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Proliferation of Mitochondria in Chronically Stimulated Rabbit Skeletal Muscle—Transcription of Mitochondrial Genes and Copy Number of Mitochondrial DNA

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Mitochondrial proliferation was studied in chronically stimulated rabbit skeletal muscle over a period of 50 days. After this time, subunits of COX had increased about fourfold. Corresponding mRNAs, encoded on mitochondrial DNA as well as on nuclear genes, were unchanged when related to total tissue RNA, however, they were elevated two- to fivefold when the massive increase of ribosomes per unit mass of muscle was taken into account. The same was true for the mRNA encoding mitochondrial transcription factor A. Surprisingly, tissue levels of mtTFA protein were reduced about twofold, together with mitochondrial DNA. In conclusion, mitochondria are able to maintain high rates of mitochondrial transcription even in the presence of reduced mtTFA protein and mtDNA levels. Therefore, stimulated mtTFA gene expression accompanies stimulated mitochondrial transcription, as in other models, but it is not sufficient for an increase of mtDNA copy number and other, yet unknown, factors have to be postulated.

KEY WORDS: Mitochondrial biogenesis; copy number; gene expression; mitochondrial transcription factor; nuclear–mitochondrial communication; stimulation; endurance training.

INTRODUCTION

The molecular mechanisms of transcription and replication of mitochondrial DNA (mtDNA) have been described in great detail *in vitro* [for review, see Shadel and Clayton (1997)]. Surprisingly little is known about the *in vivo* quantitative relations between the copy number of mtDNA and mitochondrial transcription, on the one hand, and mitochondrial particle number and functional mass, on the other hand. However, understanding these relations is essential, not only to better understand mitochondrial biogenesis, but also to understand symptoms of neuromuscular diseases caused by mutations of mtDNA. These diseases are often accompanied by a massive proliferation of mitochondria carrying mutated mtDNA in skeletal muscle fibers (ragged red fibers; Larsson and Clayton, 1995). In addition, an expansion of the population of mutated mtDNA with time has been observed in some patients, eventually even exceeding wild-type mtDNA (Weber *et al.*, 1997), and was also found during the normal aging process (Michikawa *et al.*, 1999; Wallace, 1999). This proliferation may be due to a paradoxical, selective advantage of defective mitochondria containing mutated copies or, alternatively, a vicious cycle in which defects of the respiratory chain cause mutations of mtDNA and vice versa.

Thus, in order to gain more insight into the molecular mechanisms regulating mitochondrial proliferation in skeletal muscle, we have reevaluated a well-described model, namely, increase of capacity for oxi-

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dative phosphorylation (OXPHOS) by endurance training, which can be experimentally mimicked by chronic, low-frequency electrical stimulation (CLFS; Pette and Vrbova, 1999). In this experimental model, mitochondrial fractional volume as well as activities of key enzymes of mitochondrial metabolism increase four- to sevenfold within several weeks (Reichmann *et al.*, 1985; Skorjanc *et al.*, 1998), while the equipment of isolated organelles with enzymes remains remarkably constant (Schwerzmann *et al.*, 1989). Thus, the increase of OXPHOS can be largely attributed to a true generation of more organelles (see Figs. 6 and 7 in Pette and Vrbova, 1992), *i.e.*, mitochondrial proliferation (Luis *et al.*, 1993). Increases of mRNAs for mitochondrial proteins, encoded on both nuclear chromosomes as well as on mtDNA, have been reported previously under these circumstances (Williams, 1986; Williams *et al.*, 1986) and it was shown that expression of the two genomes seems to be perfectly coordinated (Hood *et. al.*, 1989). However, the hypothesis that upregulation of mtDNA copy number is the primary and also a necessary condition for increasing mitochondrial number, proposed on the basis of the work of Williams (1986), has become a matter of debate (Nagley, 1991; Wiesner, 1992) and is still not solved. Thus, in the present study, levels of cytochrome *c* oxidase subunits (COX), the terminal enzyme of the respiratory chain, were analyzed representing OXPHOS capacity. The time course of the expression of the mitochondrial genome and some selected nuclear genes as well as mtDNA copy number was measured in stimulated muscles. In addition, the expression and abundance of the nuclear-encoded mitochondrial transcription factor A (mtTFA), the only known regulator of mtDNA transcription and replication in mammals so far described (Shadel and Clayton, 1997), was analyzed.

MATERIALS AND METHODS

Chronic Low-Frequency Stimulation

New Zealand Red rabbits were anesthetized with pentobarbitone sodium (Macarthys Laboratories Ltd. Romford, Essex). Stimulating electrodes (coiled multistrand stainless-steel wire) were fixed on each side of the left lateral peroneal nerve and secured to the surrounding tissue in order not to disturb the nerve mechanically. Animals received antibiotics until their wounds had healed (usually 3–5 days after the operation). Stimulation via a telemetric system was performed at a frequency of 10/s, an intensity of 2 to 9 V, and a duration of 0.15 ms for 12 h per day with settings 1 h ON–1 h OFF. The animals were thus able to move freely in their cages (Skorjanc *et al.*, 1998). After 0, 8, 14, and 50 days of chronic electrical stimulation, three animals were euthanized. Both the stimulated and the unstimulated, contralateral tibialis anterior muscles were isolated and stored at −80°C until used for extraction and analysis of RNA, DNA, and proteins. These experiments were done in the laboratory of D. Pette, Konstanz, and further data on the same animals have been previously published (Skorjanc *et al.*, 1998).

Immunoblotting

Small pieces of frozen muscle tissue were homogenized in hot (95°C) 50 mM Tris (pH 6.8), 4 M urea, 10% SDS, 15% glycerol, and 20 mM dithiothreitol in a small Potter homogenizer at approximately 10 mg/100 μl. Equal amounts of protein, measured by the method of Bradford (1976), were loaded on 12.5% polyacrylamide–SDS gels prepared according to Laemmli (1970) and run at 60V and 15 mA at room temperature. Proteins were transferred to nitrocellulose in 154 mM glycine, 20 mM Tris (pH 8.3), and 20% methanol at 12V and 100 mA for 75 min at room temperature. Blots were blocked in TBST [200 mM NaCl, 50 mM Tris (pH 7.5) and 0.5% Tween] and 2% bovine serum albumin for 2 h. The 24-kD mtTFA subunit was visualized by an antibody raised against mouse mtTFA (kindly donated by Dr. D.A. Clayton, Stanford University School of Medicine, California). The antiserum was diluted 1:5000 in TBST containing 2% bovine serum albumin. Subunits of cytochrome *c* oxidase (COX) were visualized by an antiserum (diluted 1:3,000) raised against the human COX holoenzyme (kindly donated by Dr. H. Spelbrink, Amsterdam). Goat anti-rabbit immunoglobulin G (diluted in TBST 1:10,000 and containing 2% bovine serum albumin) was used as the secondary antibody coupled to horseradish peroxidase and visualized with the ECL chemoluminescence system (Amersham) by exposure to X-ray film. Lanes were scanned by densitometry.

Extraction and Blotting of RNA for Hybridization

For estimation of RNA content, total RNA was extracted by the acid guanidinium isothiocyanate pro-

cedure (Chomczynski and Sacchi, 1987) with 100–200 mg of tissue pulverized under liquid nitrogen. RNA was checked for intactness by visualization of sharp 28 and 18 S ribosomal RNA bands in formaldehyde–agarose gels stained with ethidium bromide (Sambrook *et al.*, 1989). RNA was blotted onto nitrocellulose membranes in three dilutions (2.5, 1.25, and 0.6 μg) in a filtration apparatus according to the manufacturer's instructions (Slot Blots, Schleicher and Schuell).

Extraction and Blotting of DNA for Hybridization

For estimating the relative abundance of mtDNA, 20–50 mg tissue were thawed, minced on ice with 500 μl lysis buffer (10 mM Tris, 100 mM NaCl, 25 mM EDTA, 1% SDS, and 10 mg/ml proteinase K), and incubated in a gyratory water bath shaker at 48°C overnight. RNase A (50 μg/ml) was added and the incubation continued for 30 min at 37°C. The mixture was extracted twice with phenol–chloroform–isoamyl alcohol (24:24:1) and once with chloroform–isoamyl alcohol (24:1), and the DNA precipitated at −20°C, centrifuged, and dissolved in 10 mM Tris–1 mM EDTA (Sambrook *et al.*, 1989). To linearize the closed circular mtDNA molecule, 2.5 μg of DNA were digested with *Bam*HI and *Xba*I under conditions recommended by the manufacturer. DNA was denatured by acid treatment, neutralized, and blotted in three dilutions (1.25, 0.63, and 0.31 μg) to nitrocellulose membranes.

Hybridization of DNA and RNA Blots

Blots were prehybridized for 2 h and hybridized overnight at 42°C (prehybridization: 40% formamide, 5x SSC, 50 mmol/L phosphate buffer, pH 7.4, 10x Denhardts solution, 0.2% SDS, 500 μg/ml salmon sperm DNA; hybridization: 50% formamide, 3x SSC, 10 mmol/L phosphate buffer, pH 7.4, 2x Denhardt's solution, 0.2% SDS, 170 μg/ml salmon sperm DNA). cDNA probes for COX subunits I, III, V*b*, and VI*c* as well as 12 S rRNA isolated from appropriate plasmids were labeled to high specific radioactivity by the random priming method. Probes were described in detail previously (Wiesner *et al.*, 1992a). After hybridization, blots were washed at 42°C (2 × 15 min in 2x SSC, 0.1% SDS followed by 2 x 15 min in 0.1× SSC, 0.1% SDS). Between hybridizations, the previously used probe were stripped from the blots (4 × 5 min incubations in boiling 0.01x SSC, 0.01% SDS). Finally, they were hybridized to a probe for cytosolic 28 S rRNA. For this probe, hybridization temperature was 44°C and the last two washing steps were performed at 50°C. In addition, a 1900-bp probe for rat cytochrome *c* cloned into the *Kpn*I–*Bam*HI sites of the plasmid RC4–4B (Virbasius and Scarpulla, 1990) was used under the same conditions. In order to measure mtTFA mRNA, a 1550-bp probe-encoding mouse mTFA cloned into the *Eco*RI site of pBS–KS was used (Garman and Clayton, unpublished); in this case, hybridization temperature was 38°C, yeast t-tRNA was used instead of salmon sperm DNA for blocking, and only two washes were employed after hybridization (2 x 15 min, 0.1x SSC, 0.1% SDS, 38°C). Blots were exposed to X-ray films.

Densitometric Evaluation of Hybridization Experiments

Levels of RNAs and mtDNAs were estimated from autoradiograms of slot blots. The absorbance of the autoradiograms was measured with a scanning densitometer and arbitrary numbers for RNA and mtDNA levels were used only from the densitometric values of those dilutions, which were in the linear range. For normalization, densitometric data for transcripts and mtDNA were normalized to the 28 S rRNA signal, also taking care that the signal was in the linear range. Finally, values were expressed as the ratio of stimulated vs. unstimulated contralateral muscle of the same animal.

Statistical Evaluation of Results

Results of stimulated versus unstimulated contralateral muscles were compared by Student's *t* test and $P < 0.05$ was assumed to be statistically significant.

RESULTS

Changes in Cytochrome *c* Oxidase Subunits

Cytochrome *c* oxidase is the terminal enzyme of the respiratory chain consisting of 13 subunits. In the

gel system used here (Fig. 1), the three subunits encoded on mitochondrial DNA (I–III) were easily separated and detected by the antiserum raised against the human holoenzyme. The apparent molecular mass of subunit I is considerably lower than the calculated mass in the presence of SDS. Unfortunately, no antibody against this subunit is available at present; however, by its complete absence in cells depleted of mtDNA (ρ0 cells), we have shown that the protein migrating at approx. 44 kDa is indeed COX subunit I (Spitkovsky and Wiesner, unpublished data). Of the ten subunits encoded in the nucleus, subunit IV could easily be resolved in the muscle homogenate, while the other subunits closely migrated together in two groups (V*a*/V*b* and VI–VIII). However, it is obvious that after 50 days of electrical stimulation, an increase of all subunits had occurred. Densitometric analysis showed that all COX subunits were increased three- to fourfold in stimulated muscles.

Changes of RNAs Encoded by mtDNA and by Selected Nuclear Genes

The mRNAs for mitochondrial proteins as well as 12S rRNA were quantitated by slot–blot hybridization assays using probes that had been shown to be highly specific by Northern blotting (not shown). After 50 days of electrical stimulation, the transcripts encoded on mitochondrial DNA, mRNAs for subunits I and III of COX, as well as 12 S rRNA, were significantly elevated over control levels (Fig. 2A). mRNAs for nuclear-encoded subunits of COX, for cytochrome *c*, as well as the mitochondrial transcription factor A, were not significantly increased at any time point (Fig. 2B). However, it is essential to note that total RNA per unit mass of muscle massively increases after CLFS (Seedorf *et al.*, 1986). In order to take this into consideration, values obtained by our hybridization analysis were multiplied by the values reported by Seedorf *et al.* (1986) (Figs. 2A and B, inserts). Thus, when expressed per unit mass of muscle, the true abundance

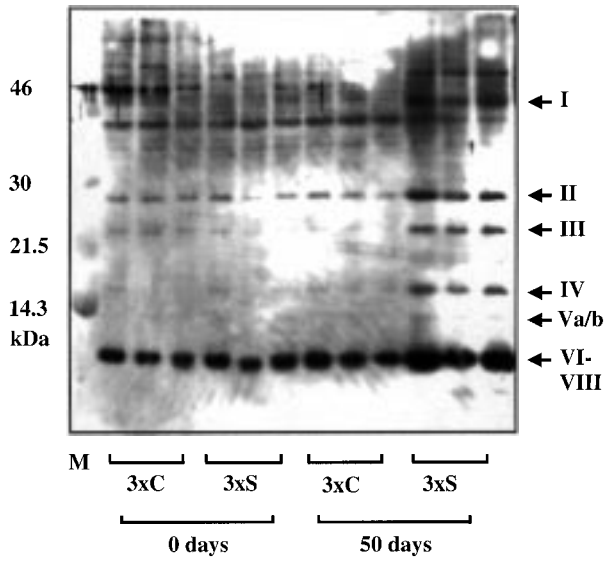

Fig. 1. Immunoblot analysis of COX subunits in control (C) and stimulated (S) rabbit tibialis anterior muscles after 0 and 50 days. Each lane contains the sample from one individual muscle. The positions of subunits I–III (mitochondrial), subunits IV and V*a*/*b* (nuclear), as well as other nuclear subunits that closely comigrate (VI–VIII), are given. M, molecular weight marker cross reacting with antiserum.

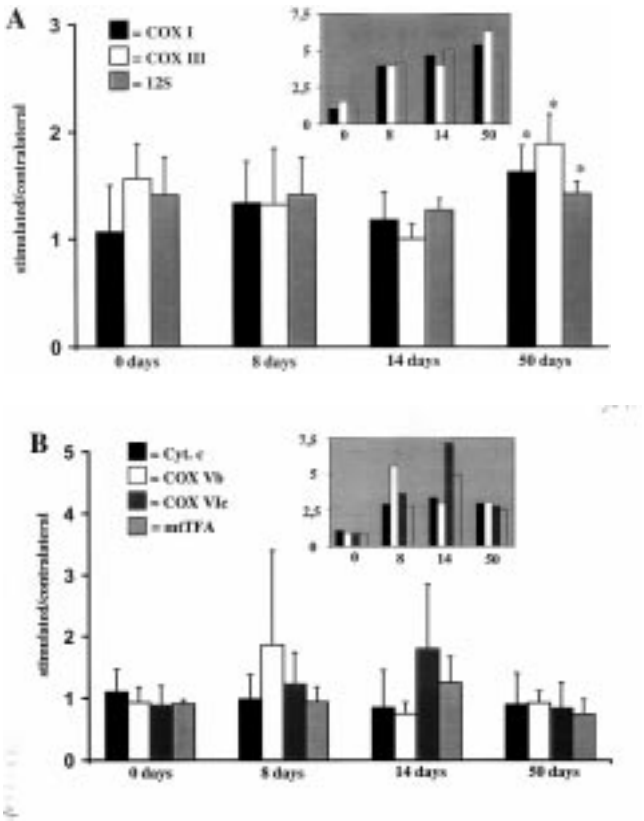

Fig. 2. Levels of transcripts encoded by mitochondrial DNA (A) or by some selected nuclear genes (B) in rabbit tibialis anterior muscles after the indicated times of stimulation. Relative densitometric numbers (stimulated/contralateral) derived from the analysis of slot-blots are given (mean ± SD, $n = 3$). The insets show the same data multiplied by the total RNA content of such muscles (Seedorf *et al.*, 1986) in order to emphasize the actual increase of these transcripts per unit mass of muscle. *, Significant difference, $p < 0.05$.

of all transcripts was indeed significantly higher in the stimulated muscles compared to unstimulated contralateral controls. The unit mass of muscle, however, is the appropriate denominator when levels of transcripts are to be compared with proteins which they encode (Fig. 1).

Changes of Mitochondrial DNA

Possible changes in the copy number of mitochondrial DNA after chronic electrical stimulation were similarly quantitated by slot–blot hybridization assays. The ratio of stimulated/contralateral muscle was 1.33 ± 1.07, 1.28 ± 0.70, 2.75 ± 2.74, and 0.38 ± 0.21 after 0, 8, 14, and 50 days of stimulation, respectively. Although the decrease after 50 days is conspicuous (−62%), it did not reach statistical significance ($p = 0.21$).

Changes of the Abundance of Mitochondrial Transcription Factor A (mtTFA)

mtTFA, functioning as a regulator of mtDNA transcription and replication, is also a highly abundant, high-affinity DNA binding protein. Thus, it is now well accepted that its tissue levels, as measured by immunoblotting, rather reflect mtDNA abundance and cannot be used as indicative of the activation state of the mitochondrial transcription apparatus.

Densitometric quantification of immunoblots (Fig. 3) showed a significant decrease of the 24-kDa protein after a stimulation period of 50 days (−46%; 3.1 ± 1.1 vs. 5.8 ± 1.1, stimulated vs. contralateral, $p < 0.05$), approximately of equal magnitude as the decrease of mtDNA. At other time points, mtTFA levels were similar in the two groups. A high molecular-weight band reacting with our antiserum was observed, however, its specificity is unclear.

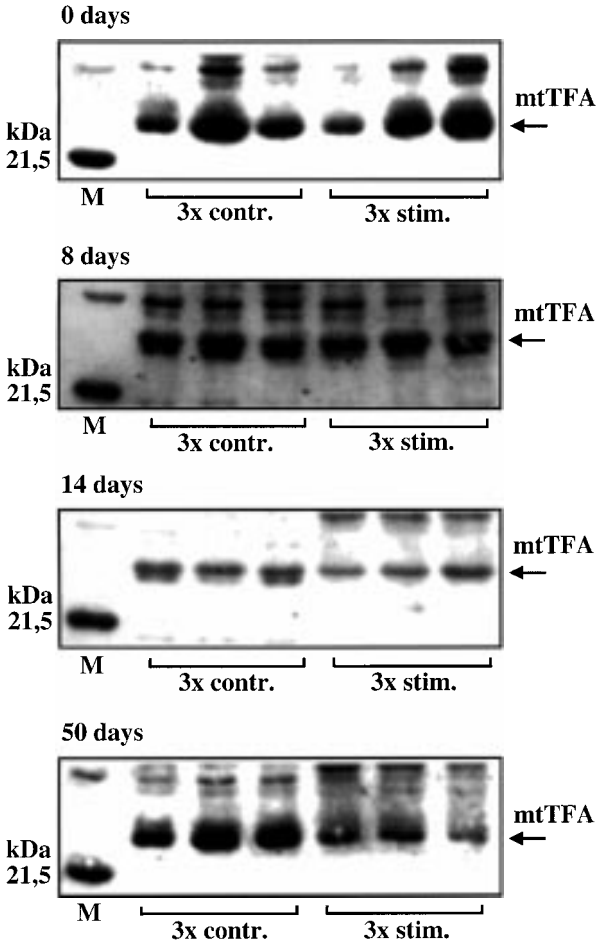

Fig. 3. Immunoblot analysis of mitochondrial transcription factor A in control (contr.) and stimulated (stim.) rabbit tibialis anterior muscles after the indicated times. Each lane contains the sample from one individual muscle, M, molecular weight marker cross reacting with antiserum.

DISCUSSION

The stimulation of OXPHOS capacity during the conversion of fast-glycolytic to slow-oxidative fibers, which is induced by CLFS in skeletal muscle, has been described in great detail in the past. However, the molecular mechanisms leading to mitochondrial proliferation are not well understood. The specific mitochondrial fractional volume of muscle cells as well as marker enzymes increase up to sevenfold, within an observation period of 4 weeks (Reichmann *et al.*, 1985, Skorjanc *et al.*, 1998). Since the equipment of isolated mitochondria with these enzymes remains remarkably constant (Schwerzmann *et al.*, 1989), the increase of OXPHOS capacity can be attributed largely to the generation of more organelles, *i.e.*, true mitochondrial proliferation. This was also supported by electron microscopic photographs (see Figs. 6 and 7 in Pette and Vrbova, 1992). As has been shown before in the analogous rat model (Hood *et al.*, 1989), relatively large increases of COX subunits are accompanied by only moderate increases of the corresponding mRNAs, when these are measured on tissue RNA blots (Figs. 2A and B). However, it has to be taken into account

that the total amount of ribosomes per unit mass of muscle massively increases after CLFS in these muscles. Polysomes are transiently elevated fivefold after 3 weeks and remain elevated twofold; poly A^+–mRNA is also twofold higher in stimulated muscle in the long-term range (Seedorf *et al.*, 1986). Both features are typical for slow oxidative when compared to fast glycolytic muscle (Gagnon *et al.*, 1991). These notable changes have to be considered if a quantitative correlation between levels of COX and its corresponding mRNAs is intended (Figs. 1 and 2A and B, inserts). Thus, a rather generalized increase of the biosynthetic capacity of the muscle in both genetic compartments seems to be the most important way for increasing OXPHOS capacity under these circumstances, strongly resembling similar mechanisms involved in mitochondrial biogenesis during hypertrophic growth of the heart (Wiesner *et al.*, 1994), as well as during cold adaptation in brown fat tissue (Klingenspor *et al.*, 1996). Little is known about the mechanisms controlling increased ribosome formation under such conditions, but the upstream binding factor of RNA polymerase I seems to be an important target (Hannan *et al.*, 1996). Virtually nothing is known about the coordination of ribosome formation with the host of genes encoding myofibrillar proteins (Pette and Vrbova, 1992), or the estimated 1000 nuclear genes for mitochondrial proteins. The transcription factor Sp1, as well as the nuclear respiratory factors NRF-1 and NRF-2 (Scarpulla, 1997), may be important candidates for coordinating transcription of nuclear-encoded mitochondrial genes as well as mitochondrial DNA under such conditions, since many of these share the consensus-binding sequence for these activator proteins in their promoter regions, among them, the genes for cytochrome *c*, COX subunit V*b*, and mitochondrial transcription factor A (for a review, see Lenka *et al.*, 1998). Thus, any further work on the specific regulation of genes during mitochondrial proliferation must take into account that mitochondrial gene expression may be masked by such a general response of the cellular transcription machinery.

The mRNA for mtTFA was also upregulated (Fig. 2B, inset), probably because the mtTFA gene also contains NRF-1, NRF-2, and Sp-1 sites in its proximal promoter region (Scarpulla, 1997). An increase of mtTFA mRNA has also been found in rat liver after thyroid hormone treatment, in brown fat tissue after cold adaptation, and in rat hearts where cellular energy flux had been impaired (Klingenspor *et al.*, 1996; Garstka *et al.*, 1994; Wiesner *et al.*, 1999). This clearly indicates that stimulation of the mtTFA gene is causally linked to the stimulation of mtDNA transcription observed under such circumstances. Thus, the mtTFA gene is indeed an important target for the not yet fully known pathway responsible for the stimulation of OXPHOS capacity under conditions of increased energy demand.

Rather surprisingly, despite of the persistent elevation of mitochondrial transcripts (Fig. 2A, inset), the mtTFA protein was significantly decreased together with mtDNA in stimulated compared to unstimulated contralateral muscle after 50 days. However, it has to be kept in mind that mtTFA is not only a transcription factor binding to sequence elements proximal to the two transcription initiation sites of mitochondrial DNA (Shadel and Clayton, 1997), but it also binds with high affinity to multiple sites in the region between the two promoters (Ghivizzani *et al.*, 1994). Although the function of the protein at these sites is unclear, tissue levels of mtTFA protein observed on Western blots rather reflect the total pool and not the activation state of the mitochondrial transcription machinery. In addition, the classical way of replication of mtDNA is also initiated by an RNA primer derived from the light strand promoter. Thus, mtTFA may also be involved in the regulation of mtDNA replication (Shadel and Clayton, 1997) and, indeed, homozygous mice lacking the mtTFA protein (Tfam −/−) lose mtDNA very early during embryogenesis and die with severe developmental defects (Larsson *et al.*, 1998). This clearly shows the importance of this protein for mtDNA maintenance. Very interestingly, in heterozygous animals (Tfam +/−), the levels of the mtTFA transcript, as well as the mtTFA protein, are gene-dosage dependent reduced together with mtDNA by approximately 50%. However, mitochondrial transcripts are reduced only marginally, if at all, and only in heart muscle, this leads to a significant reduction of OXPHOS complexes. That this tissue is most sensitive to mtTFA and, consequently, mtDNA reduction is probably due to the fact that in heart, individual organelles contain only one copy of mtDNA (Wiesner *et al.*, 1992b).

In conclusion, mitochondria are able to maintain high rates of mitochondrial transcription even in the presence of reduced mtTFA protein and mtDNA levels, in Tfam +/− mice, as well as in stimulated rabbit skeletal muscle (this study). Therefore, mtTFA protein is necessary for mtDNA maintenance and stimulated mtTFA gene expression accompanies stimulated mitochondrial transcription, but it is not sufficient for an

increase of mtDNA copy number and other, yet unknown, factors have to be postulated.

The massive proliferation of organelles observed in stimulated muscle occurs in two phases, about threefold during the first 18 days and up to sevenfold within 28 days (Reichmann *et al.*, 1985), but is obviously not accompanied by an appropriate proliferation of mtDNA molecules. The reason for even a decrease of mtDNA together with its affiliated binding protein remains unclear. However, our results emphasize again that proliferation of mtDNA is not a prerequisite for increased levels of mitochondrial transcripts and OXPHOS capacity. It has been shown before that mtDNA levels and mitochondrial fractional volume or COX activity, respectively, do not correlate at all in rat tissues (Wiesner *et al.*, 1992b). We have also calculated that in liver there is a surplus of mtDNA molecules, since only a small fraction of the mtDNA population is transcribed at any given time point (Wiesner, 1992). Thus, in the steady state as well as under conditions of stimulated mitochondrial biogenesis, the rate of transcription from a subpopulation of cellular mitochondrial DNA seems to determine the available amount of mitochondrially encoded mRNAs and mitochondrial proteins, while the copy number of mtDNA does not seem to be of major importance (Wiesner, 1992). This was also recently shown in two new models, mitochondrial proliferation during muscle development (Sogl *et al.*, 2000) as well as in rat hearts after impairment of cellular energy transfer (Wiesner *et al.*, 1999).

In summary, the activation state of the mtTFA gene seems to correlate well with the activation state of the mitochondrial DNA transcription machinery, which, on the other hand, correlates with cellular energy demand, which is high in stimulated muscle, hyperthyroid liver, or cold-adapted brown fat tissue. In these situations, sufficient mtTFA protein is provided to the proximal promoter binding sites of mtDNA to achieve high transcription rates. The copy number of mtDNA, on the other hand, is regulated in response to some other, yet unknown, demands and depends on other, yet unknown, factors. Thus, a complex interplay of mtTFA with other proteins has to be postulated to explain these discrepancies between mitochondrial transcriptional activity, mtTFA levels, and copy number of mitochondrial DNA.

Finally, it remains unclear why in some comparable experiments, specific increases of mitochondrial transcripts as well as mtDNA copy number had been observed in the past (Williams, 1986). The obvious difference between the experimental settings is that in those studies, continuous stimulation for 24 h/day was used, while here 12 h stimulation with 1 h ON/OFF periods was used and animals were able to move freely. It has been shown that degeneration of muscle fibers and regeneration from satellite cells contributes to some extent to the fast to slow conversion of rabbit muscle (Maier *et al.*, 1986). If degeneration of muscle cells exceeds regeneration during continuous stimulation, this would finally lead to a decreased number of nuclei, *i,e.*, total DNA, per unit volume of muscle. This would result in an increased amount of mtDNA on Southern blots, without a true change of mtDNA copy number.

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