastic and that old and new mRNAs decay at the same rate, at least during the time course of our experiments. The average poly(A) tail length of COX VIa-L does decrease during the chase, from approx. 100 to 60 nucleotides, but this shortening of the poly(A) tail does not increase the probability of degradation. Furthermore, the rate of poly(A) tail shortening on COX VIa-L mRNA is similar to that on α -globin mRNA, even though α -globin mRNA is severalfold more stable than COX VIa-L mRNA (Figure 5). Therefore our results suggest that the overall rate of poly(A) tail shortening *per se* is not rate limiting for the degradation of COX VIa-L mRNA, at least when compared with α -globin mRNA. It is likely that some step after the shortening of the poly(A) tail, perhaps complete deadenylation or decapping, is rate limiting for the degradation of COX VIa-L mRNA. Alternatively, the rate-limiting step in the decay of COX VIa-L mRNA could be an endonucleolytic cleavage. Further experimentation is required to elucidate the rate-limiting step in the degradation of COX VIa-L mRNA.

Analysis of the translation of COX mRNAs indicates that

there is no change in the spacing of ribosomes on either the nuclear or the mitochondrial COX mRNAs after myoblasts differentiate into myotubes. This result suggests that the translational efficiencies of these mRNAs do not change during muscle development, and that VIa isoform switching is not regulated at the level of translation during the differentiation of mouse myoblasts into myotubes.

Although the spacing of ribosomes on nuclear and mitochondrial mRNAs does not change during myoblast differentiation, the spacing of ribosomes on mitochondrial mRNAs is significantly greater than that on cytoplasmic mRNAs (250–750 compared with 60–100 nucleotides). Given that the elongation rates of mitochondrial ribosomes are estimated to be lower than those of cytoplasmic ribosomes [30,31], then this difference in ribosome spacing suggests that mitochondrial COX mRNAs are translated much less efficiently than the nuclear-encoded COX mRNAs. Cells must, therefore, accumulate 3–10-fold more mitochondrial mRNAs than nuclear mRNAs in order to synthesize equimolar amounts of mitochondrial and nuclear subunits. Analysis of the relative levels of nuclear and mitochondrial COX mRNAs shows that cells do, in fact, contain much higher levels of mitochondrial mRNAs [5,8].

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