Co-ordinate expression of cytochrome *c* oxidase subunit III and VIc mRNAs in rat tissues

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Cytochrome *c* oxidase (EC 1.9.3.1) is an enzyme which is composed of subunits derived from both the mitochondrial and the nuclear genomes. To determine whether or not the expression of these two genomes is co-ordinated at the mRNA level, we have examined the steady-state levels of mRNAs coding for cytochrome *c* oxidase subunit III (mitochondrially encoded) and subunit VIc (nuclear-encoded) in rat tissues. This was compared with the tissue concentration of the holoenzyme, which was estimated by measuring cytochrome *c* oxidase enzyme activity. The tissues (heart, brain, liver, kidney, soleus muscle and superficial white vastus muscle) possessed a 13-fold range of enzyme activity, which was highest in heart and lowest in the superficial vastus muscle. Specific subunit mRNA levels were quantified by using slot-blot hybridization of cDNA probes to total tissue RNA. The highest values for subunit III and VIc mRNA tissue contents were found in kidney, followed by liver and heart (40–60 % of that of kidney). The white vastus muscle contained the lowest subunit mRNA level (15 % of that of kidney). Although some variability was apparent within each tissue, a parallel pattern of mRNA expression of the nuclear- and mitochondrially encoded subunits was observed. Differences between muscle (heart, vastus and soleus) and non-muscle tissues were noted in the relationship between mRNA and protein levels of expression. Thus, although this suggests that tissue-specific regulatory processes operate, the steady-state expression of subunit III and subunit VIc mRNAs appears to be co-ordinately regulated.

INTRODUCTION

Cytochrome *c* oxidase (EC 1.9.3.1) is the terminal complex of the mitochondrial electron-transport chain. In mammalian tissues, the holoenzyme is made up of 13 separate subunits. The largest three of these are encoded by the mitochondrial genome, whereas the remaining ten are nuclear-encoded. The mitochondrially encoded subunits possess catalytic properties (i.e. electron transport and proton translocation), whereas the nuclear-encoded subunits are thought to have regulatory functions (Kadenbach & Merle, 1981) and exist as tissue-specific isoforms (Kuhn-Nentwig & Kadenbach, 1985). Because of its subunit composition, cytochrome *c* oxidase provides a good experimental system with which to study the interaction of the mitochondrial and nuclear genomes in the formation of a functional protein (Poyton, 1980). One way to investigate this interaction is to induce changes in the mitochondrial biogenesis of a given tissue and then to examine the alterations in specific mRNA sequences derived from both genomes. In a previous study, we have used chronic low-frequency stimulation of rat fast-twitch muscles to induce a 3–4-fold increase in mitochondrial biogenesis. Parallel 2-fold increases in mRNA levels coding for subunits III (mitochondrially encoded) and VIc (nuclear-encoded) were observed (Hood *et al.*, 1989). This provided evidence for co-ordinate expression of these two genomically separated mRNAs. In the present study we have sought further insight into the regulation of cytochrome *c* oxidase assembly by examining the steady-state levels of subunit III and VIc mRNAs in various tissues of the rat. We have selected tissues with a wide range of cytochrome *c* oxidase activities and which, in some species, display different kinetic, structural (Merle & Kadenbach, 1982) and immunological (Kuhn-Nentwig & Kadenbach, 1985) properties.

MATERIALS AND METHODS

Animals and tissues

Wistar rats (350–400 g) were housed in temperature- and humidity-controlled environments with a 12 h-light/12 h-dark cycle. Animals were killed and tissues (liver, kidney, brain, superficial white vastus muscle, soleus muscle and heart) were quickly excised, frozen and stored in liquid N₂ until further processed. Tissues were powdered in a stainless-steel mortar under liquid N₂ for subsequent RNA isolation as well as for the preparation of tissue homogenates.

Analysis of RNA

Total RNA was prepared from tissue powders by using a recently characterized (Hood & Simoneau, 1989) rapid guanidinium thiocyanate procedure (Chomczynski & Sacchi, 1987). This method is effective for the quantitative recovery of total RNA from the tissues used in the present study. It results in pure and undegraded RNA samples suitable for hybridization experiments (Hood & Simoneau, 1989). In order to quantify the tissue levels of cytochrome *c* oxidase subunit mRNAs, total RNA was applied to nylon membranes (Hybond-N; Amersham Buchler G.m.b.H., Braunschweig, Germany) using a Schleicher and Schuell Minifold II apparatus. The manufacturer's instructions were followed (Wahl, 1983).

cDNA probes

The cytochrome *c* oxidase subunit III cDNA probe was an endonuclease-*Pst*I 743 bp fragment in plasmid pBR322 originally isolated from rat liver mitochondrial DNA (Grosskopf & Feldmann, 1981). The nuclear-encoded subunit VIc cDNA was derived from a rat liver cDNA library, and was a *Pst*I 243 bp

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fragment (Parimoo *et al.*, 1984) inserted into Bluescribe (Stratagene Cloning Systems, San Diego, CA, U.S.A.). The 243 bp fragment was excised and purified before its use in the nick-translation reaction.

Nick-translation and hybridization

cDNA probes were radioactively labelled with [32 P]dCTP (Amersham Buchler) using a nick-translation kit (Boehringer). The specific radioactivities of the probes were typically 1×10^8 d.p.m./ μ g of DNA (subunit VIc) and 3×10^8 d.p.m./ μ g (subunit III). These were hybridized to slot blots in 8 ml of a solution containing 0.9 M-NaCl, 45 mM- Na_2HPO_4 , 4.5 mM- NaH_2PO_4 , 5 mM-EDTA, 50% (v/v) formamide, 0.2% (w/v) Ficoll, 0.2% (w/v) polyvinylpyrrolidone, 0.2% (w/v) BSA, 0.1% (w/v) SDS and 500 mg of calf thymus DNA/ml, pH 7.4. Hybridizations lasted 16–24 h at 40–44 °C. Blots were then washed three times at room temperature under low-stringency conditions using 0.3 M-NaCl/0.03 M-sodium citrate ($2 \times \text{SSC}$) and 0.1% SDS. This was followed by a wash of higher stringency in $0.1 \times \text{SSC}$ and 0.1% SDS at 60 °C for 30–60 min. Blots were air-dried for 15–30 min, then exposed to Hyperfilm-MP (Amersham Buchler) for autoradiography at –70 °C. Quantification of the hybridization signal was achieved using laser densitometry (LKB 2202 Ultrosan). Northern-blot analyses were also performed before the slot-blot analyses. Results (not shown) indicated that the hybridization of each cDNA probe was specific and that the RNA was undergraded.

Cytochrome *c* oxidase activity

Tissue powders were suspended (1:20, w/v) in 0.1 M- $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 7.2, containing 2 mM-EDTA and sonicated (6 $\times$ 10 s) with intense cooling on ice. This homogenate was used directly in the enzyme assay in the presence of sodium dithionite-reduced cytochrome *c* (162 μ M; Sigma Chemie G.m.b.H., Deisenhofen, Germany) and 11 mM-potassium phosphate buffer, pH 7.0. Reactions were started with the addition of homogenate, and calculations of maximal activity levels were obtained by monitoring the reaction kinetics.

RESULTS

Enzyme activity and total RNA

Table 1 illustrates the 12–13-fold range of cytochrome *c* oxidase activities which existed between the selected tissues. Heart displayed the highest, and the superficial white portion of the vastus muscle the lowest values. With respect to total RNA content, the liver possessed by far the highest tissue level. It was 2.3-fold higher than in kidney, and 9.5-fold greater than in vastus muscle.

Table 1. Cytochrome *c* oxidase activity and total RNA content of rat tissues

Values are means $\pm$ S.E.M. for three to six observations per tissue. For further details, see the text.

Tissue	Cytochrome <i>c</i> oxidase (units/g of tissue)	Total RNA (μ g/g of tissue)
Soleus	15.4 $\pm$ 1.7	829 $\pm$ 45
Vastus	4.3 $\pm$ 0.8	582 $\pm$ 42
Heart	54.8 $\pm$ 5.4	844 $\pm$ 92
Liver	36.1 $\pm$ 5.1	5533 $\pm$ 437
Kidney	33.1 $\pm$ 1.0	2415 $\pm$ 63
Brain	26.2 $\pm$ 1.1	928 $\pm$ 134

Fig. 1. Slot-blot hybridization analysis of cytochrome *c* oxidase subunit III and VIc mRNAs

Total RNA was isolated from rat brain (B) and kidney (K) and transferred to nylon membranes. Identical blots were hybridized with the cDNA probe coding for subunit III or VIc. Autoradiographs were exposed for 37 h (subunit III) or 8 days (subunit VIc).

Rat tissue mRNA level

Slot-blot hybridization analysis of rat tissues were carried out over a 4-fold range of RNA concentrations. A typical example is illustrated in Fig. 1 for kidney and brain. Only slot-blots whose autoradiograph signals were linearly related to applied total RNA concentration were used for evaluation. The results obtained from Fig. 1 and from identical single slot-blots of the other tissues were expressed as arbitrary integrated scanner units per μ g of RNA, and were averaged over the RNA concentration range (Table 2). In order to obtain an estimate of the tissue levels of nuclear- and mitochondrially encoded cytochrome *c* oxidase subunit mRNAs, and to correct for differences in the total RNA content among tissues, these values were multiplied by the total RNA content (Table 1) of each tissue to provide the results

Table 2. Quantification of cytochrome *c* oxidase subunit mRNA levels in rat tissues

Autoradiography signals were quantified by laser densitometry and expressed per μ g of total RNA and per g of tissue mass by multiplying by the total RNA content. The results are expressed as means $\pm$ S.E.M. for the four RNA concentrations (6–24 μ g); 'units' are arbitrary scanner units (see the text).

Tissue	Subunit III mRNA		Subunit VIc mRNA	
	(units/ μ g of RNA)	(units/g of tissue)	(units/ μ g of RNA)	(units/g of tissue)
Soleus	38.2 $\pm$ 9.8	31.7 $\pm$ 8.1	61.1 $\pm$ 14.1	50.7 $\pm$ 11.0
Vastus	37.5 $\pm$ 9.9	21.8 $\pm$ 5.8	31.9 $\pm$ 1.0	18.6 $\pm$ 0.6
Heart	64.6 $\pm$ 13.2	54.5 $\pm$ 11.1	99.5 $\pm$ 23.1	84.0 $\pm$ 19.5
Liver	14.6 $\pm$ 3.3	80.8 $\pm$ 18.3	14.4 $\pm$ 1.7	79.7 $\pm$ 9.4
Kidney	55.4 $\pm$ 15.9	133.8 $\pm$ 38.4	63.9 $\pm$ 3.5	154.3 $\pm$ 8.5
Brain	42.2 $\pm$ 5.1	39.2 $\pm$ 4.7	20.4 $\pm$ 1.2	18.9 $\pm$ 1.1

expressed/g of tissue weight (Table 2). Since values are expressed in arbitrary scanner units and corrections were not made for the two cDNA specific radioactivities, film exposure times or backgrounds, comparisons of the absolute values found in Table 2 between subunit III and subunit VIc hybridizations cannot be made. Only absolute value comparisons among tissues within a specific cDNA probe hybridization (subunit III or VIc) are valid from Table 2. However, it is evident from these data that the highest values of both subunit III and VIc mRNAs were found in kidney, followed by liver and heart, which ranged from 40 to 60% of the kidney value. The superficial white portion of the vastus muscle possessed the lowest subunit mRNA content (15% of the kidney value).

DISCUSSION

The present study was designed to evaluate the steady-state levels of specific mitochondrially and nuclear-encoded cytochrome *c* oxidase subunit mRNAs in various rat tissues. These were selected on the basis of their expected wide range of cytochrome *c* oxidase activities, which were used as estimates of actual tissue holoenzyme concentrations. Relationships observed when comparing protein and mRNA levels can be used to provide an insight into regulatory mechanisms of expression (i.e. transcriptional or post-transcriptional), as has previously been done (Morrison *et al.*, 1987; Hood *et al.*, 1989; Kirschbaum *et al.*, 1989). The relationship between enzyme activity and subunit mRNA levels is shown in Fig. 2. Since the mRNA levels are in arbitrary units, meaningful comparisons can only be made when the values are all referred to a common tissue. In this case liver was chosen, because of its intermediate level of cytochrome *c* oxidase activity and because the cDNA probes used for the quantification of the mRNAs were originally derived from rat liver cDNA libraries.

The most important observation provided by Fig. 2 is the relationship, within a given tissue, between the nuclear- and mitochondrially encoded subunit mRNAs. This relationship is also apparent from Table 2, which illustrates the variability in the measurements. These data indicate that these mRNAs are expressed to a similar extent within a tissue, and provide evidence for a co-ordinated regulation of the steady-state levels of mRNAs coding for these subunits. Thus low levels of cytochrome *c* oxidase activity in a tissue (e.g. white vastus muscle) are at least

partially due to the low levels of both nuclear and mitochondrial transcripts. The opposite appears to be true for heart, where cytochrome *c* oxidase activity is approx. 13-fold higher than in the white vastus muscle. It is apparent that, over a wide range of cytochrome *c* oxidase tissue contents, subunit mRNAs coded for by both genomes appear to be expressed in parallel, although some variability is also observable (e.g. heart and brain). This may in part be due to slight tissue-specific differences in the nuclear-encoded subunit VIc nucleotide sequences. It has been shown (Merle & Kadenbach, 1980; Jarausch & Kadenbach, 1982; Kuhn-Nentwig & Kadenbach, 1985) that tissue-specific isoforms of the nuclear-encoded (but not the mitochondrially encoded) subunits exist. Furthermore, some subunits are derived from separate genes in different tissues (Kadenbach *et al.*, 1982). Although this has not yet been demonstrated for subunit VIc, it may be expected that the hybridization of a cDNA probe originally derived from a rat liver cDNA library may not be identically complementary to the mRNA sequence obtained from another tissue.

In a recent study (Hood *et al.*, 1989), we have used chronic low-frequency stimulation of rat fast-twitch muscle to induce a 3–4-fold elevation in cytochrome *c* oxidase activity. Parallel 2-fold increases in both subunit III and VIc mRNAs were demonstrated. This supported the concept that the nuclear and mitochondrial genomes were co-ordinately regulated. Notably, however, the 2-fold increases in mRNAs did not match the 3–4-fold change in enzyme activity, leading to the suggestion that mechanisms operating at levels beyond transcription were additionally responsible for the increased cytochrome *c* oxidase activity observed.

A comparison of the three muscle tissues used in the present study leads to a similar conclusion. In general, mRNA levels in heart, soleus and vastus increase with the level of cytochrome *c* oxidase activity (Fig. 2). However, a line drawn through the points representing these three tissues demonstrates a slope much greater than 1, and indicates that a higher enzyme protein content is not brought about by an equal mRNA contribution. In contrast, the slope of the relationship drawn between the points representing non-muscle tissues (i.e. liver, kidney and brain) is much less than 1, suggesting that plentiful mRNA sequences are transcribed, but that they are not translated into an equivalent amount of protein. Interestingly, these three tissues also possess the highest total RNA content of the tissues investigated. Regardless of the precise mechanisms involved, different means of regulation appear to exist in muscle and non-muscle tissues. The validity of this conclusion is based on the assumption that, despite large differences in the total RNA content of the various tissues, the percentage of total RNA represented by mRNA remains constant.

The co-ordinated genomic response reported here has also been observed in skeletal muscle of hyperthyroid rats undergoing chronic electrical stimulation (D. A. Hood, A. M. Kelly & D. Pette, unpublished work). However, differences in the extent of subunit III and subunit VIc expression were observed in hypothyroid animals. This suggests that a critical level of thyroid hormone may be required for the maintenance of the co-ordinated genomic response.

The precise function of all of the subunits of cytochrome *c* oxidase is not known. This is particularly true for the smaller, nuclear-encoded subunits, which have been implicated in regulatory enzyme functions (Kadenbach, 1986). That these subunits are responsible for tissue-specific isoforms of cytochrome *c* oxidase is based mainly on immunological reactivity and electrophoretic mobility of the subunits at the protein level. Subsequent studies using Northern-blot hybridizations with specific cDNA probes encoding the nuclear subunits IV (Zeviani

Fig. 2. Relationship between tissue cytochrome *c* oxidase activity and mitochondrial and nuclear subunit mRNA content

mRNA data were taken from Table 2 and expressed as a ratio relative to the value obtained in liver. Enzyme activities are similarly expressed and taken from Table 1. Tissue symbols are: V, vastus muscle; S, soleus muscle; H, heart; L, liver; B, brain; K, kidney. ○, Mitochondrial subunit III; ●, nuclear subunit VIc. The diagonal line drawn is the line of identity.

et al., 1987), Vb (Zeviani *et al.*, 1988), VIc (Hood *et al.*, 1989; Suske *et al.*, 1987) and VIII (Suske *et al.*, 1987) have revealed single similar-sized transcripts among a variety of different tissues. Thus, at the protein level of isoform resolution, tissue specificity may arise because of post-transcriptional modifications. The importance of these nuclear-encoded proteins for holoenzyme function has been elegantly demonstrated by using genetic studies with strains of the yeast *Saccharomyces cerevisiae* carrying mutations in the genes encoding each polypeptide (Poyton *et al.*, 1989). Using this methodology, Dowhan *et al.* (1985) have shown that subunit IV, particularly a 38-amino-acid segment, is indispensable for cytochrome *c* oxidase assembly in yeast.

In summary, the present study demonstrates that, in tissues possessing a wide range of cytochrome *c* oxidase enzyme activities, the subunit mRNAs encoding subunits III (mitochondrial) and VIc (nuclear) are expressed to a similar extent. This lends further support to the idea that the nuclear and mitochondrial genomic sequences coding for cytochrome *c* oxidase subunits are expressed co-ordinately at the mRNA level.

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