Alteration of mitochondrial DNA and RNA level in human fibroblasts with impaired vitamin B12 coenzyme synthesis

Palmiro Cantatore[a], Vittoria Petruzzella[a], Cinzia Nicoletti[a], Francesco Papadia[b], Flavio Fracasso[a], Pierre Rustin[c], Maria Nicola Gadaleta[a,*]

[a] *Department of Biochemistry and Molecular Biology, University of Bari and Centro Studi sui Mitocondri e Metabolismo Energetico, CNR, via Orabona 4A, 70126 Bari, Italy*
[b] *Divisione Malattie Metaboliche e Genetiche, Azienda Ospedaliera Di Venere-Giovanni XXIII, Bari, Italy*
[c] *Unité de Recherches sur les Handicaps Génétiques de l'Enfant INSERM U393, Département de Génétique and Département de Pédiatrie, Hôpital Necker-Enfants Malades, 149 rue de Sevres, 75743 Paris Cedex 15, France*

Received 27 April 1998; revised version received 25 June 1998

Abstract Alterations of mitochondrial (mt) nucleic acid metabolism in methylmalonic aciduria (MMA) were studied in two cell lines from skin fibroblasts of patients with mitochondrial (GM00595) or cytosolic (GM10011) defects in the biosynthesis pathways of cobalamin coenzymes. The mtDNA level increased two-fold in GM00595 cells, which carry a mt defect in the adenosylcobalamin synthesis, whereas no appreciable change was found in GM10011 cells. The content of the two rRNAs 16S and 12S mtRNAs, normalized for the mtDNA copy number, decreased by 70% and 50% in GM00595 and GM10011, respectively. The normalized content of ND1, ND2 and CO I mRNAs decreased in GM00595, but was unchanged in GM10011. Respiratory chain complex activities measured in these two cell lines were not different from control activities. These data suggest that the maintenance of the mt function is due to doubling of mtDNA and that this compensatory response takes place only in those cells in which the greater reduction of the level of rRNA might have brought the content of these transcripts below the threshold value for optimal expression of the mt genome.

Key words: Methylmalonic aciduria; Mitochondrial RNA; Mitochondrial DNA; Human fibroblast; Cobalamin coenzyme

1. Introduction

Vitamin B12 (cobalamin, Cbl) is acquired from dietary sources and converted in two coenzyme forms, methylcobalamin (MeCbl) and adenosylcobalamin (AdoCbl). MeCbl permits the utilization of folate and catalyzes the synthesis of methionine in the cytoplasm; AdoCbl participates in the isomerization of methylmalonyl-CoA to succinyl-CoA, catalyzed by methylmalonyl-CoA mutase (MCM) within mitochondria. In humans, generally in infancy or in childhood, defects of cellular utilization and coenzyme production can cause different clinical manifestations, including neuropathy, depending on whether one or both coenzymes are impaired (for review see [1,2]). Impairment of AdoCbl and MeCbl synthesis and utilization or defects of MCM are responsible for the accumulation of methylmalonic acid and homocysteine in blood and in urine, causing diseases known as methylmalonic aciduria (MMA) or homocystinuria (HCys). These diseases are divided into two groups, called *Cbl* and *Mut*, depending on the response to treatment with cobalamin. Genetic analysis of cultured fibroblasts from responsive *Cbl* patients allowed the identification of seven complementation groups which include defects of synthesis of AdoCbl (*cbl*A and *cbl*B), MeCbl (*cbl*E and *cbl*G) and of both AdoCbl and MeCbl (*cbl*C, *cbl*D, *cbl*F).

Disorders of Cbl metabolism may affect mitochondrial (mt) functions. Methylmalonyl and propionic acid accumulation has been claimed to cause a reduction of pyruvate dehydrogenase, succinate dehydrogenase and succinyl-CoA ligase activities and a reduction in cell ATP content [3–6]. The energetic metabolism might also be impaired by the accumulation of saturated and unsaturated monocarboxylic fatty acids, as a result of ketotic episodes of organic acidemia [7]. As alterations in the mt metabolism may be associated with changes in the mt genetic system [8–10], we asked whether an impaired Cbl coenzyme biosynthesis might perturb the mt genetic system. For this purpose we measured mtDNA and mtRNA contents, and respiratory chain complex activities in cultured human fibroblasts deriving from *cbl*A and *cbl*C complementation groups. The results reported here show that only *cbl*A fibroblasts, carrying a defect in the intramitochondrial AdoCbl synthesis, have a higher mtDNA content than control and that both *cbl*A and *cbl*C cell lines exhibit alterations in the level of mature mtRNAs.

2. Materials and methods

2.1. Patients, cell lines and growth media

Skin fibroblast cell lines from patients were obtained from the NIGMS Human Genetic Mutant Cell Repository (Corriel Institute for Medical Research, Camden, NJ, USA) under numbers GM00595 (*cbl*A) and GM10011 (*cbl*C). A third cell line, GM05659, obtained from the same company, was used as control. Two other control cell lines were obtained, as described by Martin [11], from skin fibroblasts of healthy individuals. Cells were grown in Eagle's minimal essential medium (MEM) with Earle's salts (Seromed) and 2.2 g/l $NaHCO_3$, supplemented with 10% fetal calf serum, 100 U/ml of penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine.

2.2. Probes

Probes for mt genes were obtained by polymerase chain reaction (PCR). The reactions were performed in a thermal cycler in a final volume of 100 μl containing: 50–1000 ng of DNA, 0.02 μmol of each dNTP, 50 pmol of each primer, 2 U of Taq DNA polymerase (Boehringer Mannheim), in 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50

*Corresponding author. Fax: (39) (80) 5443317.
E-mail: csmmal05@area.ba.cnr.it

Abbreviations: mt, mitochondrial; Cbl, cobalamin; MeCbl, methylcobalamin; AdoCbl, adenosylcobalamin; MCM, methylmalonyl-CoA mutase; MMA, methylmalonic aciduria; Hcys, homocystinuria; HCCL, hydroxycobalamin[c-lactam]

PII: S0014-5793(98)00857-6

mM KCl. The enzyme was added after an initial denaturation step at 95°C for 5 min. The reaction was performed with 1 min denaturation at 95°C, 1 min annealing at a temperature specific for each couple of primers and 1–2 min extension at 72°C. After 25 cycles of amplification, the samples were incubated at 72°C for 10 min. The primers used were the following: ND1-For (L-3007–3023)/3.5-Rev (H-3538–3520) for probe P1; Dloop-For (L-534–553)/12S-Rev (H-1696–1677) for probe P2; 5.3-For (L-5317–5333)/CO I-Rev (H-7608–7588) for probe P3; CO II-For (L-7392–7410)/ATP1B (H-8628–8608) for probe P4; 11.7-For (L-11710–11728)/13.9-Rev (H-13950–13932) for probe P5; 13.6-For (L-13693–13711)/15.2-Rev (H-15235–15216) for probe P6. Human mtDNA nucleotide positions are according to Anderson et al. [12]. Amplification products were resolved on a 1% low melting agarose gel and visualized by ethidium bromide staining. The bands of interest were excised from the gel and eluted with Qiaquick Gel Extraction Kit (Qiagen). Total human mt DNA was purified from HeLa cells by CsCl-ethidium bromide centrifugation.

2.3. Extraction and analysis of mtDNA from cultured human fibroblasts

Cultured fibroblasts, grown to pre-confluence on 175 cm^2 ml flasks, were collected and washed with phosphate buffered saline (PBS). Cells (about 1×10^7) were resuspended in 600 μl of 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 25 mM EDTA pH 7.4 (RSB) and DNA was extracted as described by Lezza et al. [13]. For Southern blot hybridization, 15 μg of total DNA (quantified by adsorption at 260 nm) was digested with *Pvu*II, and loaded on a 0.8% agarose gel in TBE buffer (90 mM Tris-borate pH 8.0, 90 mM boric acid, 2 mM EDTA). After electrophoresis the gel was blotted onto a nylon membrane (Hybond-N, Amersham) and hybridized overnight at 65°C with 1–10 ng of labelled mitochondrial probe and with 10–100 ng of labelled 18S rDNA probe. The mitochondrial probe consisted of three fragments obtained by PCR by using the primers: 11.7-For/13.9-Rev; 5.3-For/CO I-Rev; D-Loop-For/12S-Rev. The nuclear DNA probe was a 413 bp fragment (kind gift of Dr. M. Zeviani) subcloned in pA vector (ClonTech), containing nuclear-encoded 18S rDNA sequences [14]. Both probes were labelled by random priming [15] with the Boehringer labelling kit with 50 μCi of [α-^{32}P]dATP (3000 Ci/mmol) to a final specific activity of $1–2\times10^8$ cpm/μg DNA. Blotting, prehybridization, hybridization and washing were carried out as described by Sambrook et al. [16]. The filter was exposed to a X-ray film at −70°C with an intensifying screen and the hybridization signals were quantified by densitometry with a LKB-Pharmacia Ultrascan-XL Laser densitometer equipped with a GelScan-XL-Evaluation software. In all the experiments, the filters were analyzed also by phosphorimaging with a Betascope 603 blot analyzer (Betagen). The two methods of analysis produced similar results.

2.4. Extraction and analysis of mtRNA from cultured human fibroblasts

Total RNA was isolated from about 1×10^7 pre-confluent cells by the guanidinium isothiocyanate-phenol extraction method [17]. Poly-(A$^+$) and poly(A$^-$) RNA were purified with the Dynabeads mRNA purification kit (Dynal AS, Oslo), following the producer's instructions. 10 μg of total RNA from each sample (or the corresponding amount of poly(A$^+$) RNA) was loaded on a 6.6% formaldehyde-1.2% agarose horizontal gel in 20 mM 3-(*N*-morpholino) propane sulfonic acid, 1 mM EDTA, 1 mM sodium acetate pH 7.0 (MOPS buffer), run at 3 V/cm. After the run, the gel was rinsed in water, equilibrated in 20×SSC and capillary transferred onto a nylon membrane (Hybond-N, Amersham). RNA was fixed to the membrane by baking for 2 h at 80°C and hybridized at 65°C with 100 ng of a denatured mt probe, labelled by random priming [15] with [α-^{32}P]dATP at a specific activity of $1.5–2\times10^8$ cpm/μg. Blotting, prehybridization, hybridization and washing were carried out as described by Sambrook et al. [16]. Filters were exposed for autoradiography and hybridized bands were analyzed by densitometry and phosphorimaging as above. The two methods of analysis produced similar results. Since in some experiments the same blot was used for successive hybridizations, the probe was removed by incubating the filter in a solution of 0.1% SDS for 20 min at 95°C.

2.5. Mitochondrial respiratory activities

Polarographic and spectrophotometric studies on cell lines isolated from skin fibroblasts were carried out as described elsewhere [18].

2.6. Data analysis

Hybridization data are expressed as percent of the content of each mRNA species with respect to the control. They were statistically analyzed by Student's *t*-test, with $P<0.05$ considered statistically significant. Estimation of the molecular weight on Northern blots was done using a log (size) versus mobility (cm) linear standard curve based on the mobility of size standards. These were the two cytoplasmic rRNAs 28S and 18S (5.02 kb and 1.87 kb) and the mature mtRNA species present in each gel.

3. Results

Studies on the mt genetic system in human MMA fibroblasts were performed by using cell lines GM00595 and GM10011. The GM00595 cell line derived from a *cbl*A patient with a intramitochondrial defect of AdoCbl synthesis [19]; the GM10011 fibroblasts were from a *cbl*C patient with a cytosolic defect which caused impairments of both AdoCbl and MeCbl synthesis [2]. In order to rule out variations between individual fibroblast cell lines, three cell lines from healthy individuals having the same age as the others were used as control in all the experiments. In general, in order to obtain about 1×10^7 cells two or three passages were necessary; in all cases no detectable influence of successive passages on the hybridization patterns was observed. Similarly, no detectable and reproducible differences in the growth rate between control and MMA cell lines even during successive passages were observed.

3.1. Mitochondrial DNA in human fibroblasts with MMA

The content of mtDNA from control and MMA fibroblasts was measured by digesting total cellular DNA with *Pvu*II and blot-hybridizing with a mt and a nuclear probe (Fig. 1). The mt probe detected a band of about 16.5 kb corresponding to linearized mtDNA, whereas the nuclear probe detected a 12 kb band corresponding to a *Pvu*II fragment of the nuclear rDNA. The ratio of the intensities of the two bands allowed to estimate the relative amount of the mtDNA in each cell line. Fig. 1B shows that the GM00595 cell line has a mtDNA/cell content about two-fold that of the control. No substantial variation in the mtDNA/cell was found for GM10011.

3.2. Mitochondrial transcripts in human MMA fibroblasts

The pattern of mt transcripts in MMA fibroblasts was analyzed by Northern blot hybridization (Fig. 2). The combined use of six mt probes allowed the detection of most of the mt transcripts, identified by comparison with blots of poly(A$^+$) and poly(A$^-$) RNA from control fibroblasts, hybridized with total mtDNA (Fig. 2, first panel). Probe P1 detected the 16S rRNA, the ND1 mRNA and an additional band (A1) of about 2.6 kb, having a size similar to that of 16S rRNA-$tRNA_{UUR}^{Leu}$-ND1 polycistronic transcript. This species corresponds to the RNA 19, described by Koga et al. [20] in human cells harboring the MELAS point mutation in the $tRNA^{Leu}{}_{UUR}$ gene, which caused a transcription termination deficiency at the 3′ end of 16S rRNA. Other additional bands may be ascribed to 16S rRNA degradation products, as they were identified by comparing this blot with that obtained by hybridizing the RNA with a probe containing the 16S rRNA gene only (data not shown). Probe P2 detects the 12S rRNA only. The probe P3 identifies the mature mRNAs for ND2 and CO I and an additional band of about 1.8 kb (A2) which probably represents a transcript containing CO I mRNA and

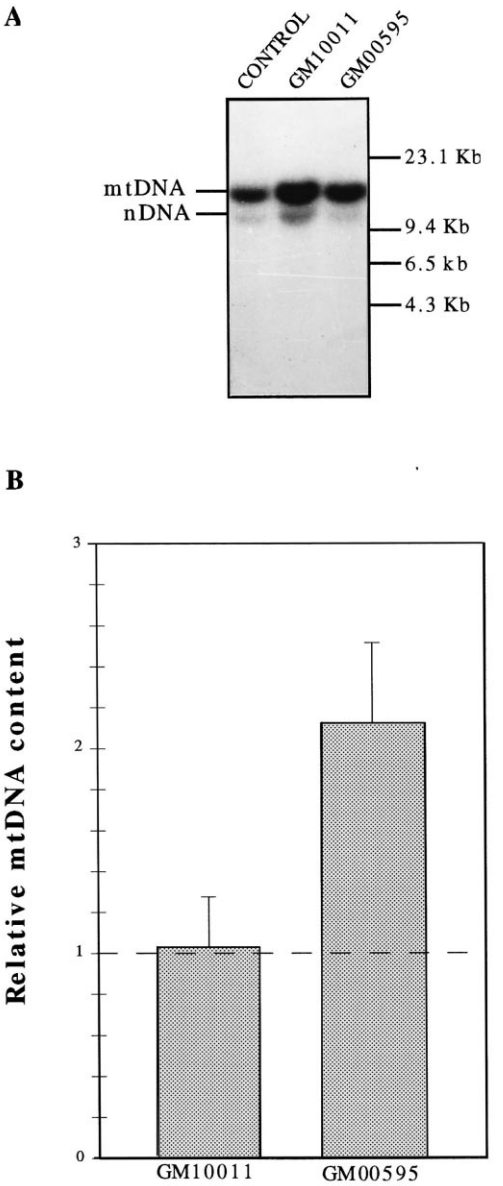

Fig. 1. Mitochondrial proliferation in human MMA fibroblasts. A: Southern blot hybridization between DNA extracted from two MMA and one control fibroblast cell lines to nuclear DNA (nDNA) and mtDNA probes. Probe identification and hybridization conditions are reported in Section 2. Positions of molecular weight markers (λ DNA×*Hin*dIII) are indicated at the right of the gel. B: Variation of the mtDNA/nDNA ratio between patient and control fibroblasts. The data were obtained by comparing, in three distinct blots, the two MMA cell lines with each control at a time. In the ordinate is reported the ratio between mtDNA and nDNA bands in the two MMA cell lines (GM00595 and GM10011) normalized with respect to the control fibroblasts (indicated by the broken line). Bars are the mean ± S.E.M. of at least four experiments each performed on mtDNA from fibroblasts isolated after 2–3 passages. Student's *t*-test statistical analysis showed that the variation observed for GM00595 ($P < 0.001$) was statistically significant.

a piece of the H-strand complementary to four L-strand coded tRNAs. This band corresponds to the RNA 6 previously described by Attardi [21] and it is visible also in the blot of poly(A^+) RNA with total mt DNA (Fig. 2, first panel: band named pCO I). Probe P4 lights up the mRNAs for CO II and ATPase 6/8 and an additional band (A3) whose size is similar to that of the polycistronic transcript covering both genes. Probe P5 identifies the mRNAs for ND4 and ND5 and probe P6 detects the mature mRNAs for cyt *b* and ND5. Longer exposure of these last two blots did not reveal any other bands except two bands with P6, which have the same size of the cytosolic rRNAs 18S and 28S that probably represent non-specific hybridization signals.

The quantitation relative to 18S and 28S rRNAs of mature and high molecular weight mtRNAs in MMA cells shows that in both MMA fibroblasts, the content of 16S and 12S rRNAs decreased by about 50% (Fig. 3A,B). As regards the mRNAs, in GM10011 they did not change significantly. In GM00595 the mRNAs for ND1, ND2 and CO I did not vary, whereas those transcribed after CO I increased about two-fold compared to control. Precursor/mature RNA ratios between MMA and control cell lines were also estimated, but they did not differ significantly (results not shown). In order to evaluate the effect of Cbl disorders on mt transcription, the steady state level of mtRNA species in control and MMA cells was next normalized with respect to their mtDNA content. While this did not affect the results obtained for GM10011 (compare Fig. 3 panels C and A), in GM00595 cells the decline of the two rRNAs reached 70% and the mRNAs for ND1, ND2 and CO I decreased significantly (Fig. 3D). The level of the other mRNAs was similar to control values.

3.3. Activity of mt respiratory enzymes

Cell respiration was polarographically measured using intact cells. Polarographic studies of mitochondrial substrate oxidation using digitonin (0.02%)-permeabilized cells were carried out by using succinate, pyruvate plus malate and glycerol-3-phosphate as substrate. We measured state 3 rate of oxidation in the presence of ADP, state 4 rate of oxidation in the presence of oligomycin and uncoupled rate of oxidation in the presence of m-Cl-CCP (carbonylcyanide *m*-chlorophenylhydrazone. In all cases no substantial decrease in patient cells as compared to control was apparent (data not shown). We also carried out a detailed spectrophotometric assessment of respiratory chain complexes: these experiments revealed normal activities in MMA cells for complexes II (malonate-sensitive succinate decylubiquinone dichlorophenolindophenol reductase), III (antimycin-sensitive decylubiquinol cytochrome *c* reductase) and IV (cytochrome *c* reductase).

4. Discussion

The data reported in this paper show that defects in the Cbl coenzyme biosynthesis are associated with alterations of mtDNA and mtRNA content in human fibroblasts. The mtDNA level increased two-fold only in GM00595 cells, carrying a mt defect in the AdoCbl synthesis. Considering the mtDNA-normalized content of mtRNAs (Fig. 3C,D), the level of the two rRNAs 16S and 12S decreased significantly in both GM00595 and GM10011, whereas ND1, ND2 and CO I mRNAs decreased in GM00595, but were unchanged in GM10011.

The doubling of the mtDNA, which takes place only in those cells (GM00595) in which there is a greater decrease of the level of the two rRNAs (the residual level was about 30% of the control value) and a decrease of the level of some mt mRNAs (compare panels B and D of Fig. 3), brings back

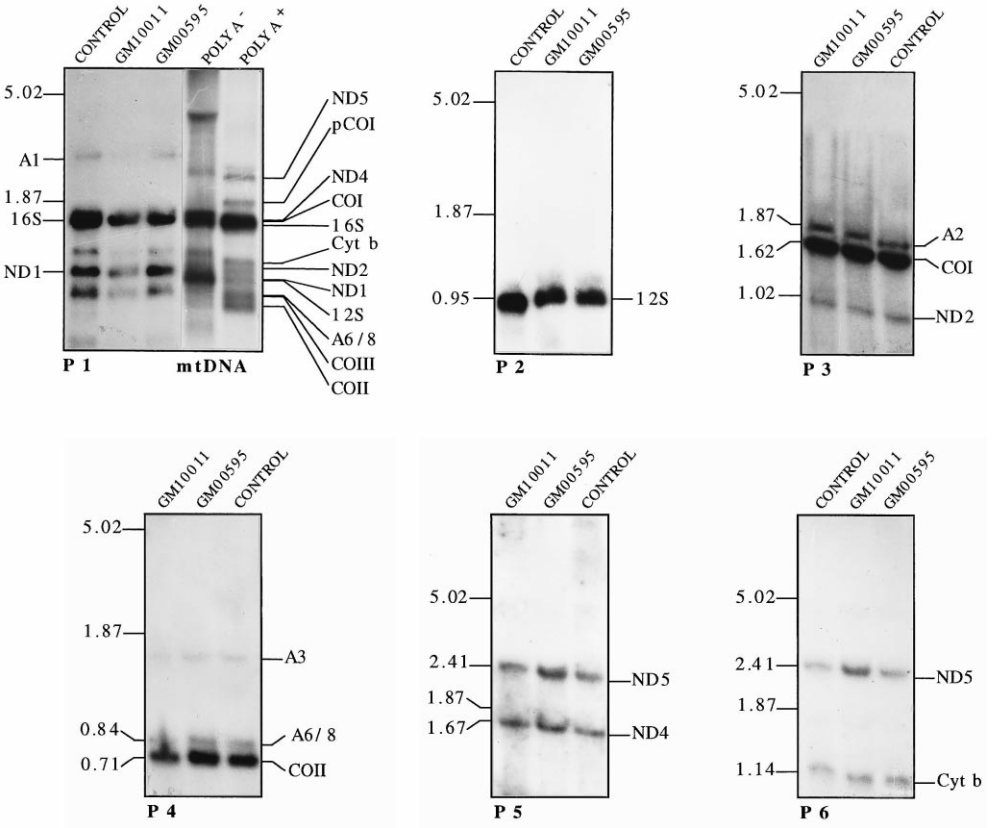

Fig. 2. Northern blot hybridization of RNA from control and patient fibroblasts with different probes. The top panels show the hybridization pattern of RNA from control and MMA cells (GM00595 and GM10011) with probes P1–P6. At the left of each panel are indicated the sizes of 28S and 18S rRNAs (5.02 kb and 1.87 kb) and of mature mtRNA species. The positions of high molecular weight RNAs (A1–A3) are also reported. The last two lanes of the first panel show the hybridization pattern of poly(A^+) and poly(A^-) RNA from control fibroblasts with total labelled mtDNA. The bottom panels show the ethidium bromide staining of the gels used for the hybridization, indicating the 28S and 18S rRNA bands used for normalization. CO I, II, III: subunits I, II and III of cytochrome *c* oxidase; ND1-6: subunits of NADH dehydrogenase; ATPase 6/8: subunits 6 and 8 of FoATP synthase; Cyt b: apoprotein of cytochrome *b*.

the cellular level of these transcripts near to control values. Mitochondrial proliferation has been reported as a common compensatory mechanism in several cases of mt disorders, including that caused by the Cbl antagonist hydroxycobalamin[c-lactam] [22,23]. In MMA cells the accumulation of intermediates of propionic acid metabolism such as propionyl-CoA and methylmalonyl-CoA, might have altered the intramitochondrial environment [1,2] probably inducing a signal to the nucleus to activate mtDNA replication. This should bring the level of the two rRNAs above the threshold value, restore the mt function at optimal levels and then explain the invariance of the activity of mt respiratory enzymes in MMA cells (see Section 3.3). This hypothesis is supported by recent work on the retrograde regulation of mt function in many systems [24,25] and by early data showing that the expression of mt genome is strictly dependent on the level of rRNAs [26–28]. The differential behavior of the two cell types studied here might be related to the fact that a mt protein (the mt cobalamin reductase) is only affected in GM00595, whereas in the GM10011 cells the blockade takes place in the cytosol and only afterwards affects the AdoCbl synthesis. This might cause only a partial reduction of mt transcripts which would be maintained above the threshold level.

The alteration of the level of mt transcripts in MMA cells, probably occurs by different mechanisms. The decrease of the two mt rRNAs in both MMA cell lines, likely depends on an effect at transcriptional level, since both rRNAs are decreased at a similar extent and rRNA synthesis is directed by an independent transcription unit. Conversely, the selective changes of a specific cluster of mRNAs cannot result from a general change of transcription, since the synthesis of the H-strand coded mt mRNAs is dependent on a single promoter (for review see [21,27,28]). Transcriptional down-regulation of a limited region of mtDNA (from 12S to CO I) could be due to transcriptional attenuation or post-transcriptional regulation mechanisms, including altered transcript processing or variation in mRNA stability. The possibility that alterations in vitamin B12 coenzyme metabolism affect the mt RNA processing is excluded by the absence of significant differences in the ratios of RNA precursors to mature transcripts, between patient and control fibroblasts (results not shown). Therefore, the change in the level of mt mRNAs, observed in MMA cells, probably depends on a segmental difference in their stability. Estimates of the rate of degradation of individual mt mRNA species from HeLa cells and rat liver indicated that most of them have a similar stability [26,29]. However, it has been occasionally reported that the rate of mt mRNA degradation can change. An early observation of England et al. [30] explained the persistence of mt protein synthesis in anucleated, transcriptionally inhibited African green monkey cells, by a stabilization of mt mRNAs. More recently Ostronoff et al. [31] showed an increase in the steady state level of

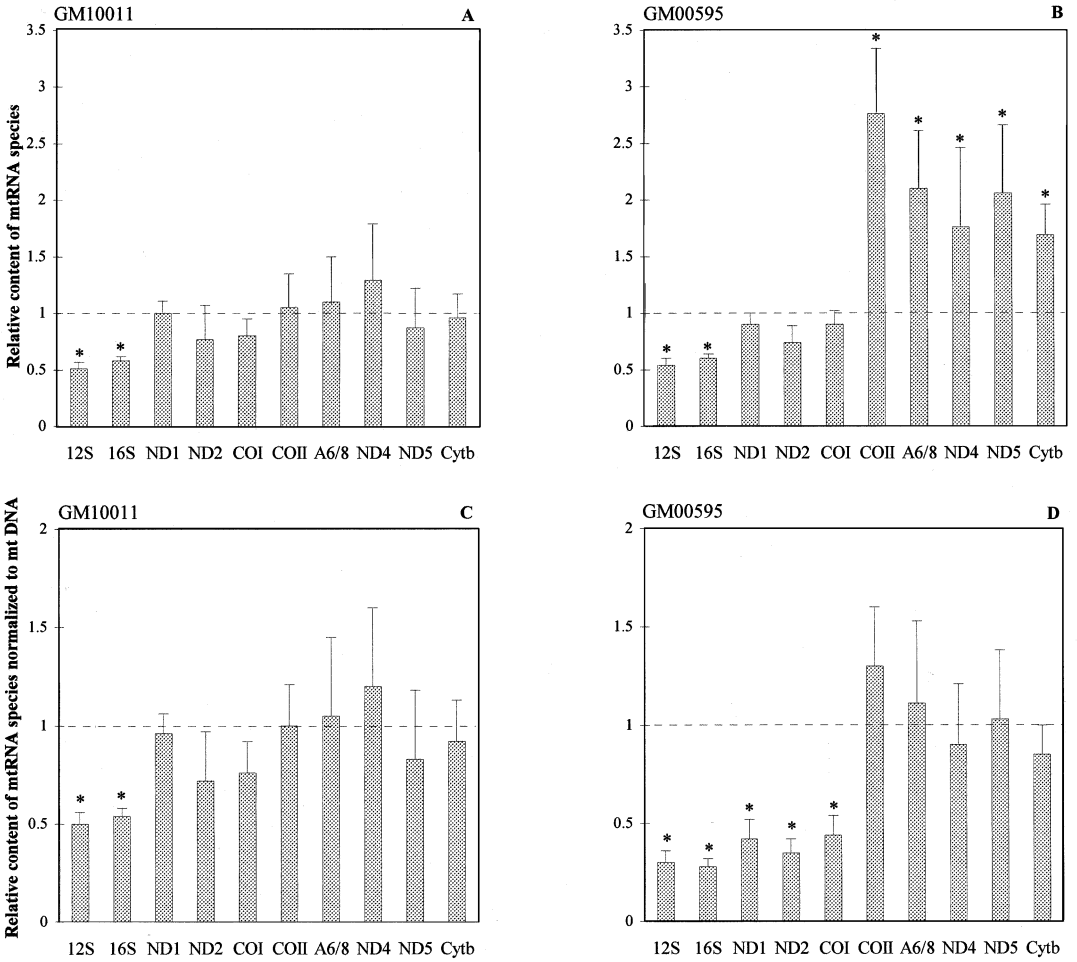

Fig. 3. Relative steady state level of mtRNAs in patient fibroblasts. The data were obtained by comparing, in three distinct blots, the two MMA cell lines with each control at a time. Panels A and B report the content of each mt transcript (normalized for the 28S+18S rRNA) in MMA fibroblasts relative to that of control cells (indicated by a broken line). Panels C and D show the relative content of each mt transcript in MMA fibroblasts, normalized for the mtDNA. Values plotted are the mean ± S.E.M. of four experiments each performed on mtDNA from fibroblasts isolated after 2–3 passages. (*$P < 0.05$, patient-control comparison).

ATPase 6/8 mRNA in developing rat liver resulting from a profound change in the stability of the mt transcripts. Specific modulation of transcription in discrete regions of mt genome has been described in several systems. They include: (a) human cells treated with pyrimethamine [32], (b) human adenocarcinoma cells treated with trehalose [33], (c) malignant breast tissues, [34], (d) adrenal cortex cells stimulated with adrenocorticotrophic hormones [35] and (e) Daudi cells treated with interferon [36].

Acknowledgements: This work was made with the financial support of Telethon Italy (Grant E192). We thank V. Cataldo for expert technical assistance.

References

[1] Fenton, W.A. and Rosenberg, L.E. (1989) in: The Metabolic Basis of Inherited Diseases (Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D., Eds.), pp. 2075–2082, McGraw-Hill, New York.
[2] Qureshi, A.A., Rosenblatt, D.S. and Cooper, B.A. (1994) Crit. Rev. Oncol. Hematol. 17, 133–151.
[3] Stumpf, D., McAfee, J., Parks, J.K. and Eguren, L. (1980) Pediatr. Res. 14, 1127–1131.
[4] Nakai, A., Shigimatsu, Y., Saito, M., Nikawa, Y. and Sudo, M. (1991) Pediatr. Res. 30, 5–10.
[5] Di Lisa, F., Menabo, R., Barbato, R. and Siliprandi, N. (1994) Am. J. Physiol. 267, H455–H461.
[6] Toyhoshima, S., Watanabe, F., Saido, I., Miyatake, K. and Nakano, Y. (1995) J. Nutr. 125, 2846–2850.
[7] Koga, Y., Yoshino, M. and Yamashita, F. (1984) J. Inher. Metab. Dis. 7, 141–142.
[8] Gadaleta, M.N., Petruzzella, V., Renis, M., Fracasso, F. and Cantatore, P. (1990) Eur. J. Biochem. 187, 501–506.
[9] Gadaleta, M.N., Petruzzella, V., Fracasso, F., Fernandez-Silva, P. and Cantatore, P. (1990) FEBS Lett. 277, 191–193.
[10] Wiesner, R.J., Kurowski, T.T. and Zak, R. (1992) Mol. Endocrinol. 6, 1458–1467.
[11] Martin, G.M. (1973) in: Tissue Culture Methods and Application (Kruse, P.F. and Patterson, M.K. Jr., Eds.), pp. 39–43, Academic Press, New York.
[12] Anderson, S., Bankier, A.T., Barrel, B.G., de Bruijn, M.H.I., Coulson, A.R., Drouin, J., Eperon, I.C., Nierlich, D.P., Roe, B.A., Sanger, F., Schreier, P.H., Smith, A.J.H., Staden, R. and Young, I.G. (1981) Nature 290, 457–465.
[13] Lezza, A.M.S., Boffoli, D., Scacco, S., Cantatore, P. and Gadaleta, M.N. (1994) Biochem. Biophys. Res. Commun. 205, 772–779.
[14] Arnaudo, E., Dalakas, M., Shanske, S., Moraes, C.T., Di Mauro, S. and Schon, E.A. (1991) Lancet 337, 508–510.
[15] Feinberg, A.P. and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13.

[16] Sambrook, J., Fritsch, E.F. and Maniatis, T., (1989) Molecular Cloning. A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[17] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[18] Rustin, P., Cretien, D., Bourgeron, T., Gerard, B., Rötig, A., Saudubray, J.M. and Munnich, A. (1994) Clin. Chim. Acta 228, 35–51.
[19] Watanabe, F., Saido, H., Yamaji, R., Miyatake, K., Isegawa, Y., Ito, A., Rosenblatt, D.S. and Nakano, Y. (1996) J. Nutr. 126, 2947–2951.
[20] Koga, Y., Davidson, M., Schon, E.A. and King, M.P. (1993) Nucleic Acids Res. 21, 657–662.
[21] Attardi, G. (1981) Trends Biochem. Sci. 6, 86–89.
[22] Leeds, F.S. and Brass, E.P. (1994) J. Biol. Chem. 269, 3947–3951.
[23] Moraes, C.T., Sciacco, M., Ricci, E., Tengan, C.H., Hao, H., Bonilla, E., Scon, E.A. and Di Mauro, S. (1995) Muscle Nerve 3, 150–153.
[24] Scarpulla, R.C., Kilar, M.C. and Scarpulla, M.K. (1996) J. Biol. Chem. 261, 4660–4662.
[25] Parikh, V.S., Morgan, M.M., Scott, L.S., Clements, L.S. and Butow, R.A. (1987) Science 235, 576–580.
[26] Cantatore, P., Flagella, Z., Fracasso, F., Lezza, A.M.S., Gadaleta, M.N. and De Montalvo, A. (1987) FEBS Lett. 213, 125–132.
[27] Cantatore, P. and Saccone, C. (1987) Int. Rev. Cytol. 108, 149–208.
[28] Attardi, G.A. and Schatz, G. (1988) Annu. Rev. Cell Biol. 4, 289–333.
[29] Gelfand, R. and Attardi, G. (1981) Mol. Cell. Biol. 1, 497–511.
[30] England, J.M., Costantino, P. and Attardi, G. (1978) J. Mol. Biol. 119, 455–462.
[31] Ostronoff, L.K., Izquierdo, J.M. and Cuezva, J.M. (1995) Biochem. Biophys. Res. Commun. 217, 1094–1098.
[32] Sprecher, H., Barr, H.M., Slotky, J.I., Tzukerman, M., Eytan, G.D. and Assaraf, Y. (1995) J. Biol. Chem. 270, 20668–20676.
[33] Lu, X., Walker, T., MacManus, J.P. and Seligy, V.L. (1992) Cancer Res. 52, 3718–3725.
[34] Sharp, M.G.F., Adams, S.M., Walker, R.A., Brammar, W.J. and Varley, J.M. (1992) J. Pathol. 168, 163–168.
[35] Raikhinstein, M. and Hanukoglu, I. (1993) Proc. Natl. Acad. Sci. USA 90, 10509–10513.
[36] Lou, J., Anderson, S.L., Xing, L. and Rubin, B.Y. (1994) J. Interferon Res. 14, 33–40.