Comparative Proteomics as a New Tool for Exploring Human Mitochondrial tRNA Disorders†

Thierry Rabilloud,‡ Jean-Marc Strub,§ Nathalie Carte,§ Sylvie Luche,‡ Alain Van Dorsselaer,§ Joël Lunardi,‡ Richard Giegé,|| and Catherine Florentz*,||

Institut de Biologie Moléculaire et Cellulaire, UPR 9002 du CNRS, 15, rue René Descartes, F-67084 Strasbourg Cedex, France, CEA-Laboratoire de Bioénergétique Cellulaire et Pathologique, UA 2019 DBMS/BECP, 17, rue des Martyrs, F-38054 Grenoble Cedex 9, France, and Laboratoire de Spectrométrie de Masse Bio-organique, UMR 7509 ULP-CNRS, Ecole de Chimie Polymères et Matériaux, 25 rue Bequerel, F-67087 Strasbourg Cedex 2, France

Received July 16, 2001; Revised Manuscript Received September 20, 2001

ABSTRACT: More than 70 different point mutations in human mitochondrial tRNA genes are correlated with severe disorders, including fatal cardiopathies, encephalopathies, myopathies, and others. So far, investigation of the molecular impact(s) of mutations has focused on the affected tRNA itself by seeking structural and/or functional perturbations capable of interfering with synthesis of the 13 mitochondrion-encoded subunits of respiratory chain complexes. Here, a proteomic approach was used to investigate whether such mutations would affect the pattern of mitochondrial proteins at a broader level. Analysis of several hundred mitochondrial proteins from sibling cybrid cell lines by two-dimensional electrophoresis, an approach that takes into account all regulatory steps of mitochondrial and nuclear gene expression, indeed reveals a number of up- and downregulated proteins when healthy and single-point-mutation-carrying mitochondria representative of either MELAS or MERRF syndrome were compared. Assignment by mass spectrometry of the two proteins which exhibit obvious large quantitative decreases in the levels of both pathologic mitochondria identified nuclear-encoded subunits of cytochrome *c* oxidase, a respiratory chain complex. This clearly shows a linkage between the effects of mutations in mitochondrial tRNA genes and the steady-state level of nuclear-encoded proteins in mitochondria. It opens new routes toward a large-scale exploration of potential proteic partners involved in the genotype−phenotype correlation of mitochondrial disorders.

The primary role of mitochondria is to provide cellular energy to living systems (*1*). Production of energy in the form of ATP requires four transmembrane respiratory chain complexes along which electrons are shuttled to oxygen. ATP synthase, also a transmembrane complex, phosphorylates ADP into ATP. A few subunits of these complexes (13 proteins for human mitochondria) are encoded by the mitochondrial genome and are synthesized within mitochondria, whereas the approximately 70 remaining subunits are encoded by the nuclear genome, synthesized in the cytosol, and imported into mitochondria. Besides these, several hundred additional proteins of importance to mitochondrial functions are nuclear-encoded and imported. These proteins also contribute to the import mechanism itself, to the maintenance and expression of the mitochondrial genome, and to various aspects of mitochondrial metabolism. Interestingly, the mitochondrial translation machinery, specifically devoted to the synthesis of the 13 respiratory chain subunits, functions with a set of 22 transfer RNAs (tRNA)[1] and two ribosomal RNAs encoded by the mitochondrial genome, and with all other factors imported from the cytosol (tRNA maturation and modification enzymes, aminoacyl-tRNA synthetases, ribosomal proteins, translation factors, etc.). Thus, mitochondrial function in general, and mitochondrial protein biosynthesis in particular, depend on the conjugated and coordinated expression of both mitochondrial and nuclear genomes.

The mitochondrial genome, and especially mitochondrial tRNA genes, are very sensitive to mutations (*2*, *3*). More than 70 different mutations have been reported over the past 10 years all over the human tRNA genes, correlated with various neuromuscular and neurodegenerative disorders. These include fatal cardiopathies, encephalopathies, myopathies, as well as deafness or diabetes, and others (*4*, *5*). Disease-related mitochondria display a decreased respiration capacity and decreased activities of respiratory chain complexes. Considering the central role of tRNA in protein biosynthesis (*6*), and hence in the synthesis of mitochondrially encoded respiratory chain subunits, it is believed that

† This investigation was supported by Association Française contre les Myopathies (AFM), European Community Grant QLG2-CT-1999-00660, and "Programme Physique-Chimie du Vivant 2000" from the Centre National de la Recherche Scientifique (CNRS).

* To whom correspondence should be addressed. Telephone: 33 3 88 41 70 59. Fax: 33 3 88 60 22 18. E-mail: C.Florentz@ibmc.u-strasbg.fr.

‡ UA 2019 DBMS/BECP.

§ UMR 7509 ULP-CNRS.

|| UPR 9002 du CNRS.

[1] Abbreviations: tRNA, transfer ribonucleic acid; COX, cytochrome *c* oxidase; MELAS, mitochondrial encephalopathy with lactic acidosis and stroke-like episodes; MERRF, mitochondrial myopathy with ragged red fibers.

10.1021/bi0114776 CCC: $22.00
Published on Web 12/07/2001

the decreased respiratory chain activities are due to the lower abundance of mitochondrial subunits or to the presence of nonfunctional subunits (*4*). So far, investigations toward unraveling the cellular perturbations induced by point mutations in tRNA genes have focused on possible effects on global mitochondrial protein synthesis and on various aspects of malfunctioning of mutated individual tRNAs. Studies on the most prevalent mutations revealed that mutations indeed may affect the rate of mitochondrial protein synthesis (*7*−*9*), and/or tRNA biogenesis [abnormal transcription (*10*), maturation (*11*), post-transcriptional modification (*12*−*14*), and stability (*15*−*17*)], and/or functional properties such as capacity for aminoacylation (*18*−*20*). In some cases, frameshifting (*21*, *22*) or slower polysome formation (*23*) has been observed. However, the effects vary both qualitatively and quantitatively according to the mutation that is being analyzed and, for the same mutation, according to the nuclear background (different cybrid cell lines, biopsies) (*24*−*26*). This highlights not only the complexity of the effects triggered by mutations in mitochondrial tRNA genes at the level of mitochondrial protein synthesis but also the absence of unifying mechanistic features so far. The situation becomes even worse when the increasing number of mutations to be investigated (*2*) and the random distribution of the mutations over the structural domains of the affected tRNAs are considered, making any functional or structural prediction for a given mutation difficult (*27*). These considerations suggest that new global approaches, besides direct investigations, are required to explore the different levels of molecular effects of individual mutations and the relationship of these effects with the phenotypic expression of the diseases.

To contribute to this understanding, we have explored the feasibility and potential input of comparative mitochondrial proteomics in searching for possible alterations in the protein content in normal and pathogenic mitochondria. MERRF (a myopathy) and MELAS (an encephalomyopathy) are the most prevalent syndromes correlated with point mutations in mitochondrial tRNA genes (*28*). Thus, the model systems investigated here were couples of cybrid cell lines representative of the MERRF syndrome, i.e., a cell line with a wild-type sequence for the $tRNA^{Lys}$ gene and a sibling cell line with an A8344G mutation in this tRNA gene, and of the MELAS syndrome with either the wild-type sequence or an A3243G mutation in the $tRNA^{Leu(UUR)}$ gene. The results show numerous differences between wild-type and mutation-carrying mitochondria, as either up- or downregulated spots in both MERRF and MELAS. On the basis of the specific initial analysis of two different proteins, two downregulated nuclear subunits of a respiratory chain complex, this study validates the comparative proteomic approach as a straightforward and promising tool for exploring mitochondrial disorders.

EXPERIMENTAL PROCEDURES

Cell Lines. The human cell lines investigated here were a kind gift of A. Chomyn and G. Attardi (California Institute of Technology, Pasadena, CA). Cell line R1C3, carrying in homoplasmic form the A to G transition at position 8344 in the mt $tRNA^{Lys}$ gene, associated with MERRF syndrome (*29*), and cell line R2-1A, carrying in homoplasmic form the wild-type version of the $tRNA^{Lys}$ gene, were isolated by transfer of mitochondria from myoblasts of a MERRF patient into human mtDNA-less (rho zero) 206 cells (*8*). The H43B cell line, carrying in nearly homoplasmic form (99%) the A to G transition at position 3243 in the mt $tRNA^{Leu(UUR)}$ gene, associated with the MELAS syndrome, and the H94I cell line, carrying in homoplasmic form the wild-type version of the $tRNA^{Leu(UUR)}$ gene, were obtained in a similar way from myoblasts of a MELAS patient and his maternal aunt (*9*).

Cell Culture and Purification of Mitochondria. Cybrid cell lines R2-1A, R1C3, H94I, and H43B were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum and 100 μg/mL bromodeoxyuridine (BrdU), plated in 10 cm Petri dishes (3.5×10^4 cells/mL for R1-2A and H94I and 8×10^4 cells/mL for R1C3 and H43B), and grown at 37 °C under 5% CO_2 until confluence as described previously (*12*). All media contained 70 μM streptomycin sulfate, 70 μM kanamycin monosulfate, and 500 units of penicillin G per milliliter. Mitochondria were isolated from broken cells according to established procedures, including EDTA treatment for removal of contaminating cytosolic ribosomes (*30*). Cells were collected by trypsinization followed by addition to the cell suspension of $^1/_{10}$ volume of calf serum and centrifugation at $250g_{av}$ for 9 min. After two washes in 130 mM NaCl, 5 mM KCl, and 1 mM $MgCl_2$, the cells were incubated for 3 min in 10 mM Tris-HCl (pH 6.7), 10 mM KCl, and 1.5×10^{-4} M $MgCl_2$ and broken in a Potter-Elvejem homogenizer with a rotating pestle up to ~80% cell breakage. The homogenate was then brought to 0.25 M sucrose and centrifuged for 5 min at $\sim 1200g_{av}$ to remove large debris and nuclei. The mitochondria were collected by centrifugation for 10 min at $8000g_{av}$ and washed in 0.25 M sucrose, 10 mM Tris-HCl (pH 6.7, 25 °C), and 1.5×10^{-4} M $MgCl_2$. A major part of the ribosomes were removed by incubation of the mitochondrial fraction in 0.25 M sucrose, 10 mM Tris-HCl (pH 6.7), and 10 mM EDTA for 10 min at 4 °C, followed by centrifugation at $8000g_{av}$ for 10 min.

Two-Dimensional (2D) Gel Electrophoresis. Mitochondrial proteins (150 μg; quantitated by the Bradford technique on mitochondria) were applied to the first-dimension gel (immobilized pH gradient of IPG, pH 4−8) by in-gel rehydration in 7 M urea, 2 M thiourea, 4% cholamidopropyldiethylammoniopropane sulfonate (CHAPS), 0.4% carrier ampholytes (pH 3−10), and 40 mM dithiothreitol (*31*). Protein-loaded IPG strips were focused for a total of 60 000 Vh (Bio-Rad Protean IEF Cell), equilibrated for 2×10 min in 0.125 M Tris-HCl (pH 7.5), 6 M urea, 30% glycerol, and 2.5% SDS, and supplemented with 60 mM dithiothreitol (first equilibration) or 150 mM iodoacetamide (second equilibration). The second dimension was a 10% acrylamide gel, cast in 0.2 M Tris-HCl (pH 8.1) and electrophoresed in a Tris-taurine (0.05 M Tris, 0.2 M taurine, and 0.1% SDS) electrode buffer containing 0.1% SDS (*32*). This allowed resolution of low-molecular mass proteins down to 6 kDa. After migration, the gels were silver stained according to the method in ref *32*.

Spot Analysis. For spot identification, micropreparative 2D gels were loaded with 500 μg of mitochondrial proteins. These gels were either stained with colloidal Coomassie blue (*33*) or transferred onto PVDF by semidry blotting (*34*). The blots were stained with Coomassie blue. The PVDF or gel

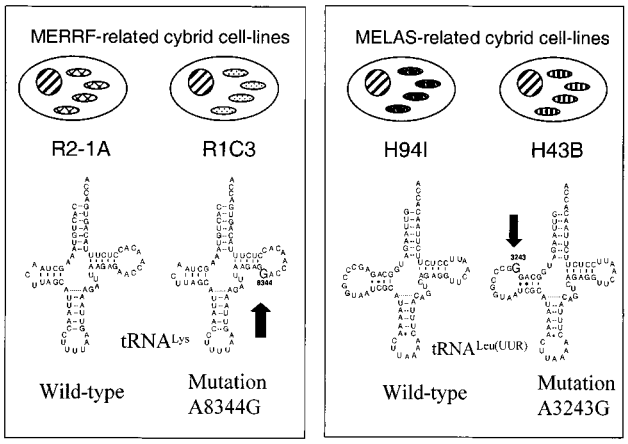

FIGURE 1: Cybrid cell lines that were investigated. The cell lines share pairwise the same nuclear background and differ at the level of a single-point mutation in one of the mitochondrial tRNA genes. R2-1A and R1C3 (left panel) have the wild-type sequences for all 22 tRNA genes, with the exception of $tRNA^{Lys}$. This gene is of wild-type sequence in R2-1A and bears the MERRF-correlated mutation A8344G in R1C3 (*8*). H94I and H43B cell lines (right panel) also have wild-type sequences for all tRNA genes with the exception of the $tRNA^{Leu(UUR)}$ gene. Whereas the sequence of this gene is the wild-type sequence in H94I, it has mutation A3243G related to the MELAS syndrome in H43B (*9*). All cell lines derive from the same parental osteosarcoma 143B cell line and thus have the same nuclear background. Mitochondria originate from MERRF or MELAS patient myoblasts. The cloverleaf structures of the tRNAs of interest are given in both panels. Sequences are derived from the corresponding genes (*46*), and pathogenic mutations are indicated with large type.

pieces of interest were then extensively destained in 40% ethanol and submitted to N-terminal Edman degradation or mass spectrometry analysis (MS), respectively. For MS analysis, the gel pieces were further washed (*35*) and the proteins were digested in-gel with trypsin (18 h). The resulting peptides were extracted with a TFA/acetonitrile/water mixture, and the peptide mixture was analyzed by MALDI-TOF using cyano-4-hydroxycinnamic acid as a matrix on a Bruker Biflex instrument with delayed extraction. Identification of the proteins using these mass fingerprinting data was carried out using the MS-Fit software (http://prospector.ucsf.edu/ucsfhtml3.4/msfit.html).

RESULTS

2D Gel Electrophoresis of Total Mitochondrial Proteins. The sibling cybrid cell lines that were studied, originating from the fusion of mitochondria-devoid host cell lines and enucleated patient cells, have the same nuclear background and differ by a single-point mutation in a specific mitochondrial tRNA gene. They thus represent an ideal experimental model for investigating the effects of the mutation (*36*). Two such paired lines were analyzed here. The first one consists of the wild-type cell line (R2-1A) and the corresponding sibling cell line with $tRNA^{Lys}$ mutation A8344G (R1C3) representative of MERRF syndrome. The second consists of a second wild-type cell-line (H94I) and a cell line with the same nuclear background, but with $tRNA^{Leu(UUR)}$ mutation A3243G in the mitochondrial genome (H43B), representative of MELAS (Figure 1). Mitochondria have been purified from the four cell lines, and their total protein content was analyzed by 2D gel electrophoresis according to established procedures (*32*). Typical silver-stained gels obtained over a pH range

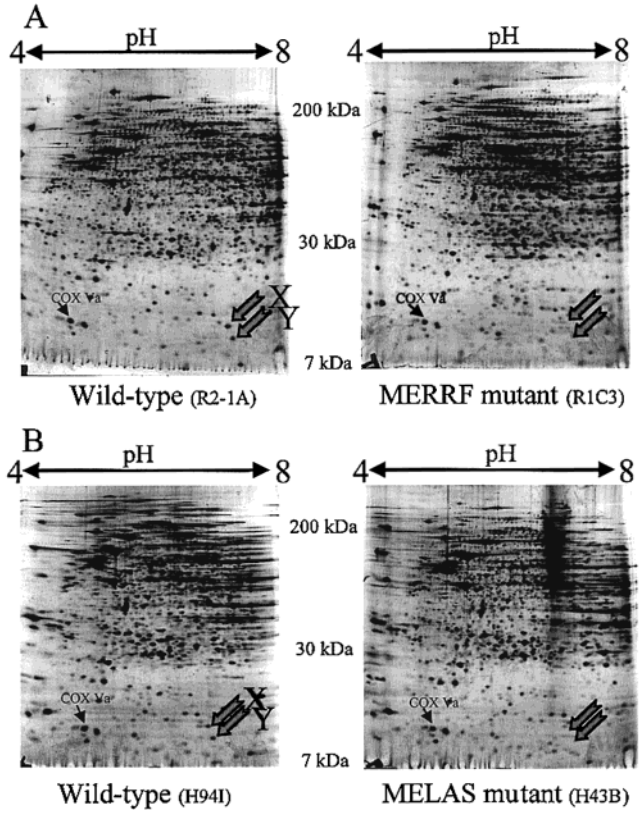

FIGURE 2: Typical 2D gel electropherograms obtained from cybrid mitochondria. Mitochondria have been purified in parallel from paired cell lines [MERRF (A) and MELAS (B)] and solubilized in the presence of a cocktail of detergents and denaturing agents to recover a large proportion of membrane proteins. Proteins have been separated in a first dimension by isoelectrofocusing followed by a second dimension of 10% polyacrylamide gel electrophoresis (see Experimental Procedures). Gels have been silver-stained. Visual comparison of two individual gels within a couple allows detection of numerous up- and downregulated proteins. The two spots undergoing the most drastic decreases in intensity in mutation-carrying mitochondria vs wild-type mitochondria are labeled X and Y. They are localized in the bottom part of the gels, where spots are well separated and easy to extract for assignment by mass spectrometry. The position of COX chain Va is also indicated.

of 4−8 are presented in Figure 2. For all four samples, more than 800 spots were detected. Complementary experiments covering the pH 3−6 and 7−10 ranges reveal only a limited number of additional spots (not shown). In all series of gel couples (four independent experiments for either the MERRF-related or MELAS-related sibling cybrid cell lines), differences can be seen by simple visual inspection of the gels. Although a complete quantitative and qualitative comparison of all pairs of gels awaits computer-assisted analysis, a very striking difference can be detected immediately (and in a reproducible manner) within all couples of wild-type and mutant gels. Whereas a large number of spots are of equal intensity in both wild-type and mutation-carrying mitochondria (especially well seen within the highly resolved bottom part of the gels), two low-molecular mass proteins leading to high intensity are present in both wild-type mitochondria but disappear almost completely in MELAS- and MERRF-related samples (spots X and Y in Figure 2). According to their relative location on the electropherograms, both spots correspond to proteins with a molecular mass of ~10000 Da and a pI in the range of 6.8−7.2.

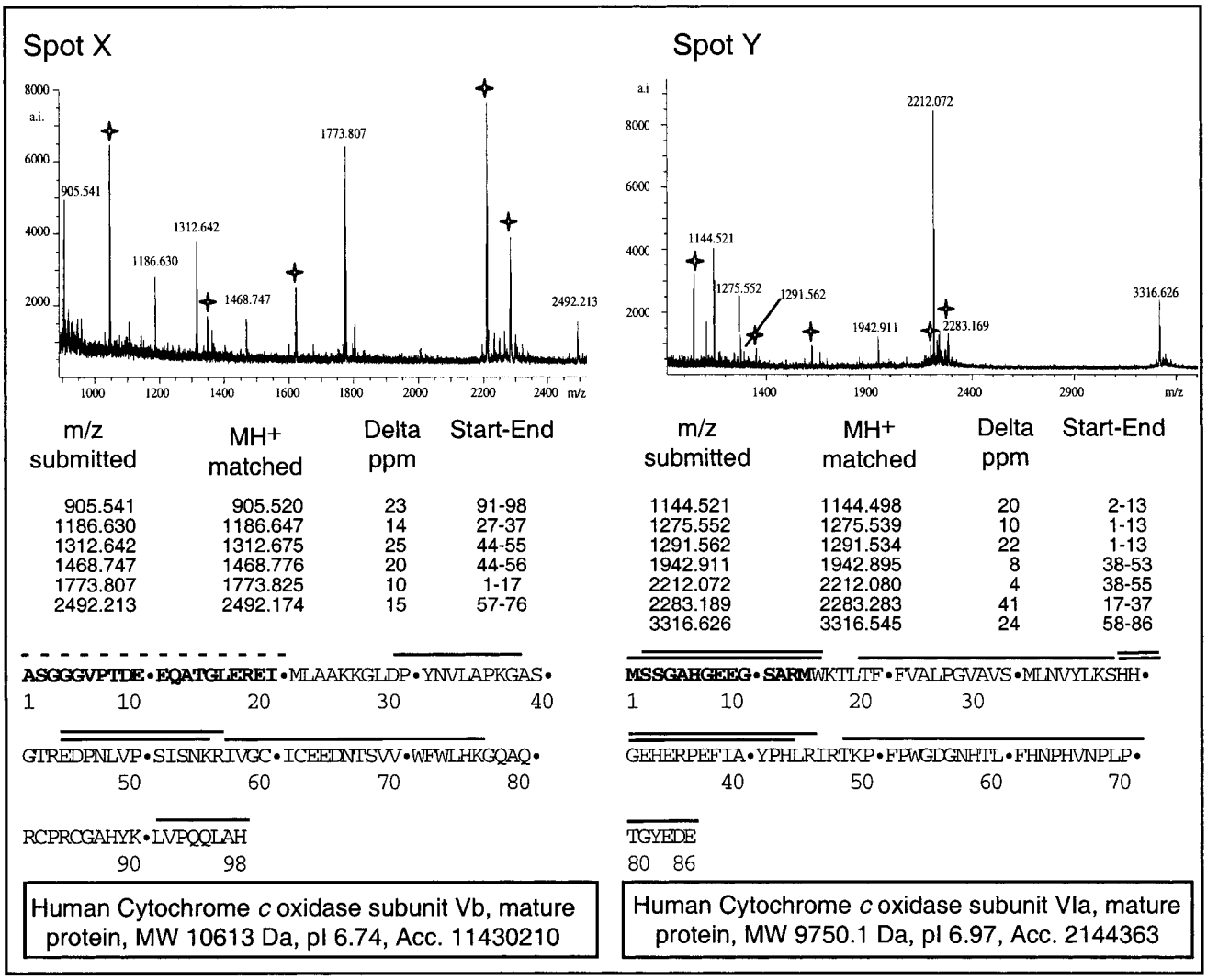

FIGURE 3: Assignment of spots X and Y by mass spectrometry and Edman sequencing of the N-terminus. At the top are MALDI-TOF mass spectra of peptides obtained by trypsic digestion. Stars denote internal mass standards. In the center are final results of the MS-Fit search in the NCBInr database. Peptides from spot X match peptides from cytochrome *c* oxidase chain Vb, and peptides from spot Y match those from cytochrome *c* oxidase chain VIa. Since Edman degradation confirms that both proteins are mature forms of the mitochondrial proteins, the sequences of the mature proteins (deprived of the mitochondrial signal peptide) are given. Bold lines represent the matched trypsic peptides measured by MALDI-TOF; dashed lines highlight the peptides sequenced by the ion trap, and bold amino acids are those detected by Edman sequencing.

Identity of Two Proteins Most Strikingly Reduced in Mutation-Carrying Mitochondria. MALDI-TOF analysis of the peptide digests of spot X, combined with a search of the NCBInr database with MS-Fit software, pointed (although with a poor score) to subunit Vb of cytochrome *c* oxidase (COX-Vb) (Figure 3). Peptides covered 34% of the precursor protein mass. Additional characterization by MS/MS on the ion trap of the nonassigned peptide with a mass of 1773.807 Da, a technique which allows direct access to the sequence of the peptide, allowed unambiguous identification of spot X as COX Vb. The analyzed peptide corresponds to residues 32−48 of the precursor protein (or residues 1−17 of the mature protein). The matched peptides now cover 60% of the protein sequence. Edman sequencing of the N-terminus of the protein present in the spot revealed that the protein was mature; i.e., the mitochondrial targeting peptide signal was absent. It must be noticed, however, that maturation was not homogeneous since 65% of the molecules started with residue 32 (1 in the mature form), 25% with residue 33 (2), and 10% with residue 31 (−1). The theoretical mass of this protein was 10 613 Da and its pI 6.74, which is in excellent agreement with the experimental values that were obtained.

Spot Y was assigned in the same way as cytochrome *c* oxidase subunit VIa (Figure 3). The matched peptides covered 94% of the mature protein. In this case as well, two peptides of 1144.552 and 1942.911 Da have been assigned by MS/MS on the ion trap as fragments of residues 2−13 and 38−53 of the mature COX VIa, respectively. Edman degradation confirmed this finding. The calculated mass and pI of the mature protein are 9750 Da and 6.97, respectively, which are also in good agreement with experimental values.

DISCUSSION

Direct Consequences of Point Mutations in Mitochondrial tRNAs. Mitochondrial tRNAs are of key importance for the synthesis of the 13 mitochondrion-encoded respiratory chain complex subunits, i.e., seven subunits of complex I, one subunit of complex III, three subunits of complex IV, and two subunits of complex V. Since there are no or only a

restricted number of isoaccepting tRNAs (one species for each of 18 amino acids and two species for leucine and serine), the fitness and specificity of each of the 22 tRNAs are of basic importance for mitochondrial translation. Point mutations in human mitochondrial tRNA genes associated with diseases, and in particular the MERRF mutation A8344G and the MELAS mutation A3243G, interfere directly with mitochondrial translation. These two mutations affect the rate and/or quality of synthesis of the respiratory chain subunits (*7*−*9*, *37*). The detailed primary molecular effects of these mutations on the tRNAs themselves are still not completely resolved but include at least incomplete post-transcriptional modification (*12*−*14*), decreased stability (*16*), and inefficient and/or incorrect aminoacylation (*18*, *23*). The restricted availability of mitochondrion-encoded subunits or the occurrence of incorrect subunits accounts for diminished activities of the respiratory chain complexes and diminished respiration capacity of the mitochondria (*4*). As a further consequence, the level of energy production in the cell is decreased. Moreover, because of the variety of biological changes observed in biopsies from patients (size and number of mitochondria, lactic acidosis, increased oxidative stress, altered membrane potential, etc.) and the various phenotypes observed for the different pathogenic mutations, additional molecular consequences of the primary effects of mutations are likely to occur.

Cascade Effects on Nuclear-Encoded Mitochondrial Proteins. Comparative proteomics, combining high-resolution 2D separation of hundreds of proteins and mass spectrometric identification, is the method of choice for revealing unpredictable differences in the protein patterns of biological systems subjected to physiological differences (*38*, *39*). The feasibility of the comparative proteomic approach in the field of human mitochondrial disorders has been investigated here using mitochondria isolated from couples of sibling cybrid cell lines sharing the same nuclear background. With the aim of looking for long-range effects of mitochondrial tRNA single-point mutations on mitochondrial protein content, this was a prerequisite for effective comparison. Although an exhaustive computer-assisted analysis of the 2D electropherograms obtained in this work still needs to be performed, it can already be concluded by visual inspection that single mutations in mitochondrial tRNA genes have long-range cellular consequences such as down- and upregulation of certain proteins. The first two proteins assigned in this study, by a combination of mass spectrometric measurements and Edman degradation, were those undergoing the most obvious decrease when comparing 2D maps from wild-type and mutation-carrying mitochondria. They turned out to be nuclear-encoded proteins, namely, COX Va and COX VIa, two subunits of cytochrome *c* oxidase. The fact that these proteins belong to the mitochondrial compartment and are not contaminants from the cytosol was demonstrated by their proteolytic maturation, i.e., the absence of their mitochondrial location signal peptide.

These results are promising for several reasons. First, they validate the comparative proteomic approach as a tool for exploring tRNA-triggered mitochondrial disorders at a technical level. Second, they also validate the approach on a biological level because a loss in COX enzymatic activity is a common parameter among mitochondrial diseases in general (*40*), a feature even sometimes used to assess a diagnosis (*41*). Third, they offer the possibility for a precise quantitative and qualitative evaluation of affected proteins (within the limit of sensitivity of the detection techniques and database content) by combining 2D mapping, computer-assisted comparative quantification, and mass spectrometry/database searches for assignment. This opens the possibility that proteins which belong to respiratory chain complex subunits will be detected as found here, or perhaps representative proteins involved in compensatory mechanisms. Such phenomena may include overexpression of proteins involved in translation. Detection of variations in the steady-state levels of the 13 mitochondrion-encoded proteins is not expected by this approach because these highly hydrophobic proteins are not resolved on 2D gels (*32*). Unfortunately, at the moment, this also holds true for a number of other membranous proteins.

Analysis of long-range cellular consequences of impaired mitochondrial protein synthesis has been investigated earlier by immunological approaches (*42*). In particular, the fate of respiratory chain complex subunits was followed by specific antibodies after complete inhibition of mitochondrial protein synthesis. Both in rho zero cells (cells with no mitochondrial genome due to extensive treatment with ethidium bromide) and in OP cells (oxidative phosphorylation-deprived mitochondria obtained by total inhibition of mitochondrial protein synthesis by treatment with doxycycline), it was shown that the steady-state levels of several nuclear-encoded subunits undergo dramatic decreases. Our present data refine these results in showing that single-point mutations in two tRNA genes are sufficient to trigger strong long-range consequences as well.

How Are Nuclear-Encoded Cytochrome c Oxidase Subunits Downregulated in MERRF and MELAS Mitochondria? A first possibility would be that import into mitochondria of COX Vb and COX VIa subunits may take place, followed by rapid degradation. It is well-known that disease-carrying mitochondria have an increased level of oxidative stress that can lead to higher rates of degradation of mitochondrial components. However, it is more likely that degradation of the two proteins may be linked to low levels of the three mitochondrion-encoded COX subunits. This would result in nonassembly of the complementary nuclear-encoded subunits, associated with their degradation (*42*, *43*). A second possibility would be that nuclear-encoded subunits are downregulated at either the transcriptional or translational level, in response to low levels of mitochondrial protein synthesis induced by the mutation. This would be a good example of direct cross-talk between the nuclear and the mitochondrial genomes and is currently under investigation. Such regulation would result in a decrease in the level of the cytosolic precursor forms and as a direct consequence in that of the mitochondrial imported mature forms. It is likely that the absence of subunits Vb and VIa would contribute in turn to the low COX activities measured with mutation-carrying mitochondria, and thus to the expression of the disease. Third, other factors that play a role in protein import into mitochondria might be involved in the nonimport of cytochrome *c* oxidase subunits. Finally, several proteins needed for proper assembly of COX (*44*, *45*) may have become altered as a consequence of the pathologic status of the cell.

Perspectives. Although many problems remain unsolved, in particular, the fate of the other nuclear-encoded subunits of COX and of the other respiratory chain complexes, the data presented here highlight the power of a comparative global proteomic approach for investigating the genotype−phenotype relationship in diseases associated with point mutations in mitochondrial tRNA genes. In particular, they demonstrate that long-range consequences of the mutations exist and that proteic partners involved in the mitochondrial disorders can be identified. These may in turn contribute in an essential way to the pathologic status of the host cell. In contrast to immunological techniques, the comparative proteomic approach will allow the investigation at one time of not only a few targeted proteins but of hundreds of proteins of mitochondrial localization that have undergone either qualitative (post-translational modifications, partial degradation) or quantitative changes as a consequence of a single-point mutation. The results of such an investigation will also shed light on the correlation between mitochondrial protein synthesis and the general metabolism of the cell. This will highlight the cascade effects which allow a single mutation in a mitochondrial tRNA gene to down- or upregulate a series of proteins.

Interestingly, these findings, showing a severe decrease in the levels of COX subunits Vb and VIa, are common to at least two independent, clinically distinct diseases associated with mutations within two distinct tRNA genes, namely, the A8344G MERRF mutation and the A3243G MELAS mutation. Furthermore, these two diseases are characterized by the presence of different negative effects of the relevant tRNA mutations on total mitochondrial protein synthesis on both a qualitative and quantitative level (*28*). Comparative proteomics on various couples of mitochondria, either representative of the same diseases but prepared from cell lines with different nuclear backgrounds or from cell lines representative of other mitochondrial disorders, may open new routes toward the comprehension of common nuclear-linked mechanistic pathways underlying the expression of mitochondrial tRNA gene mutations. Further investigations based on computer-assisted systematic qualitative and quantitative analysis of the 2D electropherograms obtained for MERRF and MELAS syndromes are now underway in an effort to further explore these possibilities.

ACKNOWLEDGMENT

We are grateful to Drs. Anne Chomyn and Giuseppe Attardi (California Institute of Technology) for the generous gift of the four cybrid cell lines. We thank Caroline Paulus for excellent technical assistance and Petra Tryoen-Tóth and Louis Levinger for critical reading of the manuscript.

REFERENCES

1. Scheffler, I. (1999) *Mitochondria*, John Wiley & Sons, New York.
2. Kogelnik, A. M., Lott, M. T., Brown, M. D., Navathe, S. B., and Wallace, D. C. (1998) *Nucleic Acids Res. 26*, 112−115.
3. Ingman, M., Kaessmann, H., Pääbo, S., and Gyllenstein, U. (2000) *Nature 408*, 708−713.
4. Wallace, D. C. (1999) *Science 283*, 1482−1488.
5. Schon, E. (2000) *Trends Biochem. Sci.*, 555−560.
6. Söll, D., and RajBhandary, U. L., Eds. (1995) tRNA: Structure, Biosynthesis and Function, pp 1−572, American Society for Microbiology Press, Washington, DC.
7. Seibel, P., Degoul, F., Bonne, G., Romero, N., François, D., Paturneau-Jouas, M., Ziegler, F., Eymard, B., Fardeau, M., Marsac, C., and Kadenbach, B. (1991) *J. Neurol. Sci. 105*, 217−224.
8. Chomyn, A., Meola, G., Bresolin, N., Lai, S. T., Scarlato, G., and Attardi, G. (1991) *Mol. Cell. Biol. 11*, 2236−2244.
9. Chomyn, A., Martinuzzi, A., Yoneda, M., Daga, A., Hurko, O., Johns, D., Lai, S. T., Nonaka, I., Angelini, C., and Attardi, G. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 4221−4225.
10. Bindoff, L. A., Howell, N., Poulton, J., McCullough, D. A., Morten, K. J., Lightowlers, R. N., Turnbull, D. M., and Weber, K. (1993) *J. Biol. Chem. 268*, 19559−19564.
11. Rossmanith, W., and Karwan, R. (1998) *FEBS Lett. 433*, 269−274.
12. Helm, M., Florentz, C., Chomyn, A., and Attardi, G. (1999) *Nucleic Acids Res. 27*, 756−763.
13. Yasukawa, T., Suzuki, T., Suzuki, T., Ueda, T., Ohta, S., and Watanabe, K. (2000) *J. Biol. Chem. 275*, 4251−4257.
14. Yasukawa, T., Suzuki, T., Ishii, N., Ueda, T., Ohta, S., and Watanabe, K. (2000) *FEBS Lett. 467*, 175−178.
15. Hao, H., and Moraes, C. T. (1997) *Mol. Cell. Biol. 17*, 6831−6837.
16. Yasukawa, T., Hino, N., Suzuki, T., Watanabe, K., Ueda, T., and Ohta, S. (2000) *Nucleic Acids Res. 28*, 3779−3784.
17. Kelley, S. O., Steinberg, S. V., and Schimmel, P. (2001) *J. Biol. Chem. 276*, 10607−10611.
18. Enriquez, J. A., Chomyn, A., and Attardi, G. (1995) *Nat. Genet. 10*, 47−55.
19. Degoul, F., Brulé, H., Cepanec, C., Helm, M., Marsac, C., Leroux, J.-P., Giegé, R., and Florentz, C. (1998) *Hum. Mol. Genet. 7*, 347−354.
20. Kelley, S., Steinberg, S., and Schimmel, P. (2000) *Nat. Struct. Biol. 7*, 862−865.
21. Masucci, J. P., Davidson, M., Koga, Y., Schon, E. A., and King, M. P. (1995) *Mol. Cell. Biol. 15*, 2872−2881.
22. Masucci, J. P., and Schon, E. A. (1996) *Mol. Biol. Rep. 22*, 187−193.
23. Chomyn, A., Enriquez, J. A., Micol, V., Fernandez-Silva, P., and Attardi, G. (2000) *J. Biol. Chem. 275*, 19198−19209.
24. Börner, G. V., Zeviani, M., Tiranti, V., Carrara, F., Hoffmann, S., Gerbitz, K. D., Lochmuller, H., Pongratz, D., Klopstock, T., Melberg, A., Holme, E., and Pääbo, S. (2000) *Hum. Mol. Genet. 9*, 467−475.
25. Jacobs, H. T., and Holt, I. J. (2000) *Hum. Mol. Genet. 9*, 463−465.
26. Janssen, G., Maassen, J., and van den Ouweland, J. (1999) *J. Biol. Chem. 274*, 29744−29748.
27. Florentz, C., and Sissler, M. (2001) *EMBO Rep. 2*, 481−486.
28. Schon, E. A., Bonilla, E., and DiMauro, S. (1997) *J. Bioenerg. Biomembr. 29*, 131−149.
29. Villani, G., and Attardi, G. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 1166−1171.
30. Attardi, B., Cravioto, B., and Attardi, G. (1969) *J. Mol. Biol. 44*, 47−70.
31. Rabilloud, T., Adessi, C., Giraudel, A., and Lunardi, J. (1997) *Electrophoresis 18*, 307−316.
32. Rabilloud, T., Kieffer, S., Procaccio, V., Louwagie, M., Courchesne, P., Patterson, P., Martinez, P., Garin, J., and Lunardi, J. (1998) *Electrophoresis 19*, 1006−1014.
33. Neuhoff, V., Arold, N., Taube, D., and Ehrhardt, W. (1988) *Electrophoresis 9*, 255−262.
34. Kyhse-Andersen, J. (1984) *J. Biochem. Biophys. Methods 10*, 203−209.
35. Rabilloud, T., Strub, J., Luche, S., Girardet, J., Van Dorsselaer, A., and Lunardi, J. (2001) *Proteomics 1*, 699−704.
36. King, M. P., and Attardi, G. (1989) *Science 246*, 500−503.
37. Dunbar, D. R., Moonie, P. A., Zeviani, M., and Holt, J. (1996) *Hum. Mol. Genet. 5*, 123−129.
38. Blackstock, W. P., and Weir, M. P. (1999) *Trends Biotechnol. 17*, 121−127.
39. Celis, J., Kruhoffer, M., Gromova, I., Frederiksen, C., Ostergaard, M., Thykjaer, T., Gromov, P., Yu, J., Palsdottir, H., Magnusson, N., and Orntoft, T. (2000) *FEBS Lett. 480*, 2−16.

40. Robinson, B. (2000) *Pediatr. Res. 48*, 581−585.
41. Tanji, K., and Bonilla, E. (2000) *Brain Pathol. 10*, 422.
42. Nijtmans, L., Spelbrink, J., Van Galen, M., Zwaan, M., Klement, P., and Van den Bogert, C. (1995) *Biochim. Biophys. Acta 1265*, 117−126.
43. Nijtmans, L., Taanman, J.-W., Muijsers, A., Speijer, D., and Van den Bogert, C. (1998) *Eur. J. Biochem. 254*, 389−394.
44. Zhu, Z., Yao, J., Johns, T., Fu, K., De Bie, I., Macmillan, C., Cuthbert, A., Newbold, R., Wang, J., Chevrette, M., Brown, G., Brown, R., and Shoubridge, E. (1998) *Nat. Genet. 20*, 337−343.
45. Tiranti, V., Hoertnagel, K., Carrozo, R., Galimberti, C., Munaro, M., Granatiero, M., Zelante, L., Gasparini, P., Marzella, R., Rocchi, M., Bayona-Bafaluy, M., Enriquez, J., Uziel, G., Bertini, E., Dionisi-Vici, C., Franco, B., Meitinger, T., and Zeviani, M. (1998) *Am. J. Hum. Genet. 63*, 1609−1621.
46. Anderson, S., Bankier, A. T., Barrel, B. G., de Bruijn, M. H. L., Coulson, A. R., Drouin, J., Eperon, J. 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.

BI0114776