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Regulation of the expression of mitochondrial proteins: relationship between mtDNA copy number and cytochrome-*c* oxidase activity in human cells and tissues

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The relationship between the relative amounts of nuclear and mitochondrial genes for cytochrome-*c* oxidase subunits and their transcripts and cytochrome-*c* oxidase activity was investigated in several human tissues and cell lines to get more insight into the regulation of the expression of this mitochondrial enzyme complex. The results show: (1) a wide range of mtDNA copy numbers; (2) constant ratios between the steady-state levels of the transcripts for the various cytochrome-*c* oxidase subunits, and (3) large variations in cytochrome-*c* oxidase activity in different tissues and cell lines that could not be related to the differences in mtDNA copy number. We conclude that the transcription of genes for both mitochondrial and nuclear cytochrome-*c* oxidase subunits is regulated coordinatedly, but also that the mtDNA copy number plays a minor role in determining differences in cytochrome-*c* oxidase activity between different cell and tissue types.

Introduction

The capacity for oxidative phosphorylation of a tissue is under both developmental and physiological control and shows, moreover, substantial variation between various cell and tissue types. Differences in capacities for oxidative phosphorylation are usually reflected in the concentrations of the mitochondrial enzyme complexes that are directly involved in oxidative phosphorylation. Four of these five enzyme complexes consist of both nuclear and mitochondrial gene products [1]. The production of the nuclearly and mitochondrially coded subunits of these enzymes requires coordination to allow efficient biosynthesis.

The results of studies in which direct relationships between the abundancy of these mitochondrial and nuclear transcripts were demonstrated [2–7] suggest coordination at the transcriptional level. This coordination might be regulated by proteins, binding to upstream sequences of nuclear genes for mitochondrial proteins that recognise comparable sequences in mtDNA [8]. The presence of the same regulatory elements in nuclear genes encoding subunits of enzymes involved in oxidative phosphorylation and in a nuclear gene that might control mtDNA replication [9] offers another regulatory mechanism. According to this model, conditions inducing a higher demand for oxidative phosphorylation would simultaneously result in enhanced expression of the nuclear genes concerned and in an increased replication of the mitochondrial genome (and thus to a higher gene dosage of subunits of mitochondrial origin). This model fully explains the direct relationship between mtDNA copy number, transcript levels for mitochondrial proteins, and the concentration and activity of the corresponding mitochondrial enzymes in muscle of experimental animals [3,6,10,11]. This model implies that the biosynthesis of mitochondrial proteins is hardly subjected to post-transcriptional regulation.

Whether mitochondrial gene dosage indeed plays an important role in modulating the oxidative phospho-

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rylation capacity [12] has been debated recently [13,14]. In view of this, we studied the relationship between mtDNA copy number and cytochrome-*c* oxidase content in a number of tissues and cell lines of human origin. Cytochrome-*c* oxidase was selected for this purpose since the mammalian enzyme is composed of three large subunits of mitochondrial origin and ten smaller, nuclearly encoded subunits [15]. In addition, tissue-specific isoforms of two of the nuclearly encoded subunits are known to exist. Our results show that the mtDNA concentration is specific for a given tissue or cell type, as is the cytochrome-*c* oxidase activity. However, a direct link between the mtDNA copy number and the cytochrome-*c* oxidase activity was absent. Variations in mitochondrial gene dosage may, therefore, only marginally contribute to the differences in capacity for oxidative phosphorylation among different cells and tissues.

Materials and Methods

Human skeletal muscle tissue was obtained from a disk-protrusion patient (adult male) who had undergone laminectomy. Human liver tissue was obtained from a liver (adult female) prepared for transplantation. Other human tissues were obtained at autopsy, and became available within 24 h post mortem. Human white blood cells were isolated from the peripheral blood by separation on Ficoll-Paque. Tissues and cells were frozen in liquid nitrogen and stored at −80°C.

HL-60 and Molt-4 cells (human leukemic cell lines), Hela S3 cells (human cervical carcinoma cell line) and GLC-4 cells (human small lung carcinoma cell line) were cultured in RPMI 1640, supplemented with 10% heat-inactivated fetal calf serum. Primary cell lines of human fibroblasts and myoblasts were cultured in Dulbecco-modified Eagle medium, supplemented with 10% heat-inactivated fetal calf serum and pyruvate (1 mM final concentration). The cells were cultured at 37°C in a humidified atmosphere of 10% CO_2 in air, harvested as described before [7,16] and stored at −80°C.

HL-60 cell populations, highly-enriched for cells in subsequent phases of the cell cycle, were obtained by counterflow centrifugation as described previously [17]. The DNA content of cells in these populations was assessed by measuring the relative fluorescence after staining with a hypotonic propidium iodide solution [18], using a flow cytometer. The percentages of the cells with a DNA content of $2n$, $2 < n < 4$ and $4n$ were calculated according to Van Egmond and Hillen [19].

Frozen tissue was ground to powder in liquid N_2. RNA was isolated from pulverised tissue or pelleted cells by a modification [7] of the guanidinium-thiocyanate/CsCl method [20]. Total DNA was isolated according to the method of Birnboim [21]. The DNA or RNA content of the resulting samples was measured spectrophotometrically. To determine the relative mtDNA copy number, agarose gel electrophoresis of DNA digested with *Eco*RI and *Xba*I was performed according to standard methods. DNA was blotted on GeneScreen Plus (DuPont) filters and hybridisation with radioactive DNA probes was performed according to Church and Gilbert [22]. A cloned *Xba*I-fragment of human mitochondrial DNA containing the entire gene of cytochrome-*c* oxidase subunit II was used to assess the relative amount of mtDNA and a human cDNA clone for the nuclearly coded subunit IV of cytochrome-*c* oxidase [23] (a kind gift of Dr. M.I. Lomax, University of Michigan, Ann Arbor, MI, USA) was used to assess the relative amount of nuclear DNA. The probes were labelled with [α-^{32}P]dCTP using the random primer technique [24]. The probe for subunit IV was labelled to a specific radioactivity as high as possible, whereas the probe for subunit II was labelled only moderately. This allowed the comparison of the hybridisation signals of the probes for nuclear DNA (subunit IV of cytochrome-*c* oxidase, a single gene copy) and mtDNA (subunit II of cytochrome-*c* oxidase, several hundred to several thousand gene copies per cell) on the same blot.

For Northern blot hybridisations, equivalent amounts of RNA from the various samples were subjected to agarose gel electrophoresis and transferred to Gene Screen Plus filters [7]. Analysis of mitochondrial and nuclear transcripts for several cytochrome-*c* oxidase subunits was also performed exactly as described before [7]. In addition to the probes described above, a battery of cDNAs was used for this purpose: cDNA for subunits VIb [25], VIc [26] (a kind gift from Dr. S. Ohta, Jichi Medical School, Tochigi-ken, Japan), VIIc [27], and for the liver-type isoforms of subunit VIa [28] and subunit VIIa [29] (kindly provided by Dr. E.A. Schon, Columbia University, New York, NY, USA). To monitor tissue-specific transcripts, bovine cDNA probes for the heart-type isoforms of subunit VIa [30] (kindly provided by Dr. R.A. Capaldi, University of Oregon, Eugene, OR, USA) and VIIa [31] (a generous gift from Dr. L.I. Grossman, Wayne State University, Detroit, MI, USA) were employed. To correct for possible unequal loading of the gels, all filters were subsequently hybridised with a 26-base oligodeoxynucleotide probe for 28S rRNA [7,32].

Autoradiographic hybridisation signals were measured using an LKB Ultroscan laser densitometer and analysed with the GelScan software programs, or with a Phosphor Imager, allowing comparison of signals with widely different intensities. The data were expressed as relative amounts: for RNA the transcript signals were compared to the signal of 28S rRNA, or to each other. For DNA, the signal of the mitochondrial genes was compared with those of nuclear genes on the same blot.

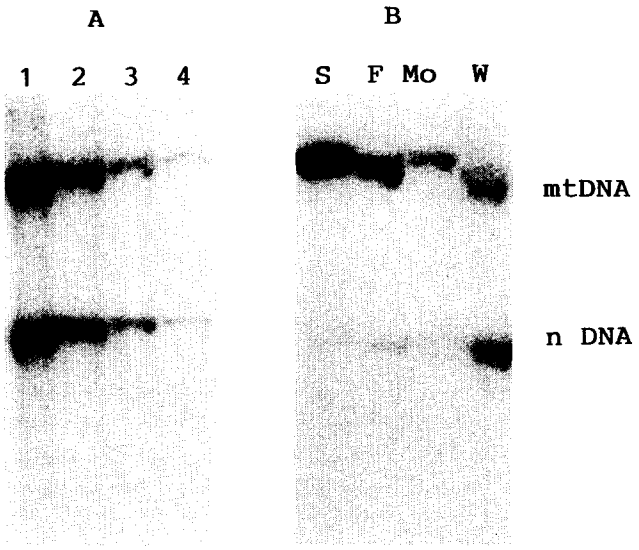

Fig. 1. Ratio of mitochondrial to nuclear DNA. Blots with *Eco*RI and *Xba*I digested total DNA from white blood cells (panel A, lane 1, 6 μg DNA; lane 2, 3 μg DNA; lane 3, 1.5 μg DNA; lane 4, 0.75 μg), from skeletal muscle (panel B, S, 0.5 μg DNA), fibroblasts (panel B, F, 0.6 μg DNA), Molt-4 cells (panel B, M, 0.5 μg DNA) and white blood cells (panel B, W, 3 μg DNA) were hybridised to cDNA probes for mitochondrial (mt DNA, subunit II) and nuclear (nDNA, subunit IV) genes for subunits of cytochrome-*c* oxidase. Panel A shows that a linear relationship exists between the size of the signals and the DNA concentration. Panel B shows a representative example of the results obtained using DNA from different types of tissues or cells.

The activity of cytochrome-*c* oxidase was measured spectrophotometrically at 20°C in 30 mM phosphate buffer (pH 7.4), using 14 μM human cytochrome-*c* [33] as substrate. The activity was expressed as the first-order reaction-rate constant, k (min^{-1}). Prior to the measurements, the enzyme was activated in 1.5% lauryl maltoside at 0°C for 15 min. The citrate synthase activity in these samples was measured according to Srere [34], and the protein and DNA contents were estimated using a modified Lowry method [35] and a fluorometric assay, respectively [36]. The activity measurements and the determination of the protein and DNA content were carried out in different portions of the same sample of homogenates of cells or tissues.

Results

Relative mtDNA levels in different cells and tissues

To obtain information about the relative mtDNA copy number, total DNA isolated from the different samples was digested with *Eco*RI and *Xba*I, electrophoretically separated and hybridised with the probes for the cytochrome-*c* oxidase subunits II and IV on the same blot. An example of the results obtained is given in Fig. 1 and the ratios between the respective hybridisation signals are shown in Table I. Large variations were found between different tissues, implying that their mtDNA copy number varied significantly. These variations were significantly less among different types of cultured cells.

Studies on the ratio between nuclear and mtDNA during the progression of the cell cycle of HL-60 cells suggested that this ratio was rather strictly regulated. This can be deduced from the data shown in Fig. 2: the ratio between mtDNA and nuclear DNA was about the same in all phases of the cell cycle. This suggests that the replication of mtDNA is linked to the replication of nuclear DNA and implies that mtDNA replication mainly occurs during the S-phase of the cell-cycle.

Relationship between mtDNA content and cytochrome-c oxidase activity

Table II shows the activities of cytochrome-*c* oxidase and of citrate synthase (a mitochondrial matrix enzyme) for the different tissues and cell lines, expressed per mg protein. A comparison of the acitvity of citrate synthase with that of cytochrome-*c* oxidase shows that large differences existed among different tissues and cells, including those regarding the ratio between both activities. The cytochrome-*c* oxidase activity was the lowest in transformed cell lines and the highest in heart tissue.

To allow a direct comparison to the mtDNA content, the cytochrome-*c* oxidase activity is also given per

TABLE I

Relative mtDNA copy number and cytochrome-c oxidase activity in human cell lines and tissues

The relative mtDNA copy number is expressed as the ratio between the hybridisation signals of mitochondrially and nuclearly encoded cytochrome-*c* oxidase genes on Southern blots. The ratio obtained for DNA from Molt-4 cells was arbitrarily set to 1. The mean ratios from three independent experiments are shown, the ratios for a given sample varied maximally 20% in different experiments. Cytochrome-*c* oxidase activity is expressed as the first-order reaction-rate constant k (per min per mg DNA). The mean of five independent measurements are shown, the variations between different experiments were less than 9% for a given sample.

Tissue/cell line	mtDNA copy number relative to total DNA	Cytochrome-*c* oxidase activity/mg total DNA	Ratio between cytochrome-*c* oxidase activity and mtDNA copy number
Heart	9.2	2180	237
Skeletal muscle	3.0	644	215
Liver	1.5	600	400
Kidney	6.2	867	140
Brain	0.6	244	407
White blood cells	0.3	147	490
Molt-4	1.0	19	19
HeLa S3	1.8	71	39
GLC4	1.0	22	22
Fibroblasts	1.6	286	179
Myoblasts	1.6	273	171

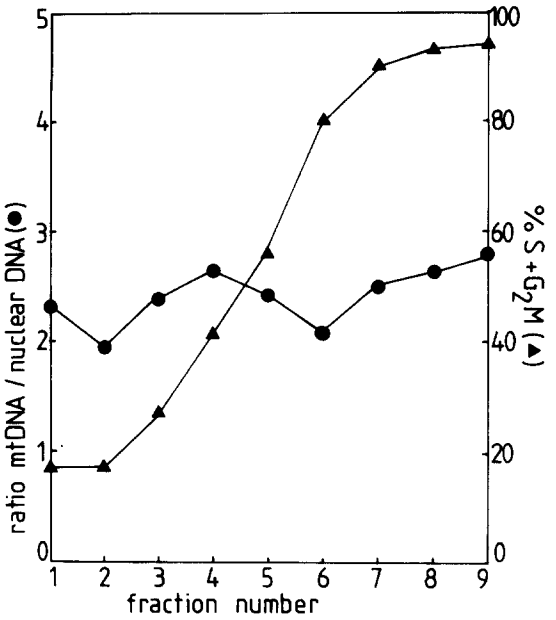

Fig. 2. Ratio of mitochondrial to nuclear DNA in subsequent phases of the cell cycle of HL-60 cells. ●, ratio between the hybridisation signals of mitochondrial and nuclear genes for cytochrome-*c* oxidase subunits on Southern blots. The ratio obtained on blots with DNA from HL-60 cells before counterflow elutriation was arbitrarily set to 2. ▲, percentage of cells in the $S+G_2M$ phase of the cell cycle. The results of one representative experiment are shown.

mg DNA in Table I. These data show that despite the fact that the transformed cell types had a mtDNA copy number that was comparable to that in liver, their cytochrome-*c* oxidase activity (on a DNA basis) was about 15-times lower. From Table I it follows that a direct relationship between the cytochrome-*c* oxidase activity and the mtDNA copy number was generally absent. This held also true during the progression of the cell cycle: the cytochrome-*c* oxidase activity increases two-fold in the G_1 stage and remains constant thereafter [17], but our present study showed that mtDNA was doubled in a later phase of the cell cycle.

Relative steady-state levels of mRNA

Blots with equivalent amounts of electrophoretically separated total RNA were prepared and hybridised with a battery of probes to compare the relative steady-state levels of the transcripts for cytochrome-*c* oxidase subunits in different samples. Because of the inavailability of healthy control material, RNA from human heart, brain and kidney had to be isolated from autopsy material. Nuclear RNA from these tissues appeared, however, to be deteriorated. The analysis of cytochrome-*c* oxidase transcripts in human tissues was therefore limited to liver and skeletal muscle. HeLa, GLC4 and Molt-4 cells were selected for transcript analysis in cultured cells of human origin. All blots were also rehybridised with an oligodeoxynucleotide probe for 28S rRNA. The signals obtained from these rehybridisations indicated that the amount of 28S rRNA per lane of a given sample did not differ more than 13%.

First, possible differences in the transcription of cytochrome-*c* oxidase genes for tissue-specific subunits were investigated. The heart-type mRNAs for subunit VIa and VIIa were only detected in skeletal muscle. The liver-type forms of both transcripts were present in liver, HeLa, GLC4 and Molt-4 cells (data not shown). As shown before [7], a small amount of the liver-type mRNA for subunit VIIa could be detected in skeletal muscle. To avoid complications in the interpretation of the results, only non-tissue-specific probes were used in succeeding hybridisations to compare the relative concentrations of cytochrome-*c* oxidase transcripts.

Hybridisation of blots with probes for mRNA of (the mitochondrial) subunit II and the mRNAs of (the nuclear) subunits IV, VIb, VIc, and VIIc, performed in duplicate with independently isolated batches of RNA, revealed a parallel pattern of steady-state levels, regardless of the origin of the transcript (see Fig. 3 for a representative result). This suggests a coordinated regulation of the transcription of both mitochondrial and nuclear genes for subunits of cytochrome-*c* oxidase. Although the relative amounts of cytochrome-*c* oxidase transcripts in tissues was subject to some variation, the relative amounts were always the lowest in liver. In skeletal muscle, the relative amounts were 24-fold

TABLE II

Specific activities of cytochrome-c oxidase and citrate synthase in human tissues and cell lines

The activity of cytochrome-*c* oxidase is expressed as the first-order reaction-rate constant *k* (per min per mg protein). The mean of five different measurements is shown, the values varied maximally 9% in different experiments with a given sample. The activity of citrate synthase is expressed as nmol product/min per mg protein. The mean of four different measurements is shown, the values varied maximally 12% in different experiments with a given sample. The last column shows the ratio between the citrate synthase activity and that of cytochrome-*c* oxidase.

Tissue/cell line	Cytochrome-*c* oxidase activity/ mg protein	Citrate synthase activity/ mg protein	Ratio between the activities of citrate synthase and cytochrome-*c* oxidase
Heart	109	917	8.4
Skeletal muscle	29	232	8.0
Liver	15	84	5.6
Kidney	26	153	5.9
Brain	10	89	8.9
White blood cells	8	206	25.8
Molt-4	1	103	103.0
HeLa S3	2	136	68.0
GLC 4	1	139	139.0
Fibroblasts	6	62	10.3
Myoblasts	6	58	9.7

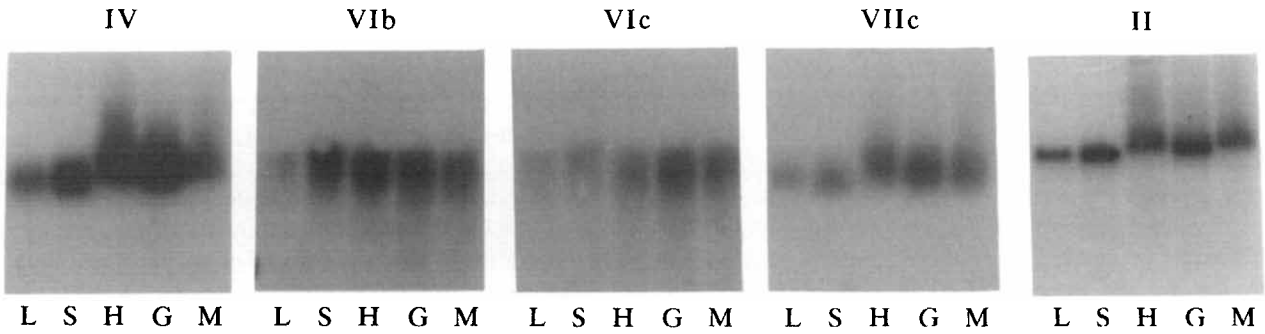

Fig. 3. Coordinated transcription of cytochrome-*c* oxidase genes. Blots with total cellular RNA (5 μg) from liver (L), skeletal muscle (S), HeLa cells (H), GLC-4 cells (G) and Molt-4 cells (M) were hybridised to DNA probes for transcripts for cytochrome-*c* oxidase subunits, as indicated. Blots were exposed to β-max film (Amersham) for 3 h (mitochondrial probe) or XAR film (Kodak) with intensifying screen for 4 days (nuclear probes).

higher than in liver, while the relative amounts in cultured cells were up to 10-fold higher than in liver.

The mitochondrial transcripts were always much more abundant than the nuclear ones. For example, the exposure time to visualise the mitochondrial transcripts was only 3 h, whereas for the nuclear transcripts 4 days were required. A detailed analysis of the intensities of the hybridisation signals of subunits II and IV, in which corrections were made for specific radioactivities and lengths of the probes, exposure times and film type, showed that the concentration of the mitochondrial transcript of subunit II was 20–25-times higher than that of the nuclear transcript for subunit IV.

Discussion

The availability of tissues and cells with a wide range of cytochrome-*c* oxidase activities offers a possibility to study aspects of the regulation of the synthesis of the enzyme. In the present study the possibility was tested that variations in cytochrome-*c* oxidase activity between different cells and tissues of human origin are related to differences in mtDNA copy number.

Firstly, the results demonstrate that large differences exist in the specific activities of cytochrome-*c* oxidase and citrate synthase from several human tissues and cells. This most likely reflects well-known differences in metabolic demands and pathways among various tissues. Moreover, the differences in tissue-specific ratios of both enzymes (Table II) were similar to those described before for several tissues from the mouse [16]. However, the specific activity of cytochrome-*c* oxidase was about 4–10-fold lower in tissues or cell lines of human origin as compared to their mouse equivalents, whereas the citrate synthase activity showed only a 2-fold reduction. This illustrates species-specific differences in oxidative phosphorylation capacity.

Secondly, our study shows that also variations exist in mtDNA copy number between various cells and tissues of human origin (Table I). These variations were not directly related to the differences in content of cytochrome-*c* oxidase as was demonstrated by comparing the relative mtDNA copy number and the cytochrome-*c* oxidase activity. Since the total amount of DNA in mammalian cells consists for only 1–2% of mtDNA, the variation in DNA content per cell will be negligible, in contrast to variations of the protein content (compare Tables I and II). Therefore, we expressed the enzyme activity on a DNA basis (Table I) to obtain the relation between the cytochrome-*c* oxidase activity and the dosage of the mitochondrial genes. We have shown before that there is a linear relationship between the cytochrome-*c* oxidase activity and its concentration in normal human tissues and cells [33,37].

Variations in mtDNA copy number have been described for several tissues of the rat [38], and also for cultured primary cell lines derived from various mammalian species [39]. In the former case, no direct relationship could be found between the mtDNA copy number and the cytochrome-*c* oxidase activity, in agreement with the present data for human tissues. In the latter case such a relationship was present, but we did not find this for human cultured cells. Possibly, this discrepancy might be explained by the fact that our study also included cell lines derived from tumor tissues. Recently, it has been demonstrated that the reduced content of mitochondrial proteins in tumor cells is most likely the result of enhanced degradation [4]. Variations in mtDNA content obviously do not explain the differences in cytochrome-*c* oxidase activity between the primary and the tumor cell lines in our study, since these differences were only minor.

This is in line with another study which showed that the ratio between mtDNA and nuclear DNA is the same in tumorigenic derivatives and the parent cell lines of mouse origin [40]. The results of the same study also suggest that the amount of mtDNA per cell is closely regulated within a given cell type, as a function of the total DNA content. In fact, our data indicate that this relationship might be even tighter. Using Southern blot hybridisation with mitochondrial and

nuclear probes on DNA isolated from HL-60 cells in subsequent stages of the cell cycle, mtDNA and chromosomal DNA appeared to be duplicated almost simultaneously, since their ratio remained constant (Fig. 2). This is in agreement with the report by Pica-Mattoccia and Attardi [41], but in contrast to that by Bogenhagen and Clayton [42] who found that mtDNA replication is not dependent on the cell cycle stage. The latter view is still commonly held. Unlike Pica-Mattoccia and Attardi, Bogenhagen and Clayton did, however, not use synchronised cells. This necessitated elaborate calculations and assumptions, based on the kinetics of dilution of [^{3}H]thymidine label incorporated into mtDNA. Furthermore, the present-day hybridisation techniques and methods to obtain cells at specific cell-cycle stages allowed us to reach conclusions in a more straightforward way.

The transcript analysis in the present study is interesting in view of both tissue-specific gene expression and coordination of nuclear and mitochondrial gene expression. The data show that liver-type cytochrome-*c* oxidase isoforms are expressed exclusively in poorly differentiated cultured cell lines, in line with previous data obtained at the protein level with cultured mouse cell lines [16]. The transcript levels of the nuclearly encoded subunits analysed in this study that do not have tissue-specific isoforms were found in similar relative ratios, not only in different tissues and cell lines, but also in relation to the mitochondrially encoded transcript for subunit II. Parallel changes in the transcript levels for subunit VIc and III have been described before in the rat [3,5,6]. Our data show that similar relationships exist for transcripts for subunits II, IV, VIb and VIIc in a variety of human tissues and cells. This observation strongly supports the concept of coordinated regulation at the level of transcription. The tissue-specific transcription of some genes for cytochrome-*c* oxidase subunits suggests that their transcription is under control of processes related to myofibril formation and that this control overrules the mechanism responsible for the parallel transcription of mitochondrial and nuclear genes for cytochrome-*c* oxidase subunits.

A mechanism as described in the Introduction [12], predicting a direct relationship between the concentration of the transcripts and the mtDNA gene dosage, offers an attractive explanation of how coordination might be achieved. From our data, it cannot be deduced exactly whether or not a direct relationship between the mtDNA copy number and the absolute concentration of the transcripts exists. Because of the qualitative and quantitative variations in the RNA populations from various origins, it is difficult to compare the transcript levels in different cells and tissues in an absolute way. It is, therefore, not possible to relate these to the mtDNA copy number. Despite this, it can be assumed that cultured cells contained far higher transcript levels than liver. Also rat hepatoma cells appeared to have a higher abundancy of transcripts for mitochondrial proteins than rat liver [4]. Our study also showed, however, that the relative mtDNA copy numbers in liver and cultured cells were similar. This does not exclude a direct relationship between mtDNA dosage and the transcript levels of cytochrome-*c* oxidase subunits, but implies at least that tissue-specific differences in this relationship exist. Nonetheless, the large variety in the relationship between mtDNA copy number and the cytochrome-*c* oxidase activity, shown in Table I, can hardly be explained on the basis of differences in steady-state transcript levels only. Tissue-specific differences in the regulation of the replication of mtDNA, of transcription, translation and degradation of cytochrome-*c* oxidase subunits, most likely contribute to tissue-specific and cell-type specific variations of the cytochrome-*c* oxidase activity. Such an explanation does not exclude that mitochondrial gene dosage is the main regulatory mechanism in modulating mitochondrial biogenesis in a given tissue, e.g., muscle.

The finding that the concentration of mitochondrially encoded transcripts for cytochrome-*c* oxidase subunits was at least 20-times higher than that of the nuclear ones presents a paradox, since the subunits in the assembled enzyme complex are present in equimolar amounts. One possible explanation is that mitochondrial transcripts and translation products are always made in excess. If this were the case, regulation of the synthesis of cytochrome-*c* oxidase would solely take place outside the mitochondria. An alternative explanation is that the process of mitochondrial protein synthesis requires higher transcript concentrations to synthesize proteins at a rate comparable to that of the cytoplasmic system. This might, for instance, be due to the absence of a 5′-leader sequence complementary to mitochondrial 12S rRNA [43], which could result in less efficient initiation of translation. Further experiments on the biosynthesis of individual cytochrome-*c* oxidase subunits are now in progress to settle this matter.

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