Vol. 200, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1994 Pages 619-626

STOICHIOMETRY OF MITOCHONDRIAL TRANSCRIPTS AND REGULATION OF GENE EXPRESSION BY MITOCHONDRIAL TRANSCRIPTION FACTOR A

Heike L. Garstka, Michael Fäcke[1], Javier Ramos Escribano and Rudolf J. Wiesner

Department of Physiology II, University of Heidelberg, Heidelberg, Germany
[1]Deutsches Krebsforschungszentrum, Angewandte Tumorvirologie, Heidelberg, Germany

Received March 4, 1994

Summary: **The steady state concentration of cytochrome c oxidase subunit I mRNA and 12 S rRNA, respectively, measured by a quantitative reverse transcription/polymerase chain reaction method, was 4 and 15 molecules per molecule of mt DNA in rat liver and 2 and 9 molecules in rat muscle, respectively. These results imply that in the mitochondrial compartment, the molar concentration of all thirteen mRNAs by far exceeds the concentration of ribosomes, a situation fundamentally different from the cytosolic compartment. Following thyroid hormone treatment, both mitochondrial transcripts increased, in parallel with the mRNA encoding mitochondrial transcription factor A. We conclude that this transcription factor might be the rate limiting factor for mitochondrial transcription *in vivo*, at least under these conditions.**

Transcription of vertebrate mt DNA starts from a bipartite promoter located in the so-called D-loop region. Both strands of the circular molecule are transcribed into polycistronic transcripts, which are processed giving rise to 2 rRNAs, 13 mRNAs and 22 tRNAs (1). Due to premature termination after reading through the two rRNA genes, which are located at the 5'end of the heavy-strand-transcript, the rates of synthesis and, consequently, the final steady state concentrations of the two rRNA species are higher than the concentration of mRNAs (2,3). In rat liver, heart and brain, five to ten times more 12S rRNA molecules have been measured compared to each of some selected mRNAs, which, on the other hand, are all present in about equimolar concentrations (4,5). This stoichiometry is surprising. It implies that in the mt compartment, the molar concentration of the total mRNA pool exceeds the concentration of ribosomes, a situation being essentially different from the cytosol. Given the importance of this observation and its potential implications for the process of mt translation, we used a recently developed, quantitative

Abbreviations used: bp, base pairs; COX I, cytochrome c oxidase subunit 1; T3, 3,3',5-Triiodo-L-Thyronine; mt, mitochondrial; mtTFA, mitochondrial transcription factor A; PCR, polymerase chain reaction; RT, reverse transcription.

0006-291X/94 $5.00

RT/PCR method (6) to reinvestigate the stoichiometry between mt RNA classes, in the steady state, but also after experimental manipulation of mt transcript levels by thyroid hormone (T3).

In addition, the possible role of mitochondrial transcription factor A (mtTFA; 7) in regulating mt gene expression was investigated, based on the current concept that transcription factors determine to what extent a gene is transcribed. *In vitro*, mt RNA polymerase and this single additional protein have been shown to be necessary and sufficient for faithful and efficient transcription of vertebrate mt DNA (8). However, although mtTFA is certainly an essential part of the mt transcription complex (9), until now it has not been established that its abundance is regulating the rate of transcription *in vivo*. Thus, in a first approach we have correlated expression of the mtTFA gene with changes of mt gene expression which have been well described in the past in rat liver in different thyroid states (e.g. 10, and references quoted therein).

MATERIALS AND METHODS

The procedure as well as data on the heart weight/body weight ratio, as indicator of thyroid status, as well as circulating T3 levels in animals from which RNA was used in the present study has been described previously (10). Briefly, rats were made hypothyroid by feeding them a diet containing propylthiouracil for three weeks. Some animals stayed hypothyroid, while others were injected with T3 (12.5 mg/100 g daily) for up to five days in order to induce hyperthyroidism. RNA was extracted from the liver and the oxidative part of the M. gastrocnemius by the acid guanidinium isothiocyanate method (11). For quantitation of transcript levels, RNA was blotted on nitrocellulose membranes in three dilutions (1, 2 and 4 μg) using a slot blot filtration apparatus.

A clone for human mtTFA was established in our laboratory using the published sequence (12), human RNA as template and appropriate primers flanking the coding region (MTF1: 5`-CCTCTAGGCGATGGCGTTTCTCCGAA-3`-position 148 and RevMTF1: 5`-CCATCGATCCATTGTGAACACATCTC-3`-position 886). These primers match the sequence over a length of 20 bp and introduced restriction sites for XbaI and ClaI, respectively, at their 5`ends. After RT/PCR, the 783 bp PCR product was subcloned into pBluescript II KS (Stratagene). E. coli was transformed, plasmid DNA from individual clones was prepared and the sequence ot the entire mtTFA insert was confirmed by dideoxy-sequencing (13).

The mtTFA insert, a cDNA for 28 S ribosomal RNA (14) as well as PCR products derived from mt COX I mRNA and mt 12 S ribosomal RNA, respectively, generated as described below, were labelled by the random priming method and α-^{32}P-dCTP. These probes were used to analyze levels of the appropriate transcripts on Northern blots or RNA slot blots by hybridization in formamide containing buffers (13). Blots were hybridized consecutively with the probes for mtTFA, COX I, 12 S and 28 S and were stripped by boiling in 0.01x SSC, 0.01% SDS before proceeding to the next probe. The absorbance of autoradiogramms of the slot blots was quantitated with a scanning densitometer, and arbitrary numbers for RNA levels were obtained from the densitometric values of one or two dilutions, making sure that these were in the linear range. In order to correct for unequal blotting, values for mtTFA, COX I and 12 S were corrected using the data derived from hybridization to 28 S rRNA (14).

Since Northern blot analysis is unable to tell the true stoichiometry between the two mt transcripts, COX I mRNA and 12 S rRNA, their tissue levels were quantitated by a method developed recently in our laboratory (6). Briefly, after RT using two complementary primers specific for COX I (positions 5898-5919) and 12 S (positions 609-628 of the published rat sequence, 15), an aliquot of the RT reaction is subjected to PCR in the presence of two specific forward primers (5538-5559 and 310-329, respectively) and 10 μCi of α-^{32}P-dCTP. Aliquots are taken from the reaction in consecutive cycles and loaded on an 1.5% agarose gel. The product bands (318 bp and 381 bp for 12 S and COX I, respectively) are cut from the gel, dried overnight at 80^{o}C and the

incorporated radioactivity (cpm) is measured by liquid scintillation counting. Product accumulation (N_n) in consecutive cycles (n) is calculated in molar terms from these data and the specific radioactivity of the precursor, dCTP (cpm/mol). The molar concentration of target (N_o) can then be calculated by analysing these data by linear regression analysis of the transformed equation describing product accumulation in the PCR, $\log N_n = \log \text{eff} \times n + \log N_o$ (eq. 1), with eff being the amplification factor in the exponential phase of the reaction.

After correction for the first cycle, in which the single stranded cDNA, which is equimolar to the RNA target, is converted to double-stranded PCR products/targets (6), the result, moles of 12 S and COX I cDNAs per aliquot of the PCR reaction, is converted to molecules per μg RNA used in the RT reaction using Avogadro's number and taking into account the appropriate dilution factors. This method has been shown to measure faithfully and accurately the amount of template introduced into a PCR reaction (16). It also measures RNA concentrations correctly, since we have shown that decreasing the concentration of substrate RNA from 1 μg down to 30 ng by two-fold dilution steps resulted in the same incorporation of radioactivity into the products, however one cycle later for each dilution step. Thus, cDNA synthesis does not depend on the primer-template ratio (about 50.000 under our assay conditions), which would have been expected if the efficiency of RT were less than 100% (6; and data not shown). In addition, RT has indeed been demonstrated to be complete under similar conditions in another system (17) and about 50% of purified globin mRNA were reverse transcribed into full length transcripts when only a threefold excess of primer was used (18).

RESULTS

The principle of the PCR method used here to quantitate mt transcripts is best illustrated by plotting product accumulation in the PCR reaction (N_n, in moles/μl) vs. cycle number (n; Figure 1). The efficiency of amplification, eff, is given by the slope of the lines, whereas the molar concentration of cDNAs at cycle zero, i.e. before amplification, could be obtained graphically by extrapolation. More conveniently, these values are calculated by linear regression analysis. Results are shown in Table I. In order to make them more transparent, the data have been converted to molecules per molecule of mt DNA (Table II), using previously published values on the content of RNA and numbers of mt DNA molecules, respectively, in these tissues (14,16).

In control rats, mitochondria contain about four times more 12 S rRNA molecules per mt DNA than COX I molecules, both in liver and skeletal muscle (Table II). These numbers are

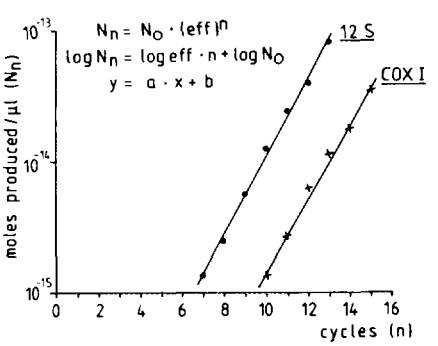

Figure 1. Exponential accumulation of two products derived from 12 S rRNA and COX I mRNA after RT in the PCR. The inset shows the transformed equation describing product accumulation in the PCR which can be used to calculate the concentration of target molecules at cycle zero (N_o), i.e.,before amplification, by linear regression analysis.

Table I
Numbers of COX I mRNA and 12 S rRNA molecules per unit mass of tissue RNA in liver and red muscle of rats in different thyroid states

	LIVER		RED MUSCLE	
	COX I	12 S	COX I	12 S
control	1.4 ±0.6	5.9 ±1.2	0.9 ±0.7	5.3 ±4.0
hypothyroid	1.7 ±0.8	7.0 ±3.9	0.4 ±0.5	1.9 ±1.2
hyperthyroid	3.8 ±3.3	30.7 ±6.0	n.d.	n.d.

The numbers are molecules x 10^8 per μg of tissue RNA (means ± S.D.; n = 5). Hyperthyroid samples are samples taken from animals after five days of T3 injection.

essentially unchanged in hypothyroid liver, but are both severely reduced in hypothyroid muscle. After five days of T3 injection into hypothyroid rats, in the liver COX I mRNAs increased 2.5-fold, while 12 S rRNAs increased 4-fold compared to hypothyroid animals. Since there is little effect of this treatment on mt transcripts in skeletal muscle (10), this tissue was not investigated further. In summary, COX I mRNA, but also 12 S rRNA are regulated by T3 in a tissue specific manner, as described previously for COX II and COX III mRNAs (10).

In order to investigate the role of mtTFA in mt transcription, hybridization analysis was performed, comparing mtTFA gene expression to expression of COX I and 12 S transcripts in rat livers in different thyroid states. On Northern blots, the mtTFA cDNA mainly hybridized to a transcript of the expected size of about 2 kb (12; not shown). Both the 12 S rRNA and COX I mRNA were largely unchanged in hypothyroid animals and were increased 2-3fold after T3 treatment (Figure 2), in accordance with results obtained recently for COX II and COX III

Table II
Numbers of COX I mRNA and 12 S rRNA molecules per molecule of mitochondrial DNA in liver and red muscle of rats in different thyroid states

	LIVER		RED MUSCLE	
	COX I	12 S	COX I	12 S
control	4	15	2	9
hypothyroid	4	18	1	3
hyperthyroid	10	79	n.d.	n.d.

Numbers of molecules were calculated using the data given in Table I, and the RNA content (mg/g wet weight) and the numbers of molecules of mt DNA (molecules/g wet weight), respectively, which were published previously for these tissues (10, 14, 16).

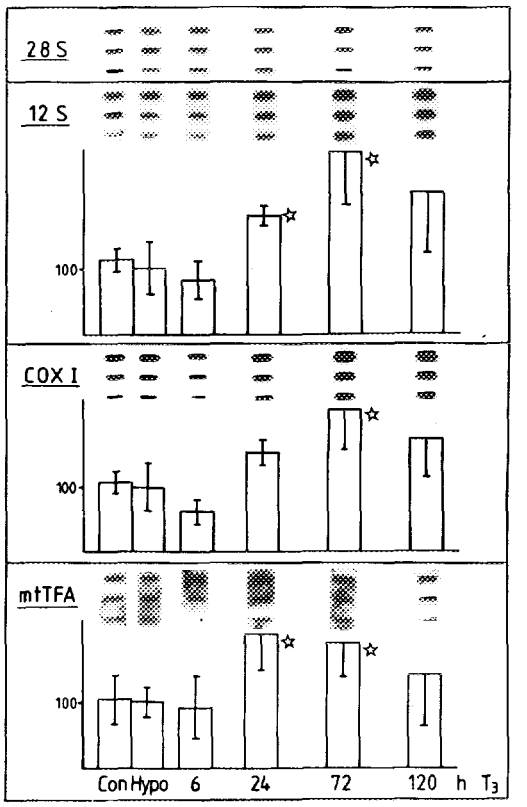

Figure 2. Levels of 12 S, COX I and mtTFA transcripts in livers of untreated control rats, hypothyroid rats and hyperthyroid rats after the indicated times of T3 treatment. Values are arbitrary densitometric units derived from the autoradiographic signals of one or two different amounts of blotted tissue RNA (slot blots; means ± S.D.; n = 5). Representative exposures are shown for each experimental group and each probe (1, 2 and 4 μg of blotted RNA). The same slot blots are also shown after probing with a 28S cDNA; data derived from these measurements were used to correct for unequal loading.

mRNAs (10). These increases were also in good agreement with the results obtained by quantitative RT/PCR (Table II). The rise in 12 S rRNA seemed to precede the rise in COX I mRNA, which was significant only after three days, again in agreement with previously published data for COX II and COX III mRNAs (10).

Like mt transcripts, mtTFA mRNA was unchanged in hypothyroid liver. It was increased 2-fold one day after T3 treatment, and started to decline later, but was still significantly elevated at day three. Thus, levels of mtTFA transcripts were regulated by T3 in an analogous way compared to mt transcripts.

DISCUSSION

The present study had two objectives: First, to reinvestigate the ratio between tissue levels of the two mt transcript classes, rRNAs and mRNAs, which seems to be surprisingly low in differentiated tissues according to previous studies (4,5). Secondly, to study the possible regulatory

role of mtTFA on mt transcription, using modulation of the thyroid state in rats as a well established model (e.g. 10).

Our results on the abundance as well as the stoichiometry of mt transcripts are in perfect agreement with data reported previously using a completely different assay, namely quantitative solution hybridization: Cantatore et al. (4) have reported a ratio of five between 12 S rRNA and COX I mRNA in rat liver, while we find a ratio of four (Table II) in rats of similar age (about six weeks). Whether this is truly different from the ratio of ten reported more recently in liver, heart and brain from older rats (eight months; 5), or due to the inherent variation of these rather complex measurements, remains to be elucidated. However, the main finding is that in differentiated rat tissues, the ratio between one selected rRNA and one selected mRNA is surprisingly low. Both the 2 rRNAs on the one hand as well as the 13 mRNAs on the other hand have been shown to be present in approximately equimolar concentrations (2,4). Thus, rat liver would contain fifteen 12 S and 16 S molecules, respectively, and 13 x 4 = 52 mRNA molecules per molecule of mtDNA (Table II). Using the number of 3 mt DNA molecules per rat liver mitochondrion estimated previously (16), at the maximum, 3 x 15 = 45 assembled ribosomes would face an excess of 3 x 52 = 156 mRNA molecules in the enclosed mt compartment. This is certainly fundamentally different from the cytosolic compartment, which can be easily shown by some simple calculations: Cellular RNA, composed almost entirely of cytosolic RNA, contains about 1-5% mRNA and 90-95% rRNA (weight/weight; 14,19). Thus, the rRNA/mRNA ratio in the cytosolic compartment is in the range of 15 to 20. In the mt compartment, however, it is much lower. A molecular weight of 8×10^5 d for a fictitious 2500 bp 12 S + 16 S rRNA molecule, present in 15 copies per mt DNA, can be assumed. Likewise, a fictitious 12000 bp polycistronic mRNA molecule, excluding tRNAs and the D-loop region, has a molecular weight of 4×10^6 d, and is present in four copies per mt DNA. Thus, the weight/weight ratio of rRNA to mRNA in rat liver mitochondria would be 0.75 ($1.2 \times 10^7 / 1.6 \times 10^7$). It should be noted that in contrast to these results, in exponentially growing HeLa cells, grown in suspension culture, the rRNA/mRNA ratio was reported to be much higher (2).

The implications of a low ribosome-to-message ratio for the process of mt translation in differentiated tissues *in vivo* are far-reaching, but remain speculative for the moment. Mitochondrial mRNAs contain no or very short 5'untranslated regions (20), they are not "capped" like cytosolic mRNAs (21) and contain no conserved adapter sequence like prokaryotic mRNAs (22). We propose that the lack of any particular recognition signal for the ribosome makes a high intramitochondrial concentration of mRNAs necessary in order for translation initiation to occurr at a reasonable rate for each individual mRNA. In accordance with our proposal, polysomes have never been observed in animal mitochondria (23) and a very low translational efficiency, i.e. less than 10% of COX I mRNAs translated into COX I protein at any time, has been calculated previously (3).

For an efficient augmentation of mt protein synthesis, both the concentrations of mRNAs as well as ribosomes should increase upon a stimulus, again in contrast to the cytosolic compartment, where enhanced synthesis of a certain protein is achieved simply by increasing its mRNA in many cases. Our results support this proposal, since increased synthesis of mt proteins like COX

following T3 treatment (10) are indeed accompanied by increases of both COX mRNAs as well as 12 S rRNA (Table II and Fig. 2). Thus, efficient stimulation of mt biogenesis not only requires the concerted synthesis of structural proteins and enzymes encoded in the two genetic compartments, but also the enhanced provision of the numerous ribosomal proteins and factors involved in mt translation (24).

These data thus raise more questions than they solve. In contrast, the results shown in Fig. 2 clearly indicate that mtTFA might regulate mt transcription *in vivo*, at least under these circumstances. It has been shown unequivocally that T3 indeed increases the rate of mt transcription in rat liver (25,26). We now demonstrate that expression of mtTFA is also regulated by the hormone. Not surprisingly, a thyroid receptor binding element (TGACC) located upstream of a GC-rich region can be found when scanning the upstream region of the human mtTFA gene published recently (position -375 to -371, 27). This arrangement of sequence elements should confer T3 responsiveness to the gene according to the data of Voz et al. (28). Thus, we postulate that mtTFA may be the long sought missing link between T3 and mt proliferation (29). It remains to be determined whether it is also the general, rate limiting factor regulating mt gene expression *in vivo* upon other stimuli.

Acknowledgments: The skilful technical assistance of Christl Bletz is greatfully acknowledged. This work was supported by The Deutsche Forschungsgemeinschaft, SFB 320, C5. J.R.E. was supported by the ERASMUS program (Heidelberg-Salamanca).

REFERENCES

1. Clayton, D.A. (1991) Annu. Rev. Cell Biol. 7, 453-479.
2. Gelfand, R. and Attardi, G. (1981) Mol. Cell. Biol. 1, 497-511.
3. Cantatore, P., Flagella, Z., Fracasso, F., Lezza, A.M.S., Gadaleta, M.N. and Montalvo, A. (1987) FEBS Lett. 213, 144-148.
4. Cantatore, P. Gadaleta, M.N. and Saccone, C. (1984) Biochem. Biophys. Res. Commun. 118, 284-291.
5. Gadaleta, M.N., Petruzella, V., Renis, M., Fracasso, F. and Cantatore, P. (1990) Eur. J. Biochem. 187, 501-506.
6. Wiesner, R.J. (1992) Nucleic Acids Res. 20, 5863-5864.
7. Shadel, G.S and Clayton, D.A. (1993) J. Biol. Chem. 268, 16083-16086.
8. Fisher, R.P., Topper, J.N. and Clayton, D.A. (1987) Cell 50, 247-258.
9. Parisi, M.A., Xu. B. and Clayton, D.A. (1993) Mol. Cell. Biol. 13, 1951-1961.
10. Wiesner, R.J., Kurowski, T.T. and Zak, R. (1992) Mol. Endocrinology 6, 1458-1467.
11. Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156-159.
12. Parisi, M., Clayton, D.A. (1991) Science 252, 965-969.
13. Sambrook, J., Fritsch, E.F. and Maniatis, T.: Molecular Cloning - A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989.
14. Gagnon, J.T., Kurowski, T.T., Wiesner, R.J. and Zak, R. (1991) Mol. Cell. Biochem 107, 21-29.
15. Gadaleta, G., Pepe, G., DeCandia, G., Quagliarello, C., Sbisa, E. and Saccone, C. (1989) J. Mol. Evol. 28, 497-516.
16. Wiesner, R.J., Rüegg, J.C. and Morano, I. (1992) Biochem. Biophys. Res. Comm. 183, 553-559.
17. Delidow, B.C., Peluso, J.J. and White, B.A. (1989) Gene Anal. Techn. 6, 120-124.

18. Berger, S.L., Wallace, D.M., Puskas, R.S. and Eschenfeldt, W.H. (1983) Biochemistry 22, 2365-2372.
19. Lewin, B. Genes III. John Wiley & Sons, New York, 1987.
20. Grohmann, K., Amalric, F., Crews, S. and Attardi, G. (1978) Nucleic Acids Res. 5, 637-651.
21. Shatkin, A.J. (1976) Cell 9, 645-653.
22. Shine, J. and Dalgarno, L. (1974) Proc. Natl. Acad. Sci. (USA) 71, 1342-1346.
23. Liao, H.X. and Spremulli, L.L. (1990) J.Biol.Chem. 265, 11761-11766.
24. Denslow, N.D., Anders, J.C. and O'Brien, T.W. (1991) J. Biol. Chem. 266, 9586-9590.
25. Gadaleta, M.N., DiReda, N., Bove, G. and Saccone, C. (1975) Eur. J. Biochem. 51, 494-501.
26. Barsano, C.P., DeGroot, L. and Getz, G.S. (1977) Endocrinology 100, 52-60.
27. Tominaga, K., Akiyama, S., Kagawa, Y. and Ohta, S. (1992) Biochim. Biophys. Acta 1131, 217-219.
28. Voz, M.L., Peers, B., Wiedig, M.J., Jacquemin, P., Belayev, A. and Martial, J.A. (1992) Mol. Cell. Biol. 12, 3991-3997.
29. Tata, J.R. in: Regulation of Metabolic Processes in Mitochondria. (Tager, J.M. et al., eds). 489-507, Elsevier, Amsterdam, 1966.