Volume 292, number 1,2, 79–84 FEBS 10322 November 1991

ADONIS 0014579391010320

Switching of bovine cytochrome *c* oxidase subunit VIa isoforms in skeletal muscle during development

G.D. Ewart, Y.-Z. Zhang and R.A. Capaldi

Institute of Molecular Biology, University of Oregon, Eugene, OR 97403, USA

Received 30 August 1991; revised version received 9 September 1991

A cDNA encoding the liver isoform of bovine cytochrome *c* oxidase subunit VIa (VIaL) was cloned from bovine liver RNA by reverse transcription and the polymerase chain reaction. The nucleotide and deduced amino acid sequences show high conservation with the corresponding rat and human liver subunits. The sequence similarity between beef heart and beef liver VIa is 60%. Northern analyses of the steady-state levels of the VIa-heart (VIaH) and VIa-liver (VIaL) transcripts showed that adult liver and brain contained only VIaL transcripts, the VIaH transcript predominated in heart with a small amount of VIaL also present, while in adult skeletal muscle VIaH was present exclusively. The VIaL transcript was found in fetal heart and skeletal muscle from 104–215-day-old fetuses, in as much as 25% of the amount of VIaH transcript. The down-regulation of VIaL transcript in skeletal muscle at or close to birth may be correlated with a change in amount of cytochrome *c* oxidase relative to the bc_1 complex (complex III) observed spectrally when fetal and adult muscle samples were compared.

Cytochrome *c* oxidase; Isoform switching; Development

1. INTRODUCTION

Cytochrome *c* oxidase (EC 1.9.3.1) is the terminal enzyme of the mitochondrial respiratory chain, catalyzing the reduction of molecular oxygen with electrons from reduced cytochrome *c* and concomitantly conserving the reaction energy by pumping protons across the inner mitochondrial membrane (for recent reviews see [1,2]). In mammals the enzyme consists of 13 non-identical polypeptides, the three largest of which contain the prosthetic groups that are involved in catalysis (two copper atoms and hemes *a* and a_3), and make up the functional core of the complex. These subunits are encoded on mitochondrial DNA and are homologous to the polypeptides found in aa_3-type cytochrome *c* oxidases of prokaryotes such as *Paracoccus denitrificans* [3]. As yet, no functional roles have been established for the ten nuclear-coded subunits of the mammalian enzyme, although regulatory functions have been postulated (see [4]). Work with the yeast oxidase has shown that some of the nuclear-coded subunits are essential for the assembly of the complex [5–7].

Mammalian cytochrome *c* oxidase occurs as tissue-specific isoforms [8–10]. This was first demonstrated by differences in the migration of several of the nuclear-encoded subunits when enzyme from different tissues was compared by $NaDodSO_4$ electrophoresis [11]. In beef, amino acid and cDNA sequencing has confirmed that subunits VIa, VIIa and VIII each occur as two forms; an H or heart form and an L or liver form [10,12]. Interestingly, the number of tissue specific subunits appears to be different in other mammals: in rat there may only be one form of VIIa (the L-form by homology with beef) [13] and in humans there may be only one isoform of VIII (the L-form) [14]. More recent protein chemical studies have suggested the occurrence of oxidase isoforms containing combinations of both the H and L subunits in tissues such as brown fat and smooth muscle [15].

Cloning and sequencing of the genes for the mammalian cytochrome *c* oxidase has progressed rapidly. The cDNAs for both the H- and L-form of VIa in rat [16] and VIII in beef [10] have been reported along with the cDNAs for the L-form of VIa, VIIa and VIII of human [17–19]. In addition, cDNAs for subunits IV, Va, Vb, VIb, VIc, VIIb and VIIc of beef and human have been cloned (see [2] for a review), and in this paper, we report the cloning and sequence of the L-form of bovine VIa. With cDNAs available as probes, it is now possible to study the biogenesis of the mammalian cytochrome *c* oxidase at the level of transcription of subunits. Here we describe experiments on the transcription of the subunit VIa isoforms which show that the isologues of this subunit, and by inference the enzyme as a whole, are not only tissue-specific but also regulated differently in different tissues during development.

2. MATERIALS AND METHODS

2.1. *General materials*

All chemicals were of the highest purity available. Taq polymerase for the polymerase chain reaction was from Perkin Elmer Cetus. AMV

Correspondence address: R. Capaldi, Institute of Molecular Biology, University of Oregon, Eugene, OR 97403, USA. Fax: (1)(503) 3465891.

(avian myeloblastosis virus) reverse transcriptase was from Bethesda Research Laboratories. Other enzymes used in the routine manipulation and cloning of DNA were purchased from Boehringer-Mannheim and used under the conditions recommended by the supplier or as described by Maniatis et al. [20]. Radiochemicals, [α-^{32}P]dCTP (6000 Ci·mmol^{-1}) and [α-^{35}S]dATP (> 1000 Ci·mmol^{-1}), were obtained from Amersham.

2.2. *Isolation and sequencing of a cDNA encoding subunit VIa-liver isoform*

The cDNA for subunit VIaL was obtained from bovine liver RNA (6 μg) by the polymerase chain reaction using standard methods [21] with a COY Tempcycler model 50 (COY Laboratory Products Inc., Ann Arbor, MI). Two mixed oligonucleotides were synthesized as primers. One corresponded to possible cDNA sequences derived from N-terminal amino-acid sequence data of bovine liver subunit VIa:

```
N-terminal        S  S  G  A  H  G  E  E  G
  sequence:
  5′ oligo: (5′)-TCGAGTGGCGCCCAYGGNGARGARGG-(3′)
```

where N is any nucleotide, R is either purine and Y either pyrimidine. The other was complementary to the consensus cDNA sequence from the rat and human liver isoforms of this subunit in a highly conserved region encompassing the codon for termination of translation:

```
  3′ oligo: (3′)-CCGATRCTTCTACTYATTTCTCTTAAGGA-(5′)
 Rat/Hum (5′)-GGCTAYGAAGATGARTAAAGAGAA-(3′)
consensus:
 Predicted        G  Y  E  D  E  *
  sequence:
```

The *Eco*RI restriction site (underlined) was added to the 5′ end of the latter oligonucleotide to aid subsequent insertion of amplification products to pBluescript (Stratagene).

The 270-bp fragment corresponding to the coding region of the VIa liver gene was excised from agarose gels, purified by phenol extraction and ethanol precipitation, and ligated into pBluescript which had been prepared by treatment with *Eco*RI, *Sma*I and alkaline phosphatase.

Three independent clones were sequenced using the dideoxynucleotide chain termination method of Sanger et al. [22]. Sequencing reactions were carried out as detailed in the protocols supplied with the Sequenase (United States Biochemical Corp.) or the TaqTrack (Promega) sequencing kits using [α-^{35}S]dATP as label and substituting 7-deaza dGTP for dGTP. Alkaline-dissociated double-stranded DNA was used as template and T7 or T3 promoter primers were used to initiate the sequencing reactions.

2.3. *Cloning of a VIa-heart cDNA*

The cDNA fragment used in Northern analyses, encoding the heart isoform subunit VIa, was cloned from bovine heart RNA. The oligonucleotides used were:

```
                          A  S  A  A  K  G  D
5′ oligo: (5′)-TCGGATCCCTGGCCAGTGCGGCCAAGGGAGAC-(3′)
            (BamHI)
3′ oligo: (3′)-TCGGAACTCCGAACCGACTACG-(5′)
                          *
```

where the asterisk indicates the position of the codon for termination of translation of the subunit. The sequence data on which the oligonucleotides were based were kindly provided before publication by Dr M. Lomax (University of Michigan). Again, the *Bam*HI site was added to one oligonucleotide to aid in the subsequent cloning of the PCR products.

2.4. *RNA isolation and Northern blot analyses*

Adult and fetal heart, muscle, brain and liver tissue were collected from freshly slaughtered cows (15–30 min after death) from a local slaughterhouse. The age of the fetuses was estimated from the crown to rump length [23]. For this study, tissue was collected from fetuses at approximately 104, 125, 134 and 215 days of development. Samples to be used for RNA isolation were immediately frozen on dry ice and stored at -70°C until use.

Total cellular RNA was isolated from 1–4 g of fresh bovine tissues by the method of Chirgwin et al. [24]. RNA in the preparations was quantitated by measuring their absorption at 260 nm using a conversion factor of 20 μg/ml = 1.0 optical density. The ratio of A_{260} to A_{280} was routinely between 2 and 2.1, indicating high purity of the RNA and thereby allowing accurate quantitation. Aliquots (20 μg) were electrophoresed through 1.5% agarose gels and transferred to nylon membranes (Nytran, Schleicher and Schuell) as described previously [10].

DNA probes were prepared using a random-priming kit (Amersham). For use as templates in the labeling reaction, DNA fragments of the liver (274 bp, 57% GC) or heart (171 bp and 124 bp, 45% GC) isoforms of subunit VIa were cut from their respective pBluescript plasmids with *Eco*RI and *Bam*HI and purified from agarose gels. The heart cDNA was isolated as two fragments due to an internal *Bam*HI site. Specific activities of 7×10^9 and 6×10^9 dpm·μg^{-1} were calculated for the liver and heart probes, respectively.

Hybridization was performed at 45°C for 16–20 h in 50% formamide, 2% NaDodSO$_4$, 0.5% milk powder (Blotto, Diploma non-fat skim-milk powder), 0.5 μg·ml^{-1} yeast RNA, 5 × SSPE (1 × SSPE: 0.18 M NaCl, 0.01 M sodium phosphate, pH 7.7, 0.001 M EDTA). The most stringent wash was at 55°C in 0.2 × SSPE, 1% sodium dodecyl sulfate. Washed membranes were exposed to Kodak XAR-5 film at -70°C in the presence of a Cronex intensifying screen. Sizes of the isoform encoding transcripts were estimated by comparison with the mobility of known molecular weight RNA molecules (0.2–0.9 kb ladder from Bethesda Research Laboratories). Transcript levels were quantitated by counting the radioactivity in lanes from the Northern blots directly, using the Ambis Radiolytic Imaging System (Model 0015060-1).

2.5. *Spectral analysis of mitochondrial preparations*

For liquid nitrogen temperature spectroscopy, samples of 600 g supernatant (4–10 mg of total protein), suspended in 1 ml of STE (0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, 5 mM EDTA) buffer/0.5% Triton-X100, were diluted with an equal volume of glycerol and dithionite-reduced minus air-oxidized absorption spectra recorded using an SLM-Aminco DW2000 spectrophotometer fitted with the low temperature accessory (part FA-051). The relative levels of hemes aa_3, b and bc_1 were estimated from the absorption maxima at 445 nm and 603 nm for cytochromes aa_3, 562 nm for cytochrome b and 422 nm for combined cytochromes bc_1 [25,26]. Protein concentrations in cell extracts were determined by the method of Bradford [27] using bovine serum albumin as the reference standard.

3. RESULTS

3.1. *Isolation and characterization of a cDNA encoding the liver isoform of subunit VIa*

The coding region of the gene of beef cytochrome *c* oxidase subunit VI liver isoform was obtained by PCR from liver RNA.

The cDNA sequence is presented in Fig. 1A with differences between this beef and the rat and human sequences indicated. The identity of the bovine and human sequences is 90%. Of the 25 nucleotide differences, 14 are silent with respect to the amino acid sequence. The identity of bovine and rat is 88%, with 19 out of 31 nucleotide changes silent. The sequence identity between the three VIaL isoforms at the amino

A. Mammalian VIa-Liver Form cDNA Comparisons

```
            S   S   G   A   H   G   E   E   G   S   A   R   M   W   K
bov     1   tcg agt ggc gcc cac gga gaa gag ggc tca gct cgc atg tgg aag   45
hum                     t   c
rat                     t   c   g       t           t   t

            A   L   T   L   F   V   A   L   P   G   V   G   V   S   M
bov    46   gcc ctc acc tta ttc gtg gcg ctc cct ggg gtg gga gtg agc atg   90
hum         a t         c       c           c           c   c
rat                     ac              g   c

            L   N   V   F   M   K   S   H   H   G   E   E   E   R   P
bov    91   ctg aat gtc ttc atg aag tcg cac cac gga gag gag gag aga ccc   135
hum                 g   a   c                       c c
rat           c   c   t       c           ga      a   c c

            E   F   V   A   Y   P   H   L   R   I   R   S   K   P   F
bov   136   gag ttc gtg gcc tat ccc cat ctc cgc atc agg tcc aag ccc ttt   180
hum                 a c     c                       a       g
rat                   c     c                       a t         c

            P   W   G   D   G   N   H   T   L   F   H   N   P   H   V
bov   181   ccc tgg gga gat ggt aac cat acc cta ttc cat aac cct cat gtg   225
hum                                     t
rat                                         c       c   t       c a

            N   P   L   P   T   G   Y   E   D   E   *
bov   226   aac ccg ctt cca acc ggc tat gaa gat gag taa   258
hum           t   a         t       c           a
rat                   g   t
```

B. VIa Isoform Amino Acid Sequence Comparisons

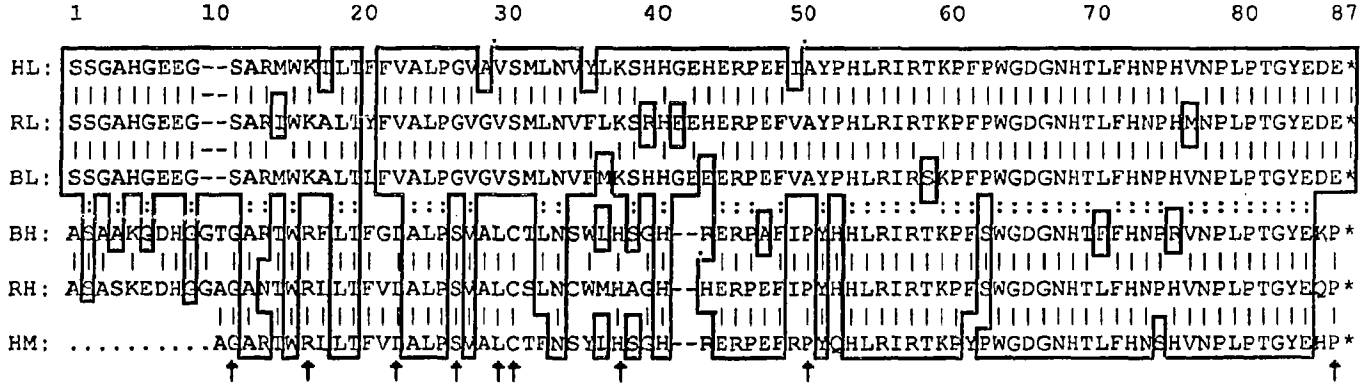

Fig. 1. (A) cDNA and predicted amino acid sequences for the L-form of bovine cytochrome c oxidase subunit VIa. (This sequence has been deposited in the EMBL data bank, Accession number M38520.) Differences between the bovine, human and rat cDNAs are indicated below the appropriate nucleotide. Parts of the sequence contributed by the oligonucleotides used in the isolation of the cDNA are underlined. (B) Amino acid homology between the known mammalian VIa isoforms. Residues common to at least two of the L-form sequences are enclosed in the box, which is extended to include the amino acids also conserved in the H-form sequences. Identical amino acids in the bovine H- and L-forms are indicated by (:); matches between other adjacent sequences are indicated by (|); and the arrows indicate positions at which three identical residues have been conserved in the L-forms which are different from three identical residues in the H-forms.

acid level are shown in Fig. 1B. The bovine sequence has 8 amino acid changes when compared to both human and rat; only one amino acid is different in all three species.

Most of the sequence similarity between the H and L isoforms of VIa is in the C-terminal half, after residue Glu[45]. This suggests that the N-terminus may comprise a tissue-specific functional domain. Fig. 1B also shows

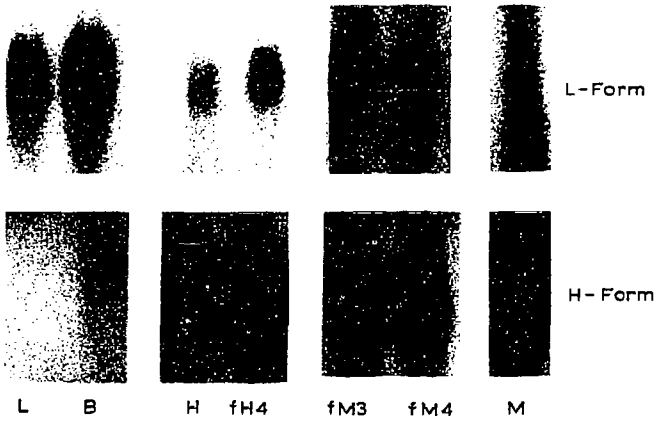

Fig. 2. Northern analysis of the subunit VIa isoform transcripts in bovine adult and fetal tissues. Blots were processed as described in section 2. The upper panels show the autoradiographs of one-half of each blot hybridized with VIa L-form-specific radioactive probes; the lower panels are the autoradiographs of the duplicate lanes probed with the VIaH cDNA. Each lane was loaded with 20 μg of total RNA isolated from adult (H, heart; M, skeletal muscle; L, liver; B, brain) or fetal (f1H or M through f4H or M) heart or hind-leg muscle. The fetal ages were estimated from crown to rump length as follows: f1, 20 cm ≈ 104 days; f2, 28 cm ≈ 125 days; f3, 32 cm ≈ 134 days; f4, 66 cm ≈ 215 days. The autoradiographs were exposed at -70°C with an intensifying screen for between 1 and 4 days.

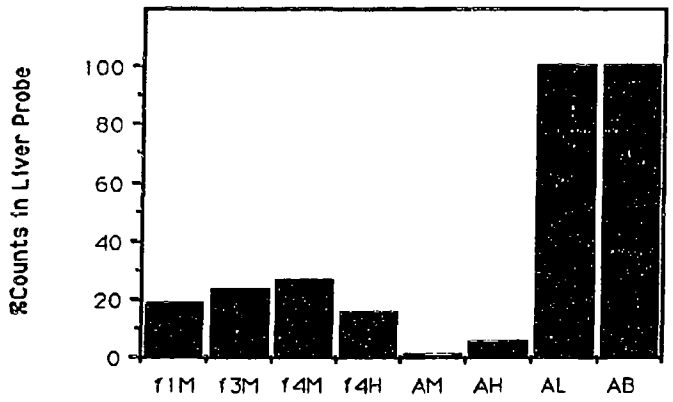

Fig. 3. Quantitation of subunit VIa H- and L-form transcripts and cytochrome content of various adult and fetal tissues. ^{32}P radioactivity was quantitated with the Ambis radiolytic imaging system as described in section 2. The values shown are the percentage of total counts in VIa transcripts (H + L) present in the L-form, calculated from the counts detected in identically sized rectangular areas of blots.

that there is greater homology between the same isoform in different species than between different isoforms in the same species. Further, the liver isoforms are more highly conserved across the three mammalian species than are the heart forms (90% identical amino acids in liver forms, cf. 82% for the H-form).

3.2. *Transcript levels of subunit VIa in fetal and adult tissues*

The H- and L-form cDNAs for subunit VIa were used in the synthesis of radioactive probes for the identification and quantitation of transcript levels in selected fetal and adult tissues on Northern blots (Fig. 2). The size of the H-form transcript was calculated as 370 nucleotides, that of the L-form transcript was 580 nucleotides. There was no cross-hybridization of either probe with the different isoform transcript under the high stringency conditions used, facilitating quantitation of isoform levels.

For direct comparisons to be made, RNA samples were run in duplicate lanes on agarose gels, and after blotting, the nylon membranes were cut to separate two identical sets of lanes; one set was hybridized with the L probe and the other with the H probe (Fig. 2). Quantitation of transcript levels was as described in section 2; appropriate corrections were made for differences in specific activity of the two probes.

As might be expected, the levels of total VIa transcripts (summed counts from the heart and liver probes) were higher in adult than in fetal tissues, as were the levels of complex III and cytochrome *c* oxidase estimated by spectroscopy. Fig. 2 shows the Northern blots for several adult tissues along with heart and skeletal muscle tissue from several different fetuses. In brain and liver, only the L form of VIa was detected using either fetal or adult tissue. In adult heart tissue the H form of VIa predominated but a significant amount of the L form of VIa (around 5% of the total transcript) was detected. In fetal heart, the levels of VIaL were higher, amounting to as much as 25% of the total transcript in the earliest fetuses examined (100–120 days). Heart tissue is heterogeneous and includes smooth muscle present as blood vesicles. Bovine smooth muscle tissue has been shown to contain a 'hybrid' cytochrome *c* oxidase, including VIaL along with VIIaH and VIIIL [15]. As smooth muscle is difficult to dissect away, particularly in small fetal hearts, in situ hybridization experiments will be required to establish conclusively whether there is VIaL transcript in heart muscle.

Results with skeletal muscle are clearer, as the tissue is more homogeneous, containing the closely-related slow twitch and fast twitch muscle cells but little or no smooth muscle. As shown in Northern blots in Fig. 2, and as quantitated in Fig. 3, fetal muscle samples contained significant amounts of VIaL RNA (20–25% of the total transcript) while at most 1% VIaL form was detected in adult muscle samples.

3.3. *Cytochrome content of adult and fetal tissues*

Several fetal and adult tissues were examined spectrally to determine the cytochrome content of their mitochondria. In liver the ratio of cytochrome *c* oxidase to complex III, based on the ratio of the absorbance at 603 to 562 nm (see section 2) was 0.9±0.1:1 (3 determinations): see also Wainio [26]. In heart the ratio of cytochrome *c* oxidase to complex III was 2.0±0.3:1 (4 determinations) for either fetal or adult tissues. Skeletal

muscle, in contrast to heart, contained different ratios of cytochromes in fetal compared with adult tissue. In the four fetal samples examined, the ratio ranged between 0.5 (for the ≈104-day fetus) to 0.9 (for the ≈215-day fetus), while in adult tissue the ratio was 1.7±0.4 (4 determinations).

4. DISCUSSION

In bovine cytochrome *c* oxidase there are isologues of subunits VIa, VIIa and VIII [4,12]. We have previously characterized the isoforms of subunit VIII and found that these show only 55% sequence identity [10]. Results presented here indicate that the H and L isoforms of bovine VIa are 60% identical, with most of the amino acid differences occurring in the N-terminal half of the polypeptide. Similar observations have been made for subunit VIa of rat [16]. We find that the H-form of VIa is the predominant or only transcript in adult heart based on Northern analyses (depending whether the 5% L-form is a contaminant or not). In skeletal muscle there is little or no L-form (1% or less).

The work presented here is the first to explore isoforms of cytochrome *c* oxidase in fetal samples. We have analyzed the steady-state levels of transcripts of the H- and L-forms of VIa in several fetuses ranging in age from around 104 to around 215 days.

Significant levels of the L isoform of VIa were found in fetal heart and skeletal muscle, i.e. 20–25% of the total VIa transcripts in both tissues, which drop to 5%, and to 1% or less, in the two adult tissues, respectively. These results suggest that the L isoform is a fetal form, which may be present in even higher proportion relative to the H-form in early fetal development of heart and muscle. We do not rule out that there is another isoform of VIa present in early development, although no gene for a third form of VIa has been identified so far.

There were possible problems of contamination with smooth muscle in heart samples, particularly from the fetuses where the heart is small. As smooth muscle cytochrome *c* oxidase contains VIaL form, no conclusions can be made about isoform switching in heart development. However, the results are clear in skeletal muscle, where there is less potential for contamination with smooth muscle. During skeletal muscle development there is switching from VIaL form (in a mixture with the VIaH form) to exclusively VIaH form in adult tissue.

The above findings further emphasize the complex regulation of the biosynthesis of cytochrome *c* oxidase in mammals. Not only is the biosynthesis of the enzyme dependent on concerted transcription from two genomes [1,2,25], but there must be regulation of tissue-specific and developmental forms in which isologues of at least three of thirteen subunits can be present in varying ratios, and possibly in different combination, in different tissues.

The significance of the presence of the various isoforms of cytochrome *c* oxidase remains unclear in the absence of clearly defined functional differences between the forms (compare results in [28] and [29]). However, the tissue specificity, and now the developmental regulation of the isoforms, is an indication that these must have important roles to play in the cell. One possibility is that the different isoforms of cytochrome *c* oxidase provide a route to regulating the amounts of enzyme in different cells. There is precedent for such a role in the work of Poyton and colleagues on isoforms of cytochrome *c* oxidase in yeast [5,30]. There are two forms of subunit V in yeast cytochrome *c* oxidase called Va and Vb. This subunit is the homologue of subunit IV of the mammalian enzyme. Subunit Va is present in cells grown under highly aerobic conditions when a large amount of cytochrome *c* oxidase is made. Subunit Vb is transcribed only in cells grown aerobically when the oxidase is present in low amounts [30]. The change in levels of aa_3 to bc_1 that accompany the switch from VIa L- to H-form transcripts in developing muscle also suggests a role of isoforms of cytochrome *c* oxidase in regulating the amount of terminal oxidase relative to other respiratory chain components.

Acknowledgement: Supported by NIH Grant HL22050.

REFERENCES

[1] Capaldi, R.A. (1990) Annu. Rev. Biochem. 59, 569–596.
[2] Capaldi, R.A. (1990) Arch. Biochem. Biophys. 280, 252–262.
[3] Raitio, M., Jalli, T. and Saraste, M. (1987) EMBO J. 6, 2825–2833.
[4] Kadenbach, B., Kuhn-Nentwig, L. and Buge, U. (1987) Curr. Top. Bioenerg. 15, 114–162.
[5] Poyton, R.O., Trueblood, C.E., Wright, R.M. and Farrell, L.E. (1988) Ann. NY Acad. Sci. 550, 289–307.
[6] Aggeler, R.J. and Capaldi, R.A. (1990) J. Biol. Chem. 265, 16389–16393.
[7] Lightowlers, R.N., Chrzanowska-Lightowlers, Z., Marusich, M. and Capaldi, R.A. (1991) J. Biol. Chem. 266, in press.
[8] Kadenbach, B., Jarausch, J., Hartmann, R. and Merle, P. (1983) Anal. Biochem. 129, 517–521.
[9] Kadenbach, B. and Stroh, A. (1984) FEBS Lett. 173, 374–380.
[10] Lightowlers, R.N., Ewart, G., Aggeler, R., Zhang, Y-Z., Calavetta, L. and Capaldi, R.A. (1990) J. Biol. Chem. 265, 2677–2681.
[11] Kuhn-Nentwig, L. and Kadenbach, B. (1985) Eur. J. Biochem. 149, 147–158.
[12] Yanamura, W., Zhang, Y-Z., Takamiya, S. and Capaldi, R.A. (1988) Biochemistry 27, 4909–4919.
[13] Kennaway, N.G., Carrero-Valenzuela, R.D., Ewart, G., Balan, V.K., Lightowlers, R., Zhang, Y-Z., Powell, B.R. and Capaldi, R.A. (1990) Pediatric Res. 28, 529–535.
[14] Van Kuitenberg, A.B.P., Muijsers, A.O., Demol, H., Dekker, H.L. and Van Beeuman, J.J. (1988) FEBS Lett. 240, 127–132.
[15] Anthony, G., Stroh, A., Lottspeich, F. and Kadenbach, B. (1990) FEBS Lett. 277, 97–100.
[16] Schlerf, A., Droste, M., Winter, M. and Kadenbach, B. (1988) EMBO J. 7, 2387–2391.
[17] Rizzuto, R., Nakase, H., Darras, B., Fabrizi, G.M., Mengel, T., Walsh, F., Kadenbach, B., DiMauro, S., Franke, U. and Schon, E. (1989) J. Biol. Chem. 264, 10595–10600.
[18] Fabrizi, G.M., Rizzuto, R., Nakase, H., Mita, S., Kadenbach, B. and Schon, E.A. (1989) Nucleic Acids Res. 17, 6409.

[19] Fabrizi, G.M., Rizzuto, R., Nakase, H., Mita, S., Lomax, M., Grossman, L. and Schon, E.A. (1989) Nucleic Acids Res. 17, 7107.
[20] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, New York.
[21] Saiki, R.K. and Gelfand, D.H. (1989) Amplifications 1, 4–6.
[22] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[23] Harris, R.M., Snyder, B.G. and Meyer, R.M. (1983) Agri-Practice 4, 18–19.
[24] Chirgwin, J.M., Przybla, A.E., MacDonald, R.J. and Rutter, W.J. (1979) Biochemistry 18, 5294–5299.
[25] Van Gelder, B.F. (1978) Methods Enzymol. 53, 125–128.
[26] Wainio, W.W. (1970) The Mammalian Mitochondrial Respiratory Chain, Academic Press, New York.
[27] Bradford, M. (1976) Anal. Biochem. 72, 248–254.
[28] Sinjorges, K.M.C., Durak, I., Dekker, H.L., Edel, C.M., Hakvoort, T.B.M. and Van Gelder, B. (1987) Biochim. Biophys. Acta 893, 251–258.
[29] Buge, U. and Kadenbach, B. (1986) Eur. J. Biochem. 161, 383–398.
[30] Trueblood, C.E., Wright, R.M. and Poyton, R.O. (1988) Mol. Cell. Biol. 8, 4537–4540.