Effects of cold environment on mitochondrial genome expression in the rat: evidence for a tissue-specific increase in the liver, independent of changes in mitochondrial gene abundance

Immaculada MARTIN, Octavi VIÑAS, Teresa MAMPEL, Roser IGLESIAS and Francesc VILLARROYA*

Unitat de Bioquímica i Biologia Molecular B, Departament de Bioquímica i Fisiologia, Universitat de Barcelona, Spain

The abundance of the mitochondrially encoded mRNA for subunit II of cytochrome *c* oxidase (COII mRNA) increases in the liver of rats exposed to environmental cold stress (4 °C ambient temperature). Only transient increases or no changes in COII mRNA levels were observed in brown fat and soleus muscle respectively. The time course of the liver COII mRNA increase was compared with the effects of cold stress on mitochondrial 16S ribosomal RNA expression and indicated that cold induces a rapid (few hours) increase in the liver mito-

chondrial mRNA levels and high levels of both messenger and ribosomal RNA mitochondrial transcripts are present after a few days of cold exposure. No changes in mitochondrial DNA abundance relative to total cellular DNA were observed in the liver of rats at any time during cold stress. It is concluded that mitochondrial genome expression is specifically increased in the liver of cold-exposed rats through different mechanisms, independent of changes in mitochondrial genome abundance.

INTRODUCTION

In the eukaryotic mammalian cell, the mitochondrial genome is a genetic system differentiated from the nuclear one. In recent years, our understanding of mitochondrial DNA structure and mechanisms of replication and transcription has increased dramatically. The whole mitochondrial DNA sequence is now available from several species, the coding regions have been fully established and regions responsible for initiation of replication and transcription are well identified. In mammals, the regions of mitochondrial DNA coding for proteins correspond to different components of the respiratory chain and F_0F_1 -ATP synthase systems. Therefore expression of the mitochondrial genome may be expected to be regulated according to the energy requirements of the cell as well as being strictly co-ordinated with the expression of nuclear genes coding for other components of the mitochondrial respiratory system. However, the mechanisms responsible for these regulatory events are poorly understood. [For reviews, see Cantatore and Saccone (1987) and Clayton (1991)].

Studies on mammalian cells in culture have shown that different biological signals, such as hormones and growth factors, cause changes in mitochondrial genome expression through changes in transcription or replication rates (Glaichenhaus et al., 1986; Van Itallie and Dannies, 1988; Van Itallie, 1990). However, much less is known about the mechanisms responsible for the control of *in vivo* mitochondrial genome expression in response to environmental and physiological determinants, probably because of the very limited number of experimentally useful models available. One of the few well-characterized physiological stimuli of respiratory capacity *in vivo* is the chronic contractile stimulation of skeletal muscle which leads to high mitochondrial genome expression as the result of an increase in mitochondrial genome abundance (Williams, 1986). Thus changes in relative mitochondrial DNA abundance are often considered to be the main mechanism involved in modulation of mitochondrial genome expression *in vivo* in the adult mammal.

The environmental temperature is an important determinant of respiratory activity in different mammalian tissues. The physiological response of small mammals to a cold environment involves an increase in respiratory activity of the naturally uncoupled mitochondria of brown fat (Nicholls et al., 1986), although there is also a substantial increase in the respiratory activity of other tissues such as liver and skeletal muscle (Behrens and Himms-Hagen, 1977; Kinnula et al., 1983; Goglia et al., 1988). In the present work we have investigated expression of the mitochondrial genome in rat tissues in response to a cold environment, in an attempt to establish a physiological model for the *in vivo* modulation of mitochondrial genome expression as well as to determine the mechanisms involved in this modulation.

MATERIALS AND METHODS

Female albino Wistar rats (*Rattus norvegicus*) from our own colony (Faculty of Biology, University of Barcelona, Spain) were used. Control rats (180–200 g body weight) were kept under standard conditions of environmental temperature (21 ± 1 °C) and light cycle (12 h light/12 h dark). The experimental animals were kept at a temperature of 4 °C, all other conditions being the same as for the control animals. Rats were killed by decapitation, and tissues (soleus muscle from both legs, interscapular brown adipose tissue and liver) were extracted and frozen using liquid N_2 .

Tissue RNA was prepared by a modified hot phenol method (Lomedico and Saunders, 1976), and the quality of RNA preparations was always checked in ethidium bromide-stained agarose gels and by the A_{260}/A_{280} ratio method (Sambrook et al., 1989). Total RNA obtained was determined by measurement of A_{260} . Slot-blot hybridization of total RNA samples using a ^{32}P -labelled oligo(dT) probe (Boehringer-Mannheim) was systematically performed to check that the relative poly(A)⁺ RNA contents in total RNA samples were not significantly different (Harley, 1987). Northern- and/or slot-blot analysis were per-

formed as already reported (Martin et al., 1989), using the pIL7 probe, corresponding to a 0.5 kb length of the cDNA for subunit II of cytochrome oxidase (COII) (Glaichenhaus et al., 1986), a 4.5 kb genomic DNA probe for rat β -actin (Nudel et al., 1983) or a probe for rat mitochondrial 16S rRNA. This last probe was constructed in our laboratory as follows: mitochondrial DNA, prepared from isolated rat liver mitochondria (Hauswirth et al., 1987), was digested with *Eco*RI. The resulting pattern of DNA restriction fragments indicated that our rat strain was the B type, according to the previously established terminology for mitochondrial genome polymorphism in Wistar rat populations (Castora et al., 1980). Thus the resulting 2.4 kb *Eco*RI fragment, corresponding to a region for the full-length 16S rRNA and a small region of the NADH dehydrogenase subunit I mRNA, was isolated by electroelution. Northern-blot analysis of rat liver RNA indicated that this fragment was useful for detecting a single band of 1.5 kb corresponding to the rat 16S mitochondrial rRNA and only longer exposure of films resulted in another signal (0.9 kb) for NADH dehydrogenase subunit I mRNA. For mitochondrial DNA-abundance studies, total DNA from livers of rats kept under different experimental conditions was prepared (Williams, 1986) and it was completely digested with the *Bam*HI endonuclease (Boehringer-Mannheim). Equal amounts (20 μ g) of digested DNA were analysed by Southern blot and hybridized by standard procedures (Sambrook et al., 1989) using the pIL7 probe. The pIL7 probe, the 4.5 kb β -actin probe and the 1.5 kb *Eco*RI fragment of rat mitochondrial DNA were labelled with [α - 32 P]dCTP (Amersham) using the random oligo-primer method. The oligo(dT) probe was labelled with [γ - 32 P]ATP (ICN Radiochemicals) using T4 polynucleotide kinase (Boehringer-Mannheim). Quantitative assessment of Northern- and/or slot-blot hybridization signals was by densitometric analysis (LKB). Statistical comparisons were performed by means of Student's *t* test.

RESULTS AND DISCUSSION

The three tissues chosen for the first part of this study, brown fat, skeletal muscle and liver, have been reported to show high respiratory activity which increases when animals are exposed to a cold environment (Kinnula et al., 1983; Nicholls et al., 1986; Goglia et al., 1988). Basal levels of mitochondrially encoded mRNA, assessed as COII mRNA abundance in control rats, were markedly different among these three tissues. As shown in Figure 1, COII mRNA concentration was severalfold higher in brown fat than in soleus muscle, and COII mRNA levels in soleus muscle were higher than in liver. The effects of exposure to a cold environment on COII mRNA abundance were also different in different tissues (Table 1). For interscapular brown adipose tissue, a significant but transient increase was detected, in accord with a previous observation (Martin et al., 1989). There were no changes in soleus muscle COII mRNA levels, whereas the liver showed a sustained increase in COII mRNA as a consequence of cold stress.

Our main attention was therefore focused on the liver as a target tissue for the effects of cold on mitochondrial genome expression. A time-course study on the short- and long-term effects of cold on mitochondrial COII mRNA levels was performed and it was compared with changes in mitochondrial rRNA expression, assessed by 16S rRNA abundance as well as β -actin mRNA as an internal control. As shown in Figure 2(a), there is a significant increase in COII mRNA levels after 6 h of cold exposure. After 1, 2 or several days of cold, liver COII mRNA levels remain significantly higher than controls. However, as depicted in Figure 3, 16S rRNA levels are not significantly

modified in the first hours of cold exposure but they rise progressively as cold treatment progresses. Thus mitochondrial messenger and ribosomal RNA levels are concurrently increased with respect to controls in cold-acclimated rats (several days of cold exposure).

In order to assess whether the observed changes (especially the long-term increase in mitochondrial messenger and ribosomal transcripts) are related to changes in mitochondrial genome abundance, the relative abundance of mitochondrial DNA with respect to total cell DNA was studied in livers of cold-exposed rats. As shown in Figure 4, Southern-blot analysis of equal amounts of total liver DNA digested with *Bam*HI and hybridized with the pIL7 probe resulted in a 10 kb band, in agreement with the known restriction map of rat mitochondrial DNA for this restriction enzyme. Relative intensities of bands from the different groups showed no differences. Thus none of the observed changes in mitochondrial transcript abundance can be attributed to modifications in the number of mitochondrial genome copies with respect to total cellular DNA.

The pattern of changes in mitochondrial genome expression observed in the liver after long-term cold exposure, i.e. parallel

Figure 1 COII mRNA expression in brown adipose tissue, soleus muscle and liver of the rat

Northern-blot analysis of 10 μ g of total RNA from control rats (environmental temperature 21 °C) probed with the pIL7 (COII cDNA) probe. B, Brown adipose tissue; S, soleus muscle; L, liver. The Figure is representative of three independent Northern-blot analyses.

Table 1 Effects of cold exposure on COII mRNA levels in different rat tissues

Results are means $\pm$ S.E.M. of densitometric scanning of independent slot-blot analyses of RNA extracted from the interscapular brown fat, soleus muscle and liver of four to five different animals for each time of exposure to an environmental temperature of 4 °C. Results are expressed as percentages of control values (environmental temperature 21 °C), the numbers of individual values are shown in parentheses and statistical significance of comparisons with respect to control values is indicated by **P* $\leq$ 0.05.

Tissue	Time at 4 °C (h)...	COII mRNA levels (%)		
		0	6	24
Brown fat		100 $\pm$ 9.0 (4)	155.0 $\pm$ 17.0 (5)*	103.6 $\pm$ 5.0 (4)
Liver		100 $\pm$ 2.2 (4)	236.8 $\pm$ 14.0 (5)*	185.4 $\pm$ 14.5 (4)*
Soleus muscle		100 $\pm$ 15.0 (4)	114.9 $\pm$ 12.8 (5)	111.81 $\pm$ 11.0 (4)

Figure 2 Time course of the effects of cold exposure on (a) liver COII and (b) β -actin mRNA levels

Results are means $\pm$ S.E.M. of densitometric scanning of four to five independent Northern-blot analyses of total RNA (10 μ g/lane) extracted from the livers of four to five different animals for each experiment group (where not shown, S.E.M. is smaller than the size of the symbol). ●, 4 °C; ○, 21 °C. Results are expressed as percentage of values for rats kept at 21 °C at 0 h. Statistical significance of differences between each cold-exposed (4 °C) group and the corresponding time-matched control (21 °C) is indicated by * $P \leq 0.05$. Gels are examples of representative Northern-blot analysis (A, controls at time 0 of cold exposure; B, C, D and E, 6 h, 24 h and 48 h and 10 days of cold exposure respectively).

changes in messenger and ribosomal RNA transcripts, is similar to various responses observed in mammalian cells in culture. Similar increases in mitochondrial mRNA and rRNA, without changes in mitochondrial genome abundance, have been reported in transformed or immortalized cell lines (Glaichenhaus et al., 1986) as well as in response to fetal calf serum (Kadowaki and Kitagawa, 1989) and are usually attributed to changes in the overall transcription rate of the mitochondrial genome. It has been reported that thyroid hormone treatment of adult rats (Mutvei et al., 1989) or hepatic cell cultures (Van Itallie, 1990) causes a pattern of changes in mitochondrial gene expression similar to that reported here. The known increase in circulating thyroid hormones as a consequence of exposure to a cold environment and the well-established mitochondriogenic effect of these hormones (Nelson, 1990) indicate that they are probably mediators of the long-term effects of cold environment observed here.

Figure 3 Time course of the effects of cold exposure on mitochondrial 16S rRNA levels

Results are means $\pm$ S.E.M. of densitometric scanning of four to five independent Northern-blot analyses of total RNA (10 μ g/lane) extracted from the livers of four to five different animals for each experimental group (where not shown, S.E.M. is smaller than the size of the symbol). ■, 4 °C; □, 21 °C. Results are expressed as percentages of values for rats kept at 21 °C at 0 h. Statistical significance of differences between each cold-exposed (4 °C) group and the corresponding time-matched control (21 °C) is indicated by * $P \leq 0.05$. The gel is an example of representative Northern-blot analysis (A, controls at time 0 of cold exposure; B, C, D and E, 6 h, 24 h, 48 h and 10 days of cold exposure respectively).

Figure 4 Effects of cold exposure on liver mitochondrial DNA levels

Southern-blot analysis of equal amounts (20 μ g) of *Bam*HI-digested DNA from the livers of rats kept at 21 °C (lane A) or 4 °C for 6 h (lane B) or 10 days (lane C). The Figure is representative of three independent analyses, probed with the pL7 (COII cDNA) probe.

The short-term response to cold obtained in the present experiments, characterized by a significant increase in mitochondrial COII mRNA, is difficult to relate to other reports but it is similar to observations from Cantatore et al. (1986) on mitochondrial genome expression during liver development. These authors reported a specific increase in mitochondrial COI mRNA not associated with any change in mitochondrial rRNA abundance in murine liver just after birth. Even though these results were originally interpreted in terms of the ontogenic determinants of mitochondrial genome expression, it should be pointed out that birth exposes the animal to dramatic cold stress. The close parallelism of these data with our present observation in the cold-exposed adult rat suggests a common mechanism in which cold stress elicits a rapid specific enhancement of mitochondrially encoded mRNAs. The molecular mechanisms that may be responsible for specific increases in mitochondrial mRNA expression, such as observed here in the short-term response to cold, are controversial. Independent control of the ribosomal

and ribosomal plus messenger transcripts at the transcription initiation level has been proposed by some authors (Attardi, 1985). Others think that the termination event at the end of 16S rRNA production may play a major regulatory role in the determination of the mitochondrial mRNA and rRNA expression relationship (Christianson and Clayton, 1988). Whatever the mechanism, rapid neurohormonal signals produced in response to cold exposure (probably sympathetic nervous system activation) must surely act through one of these proposed mechanisms and cause the short-term effects found in this study. The relative importance of a specific response to cold with respect to the general stress response in these short-term effects deserves further study. On the other hand, our present observations indicate that relative mitochondrial genome abundance is not a general regulatory mechanism of mitochondrial gene expression in the adult animal, at least in the liver. Exposure of animals to a cold environment appears to be a suitable physiological model for studying *in vivo* regulation of mitochondrial genome expression through transcriptional and/or post-transcriptional mechanisms.

Further research will be necessary to fully evaluate the physiological consequences of the increased mitochondrial genome expression in the liver of cold-exposed rats. It has been reported that cold exposure of rats leads to an increase in liver mitochondria content and respiratory activity (Goglia et al., 1988; Iossa et al., 1991). Cytochrome *c* oxidase, the terminal protein complex in the mitochondrial electron-transport chain, plays a central role in oxidative metabolism (Lomax and Grossman, 1989), and mitochondrially encoded subunits (such as COII) constitute the functional core of this enzyme complex (Cantatore and Saccone, 1987). These observations, taken together with the present findings, suggest that enhanced mitochondrial genome expression may be the mechanism that produces the adaptative increase in respiratory activity that occurs in animals living in a cold environment.

Thanks are given to Dr. N. Glaichenhaus for kindly providing the pL7 probe. This work was supported by Dirección General de Investigación Científica y Técnica (PB89-0227), Ministerio de Educación y Ciencia, Spain.

REFERENCES

- Attardi, G. (1985) *Int. Rev. Cytol.* **93**, 93–145
- Behrens, W. A. and Himms-Hagen, J. (1977) *J. Bioenerg. Biomembr.* **9**, 41–63
- Cantatore, P. and Saccone, C. (1987) *Int. Rev. Cytol.* **108**, 149–208
- Cantatore, P., Loguercio-Polosa, P., Fracasso, F., Flagella, Z. and Gadaleta, M. N. (1986) *Cell Differ.* **19**, 125–132
- Castora, F., Arnheim, N. and Simpson, M. V. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 6415–6419
- Christianson, T. W. and Clayton, D. A. (1988) *Mol. Cell. Biol.* **8**, 4502–4509
- Clayton, D. A. (1991) *Trends Biochem. Sci.* **16**, 107–111
- Glaichenhaus, N., Leopold, P. and Cuzin, P. (1986) *EMBO J.* **5**, 1262–1265
- Goglia, F., Liverini, G., Lanni, A. and Barletta, A. (1988) *Mol. Cell. Endocrinol.* **55**, 141–147
- Harley, C. B. (1987) *Gene Anal. Tech.* **4**, 17–22
- Hauswirth, W. W., Lim, L. O., Dujon, B. and Turner, G. (1987) in *Mitochondria, A Practical Approach* (Darley-Usmar, M., Rickwood, D. and Wilson, M. T., eds.), pp. 171–176, IRL Press, Oxford and Washington
- Iossa, S., Liverini, G. and Barletta, A. (1991) *J. Endocrinol.* **131**, 67–73
- Kadowaki, T. and Kitagawa, Y. (1989) *FEBS Lett.* **233**, 51–56
- Kinnula, V. L., Huttenen, P. and Hirvonen, J. (1983) *Eur. J. Appl. Physiol.* **51**, 237–245
- Lomax, M. I. and Grossman, L. I. (1989) *Trends Biochem. Sci.* **14**, 501–503
- Lomedico, P. T. and Saunders, G. F. (1976) *Nucleic Acids Res.* **3**, 381–391
- Martin, I., Giral, M., Viñas, O., Iglesias, R., Mampel, T. and Villarroya, F. (1989) *Biochem. J.* **263**, 965–968
- Mutvei, A., Kuzela, S. and Nelson, B. D. (1989) *Eur. J. Biochem.* **180**, 235–240
- Nelson, B. D. (1990) *Biochim. Biophys. Acta* **1018**, 275–277
- Nicholls, D., Cunningham, S. A. and Rial, E. (1986) in *Brown Adipose Tissue* (Trayhurn, P. and Nicholls, D., eds.), pp. 86–104, Edward Arnold, London
- Nudel, U., Zakut, R., Shani, M., Neuman, S., Levy, Z. and Yaffe, D. (1983) *Nucleic Acids Res.* **11**, 1759–1771
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) in *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Van Itallie, C. M. (1990) *Endocrinology* **127**, 55–62
- Van Itallie, C. M. and Dannies, P. S. (1988) *Mol. Endocrinol.* **2**, 332–337
- Williams, R. S. (1986) *J. Biol. Chem.* **261**, 12390–12394