Association of TCF7L2 variation with single islet autoantibody expression in children with type 1 diabetes.

Redondo, Maria J; Muniz, Jesse; Rodriguez, Luisa M; Iyer, Dinakar; Vaziri Sani, Fariba; Haymond, Morey W; Hampe, Christiane S; Metzker, Michael L; Grant, Struan F A; Balasubramanyam, Ashok

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Biochemical and molecular investigations in respiratory chain deficiencies

P. Rustin*, D. Chretien, T. Bourgeron, B. Gérard, A. Rötig, J.M. Saudubray, A. Munnich

Unité de Recherches sur les Handicaps Génétiques de l'Enfant, INSERM U393, Tour Lavoisier, Hôpital des Enfants-Malades, 149, rue de Sèvres, 75743 Paris Cedex 15, France

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Abstract

This paper describes our present strategy for the investigation of respiratory chain disorders in humans. Because very few of the underlying mutations causing mitochondrial disorders in humans are currently known, biochemical studies constitute a major tool in screening procedures for respiratory chain deficiencies. All biochemical and molecular methods described are scaled-down methods, allowing investigation in both adults and young children. Polarographic studies and/or spectrophotometric studies on whole cells (circulating lymphocytes), isolated mitochondria (skeletal muscle) and tissue homogenates are presented. Advantages and limitations of each approach, as well as useful parameters for the characterization of defects and comparison between various tissues are discussed.

Keywords: Human mitochondria; Respiratory chain defects; Mitochondria isolation; Cell respiration; Polarography; Spectrophotometry

1. Introduction

The mitochondrial respiratory chain (RC) ensures the process of electron transport from reducing equivalents (e.g. NADH, succinate) to molecular oxygen with a very large loss of free energy, much of which is conserved by the phosphorylation of ADP to yield ATP, in the process of oxidative phosphorylation. The RC is made

Abbreviations: mt, mitochondrial; RC, respiratory chain.

* Corresponding author.

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up of five multi-enzymatic complexes (Complexes I–V) encoded by both the nuclear genome and the mitochondrial DNA (mtDNA), a maternally inherited 16.5 kb circular genome found with a high copy number in the cells (Fig. 1A). As a consequence of this plurigenomic origin, RC defects can have a maternal or autosomal mode of inheritance, sporadic cases also being encountered.

Since the original report by Luft et al. [1], mitochondrial RC disorders have been essentially regarded as neuromuscular diseases. However, the process of oxidative phosphorylation is not restricted to muscles and nerves. Consequently, defects of the mitochondrial RC might potentially affect any organ or tissue [2,3]. A specific involvement of one given tissue or organ might result from the occurrence of tissue-specific isoforms of several nuclear-encoded subunits of the RC complexes [4,5]. On the other hand, it might also result from a potential mtDNA heteroplasmy (i.e. coexistence of both mutated and normal mitochondrial genomes in the cells; Fig. 1B). Indeed, variable proportions of both mutated and normal mitochondrial genomes, with tissue to tissue and cell to cell differences, are often observed in affected patients [2]. Finally, organ-specific involvement might originate from organ-specific regulation of electron fluxes in the RC [6], or from organ-specific requirements of energy supply [2]. Accordingly, RC defects display, particularly in childhood, a broad and ever-expanding spectrum of clinical features, affecting any organs or tissues [3]. As a consequence, the investigation of patients should not be restricted to muscle tissue when screening for RC disorders.

In the context of screening procedures, in vivo metabolic analyses of body fluids (blood or cerebrospinal fluid) and histological studies on muscle biopsies can provide useful information for further investigation. Hitherto, in the absence of screen-

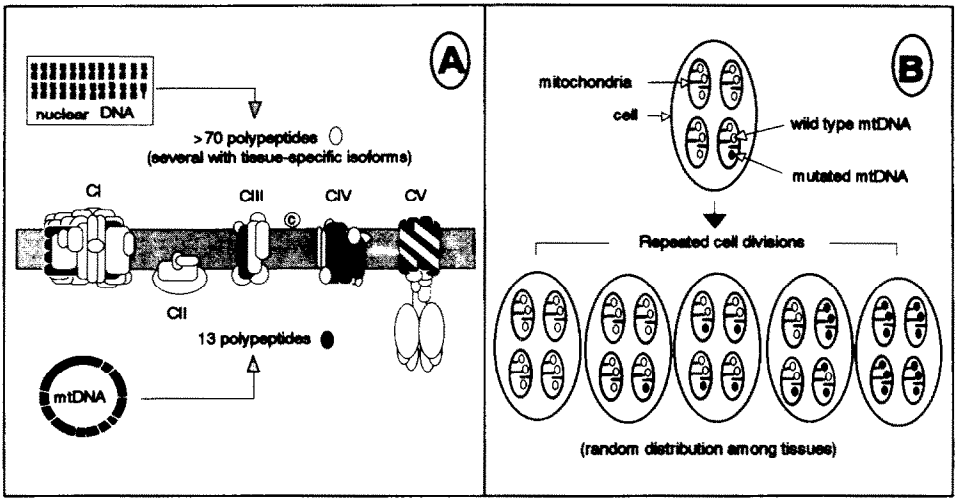


Fig. 1. Genetic features of the mitochondrial respiratory chain. (A) The plurigenomic origin of the respiratory chain polypeptides. (B) Mitochondrial heteroplasmy, with coexisting mutated and wild-type mitochondrial DNA (mtDNA). CI, CII, CIII, CIV, CV, various complexes of the respiratory chain; c, cytochrome *c*.

ing tests for the genetic defects underlying most of the RC deficiencies, biochemical characterization of RC enzymes has been the best way to recognize these conditions.

In this paper, we present our current strategy for the investigation of patients potentially affected by an RC disorder. All the methods are workable with small amounts of biological sample and therefore suitable for even very young patients.

2. Selection of patients at risk

The clinical assessment of RC disorders has proved to be a particularly difficult task, due to the very broad spectrum of symptoms associated with these diseases. It has consequently been suggested that the diagnosis of a mitochondrial disorder should be considered when one is confronted by unexplained associations of symptoms, involving seemingly unrelated organs [2]. Measurements of redox status in plasma, when carefully carried out [7], can reinforce the clinical diagnosis. Indeed, as the RC transfers reducing equivalents from NADH to oxygen, RC defects should lead to an altered oxidoreduction status (Fig. 2). This results from the functional impairment of the Krebs cycle due to accumulation of NADH, with secondary increase of blood lactate and ketone bodies together with elevated lactate/pyruvate ratios in affected children. This is particularly true in the post-absorptive period when metabolism of glycolytic substrates requires more NAD^+. Similarly, ketone body synthesis should increase after meals, instead of decreasing, owing to the channelling of acetyl CoA towards ketogenesis. When not conclusive, these tests can be tentatively substantiated by glucose (or pyruvate) loading, by exercise tests, or by examinating the redox status in the cerebrospinal fluid [2].

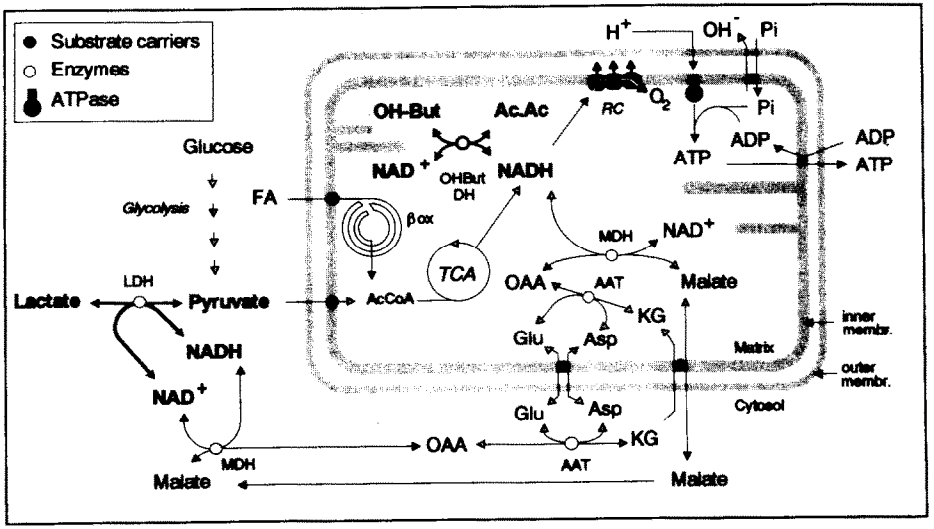


Fig. 2. Relationships between mitochondrial respiratory chain activity and redox equilibrium in the cell. AAT, aspartate aminotransferase; Ac.Ac., acetoacetate; Asp, aspartate; βox, βoxidation of fatty acids; FA, fatty acids; Glu, glutamate; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; OHBut DH, hydroxybutyrate dehydrogenase; TCA, tricarboxylic acid cycle.

Based on the clinical and metabolic features, biochemical investigations can be directed towards a given tissue (e.g. muscle, liver, heart, blood cells). However, because of its low invasiveness, study of the RC activity in circulating lymphocytes is preferred for initial investigations.

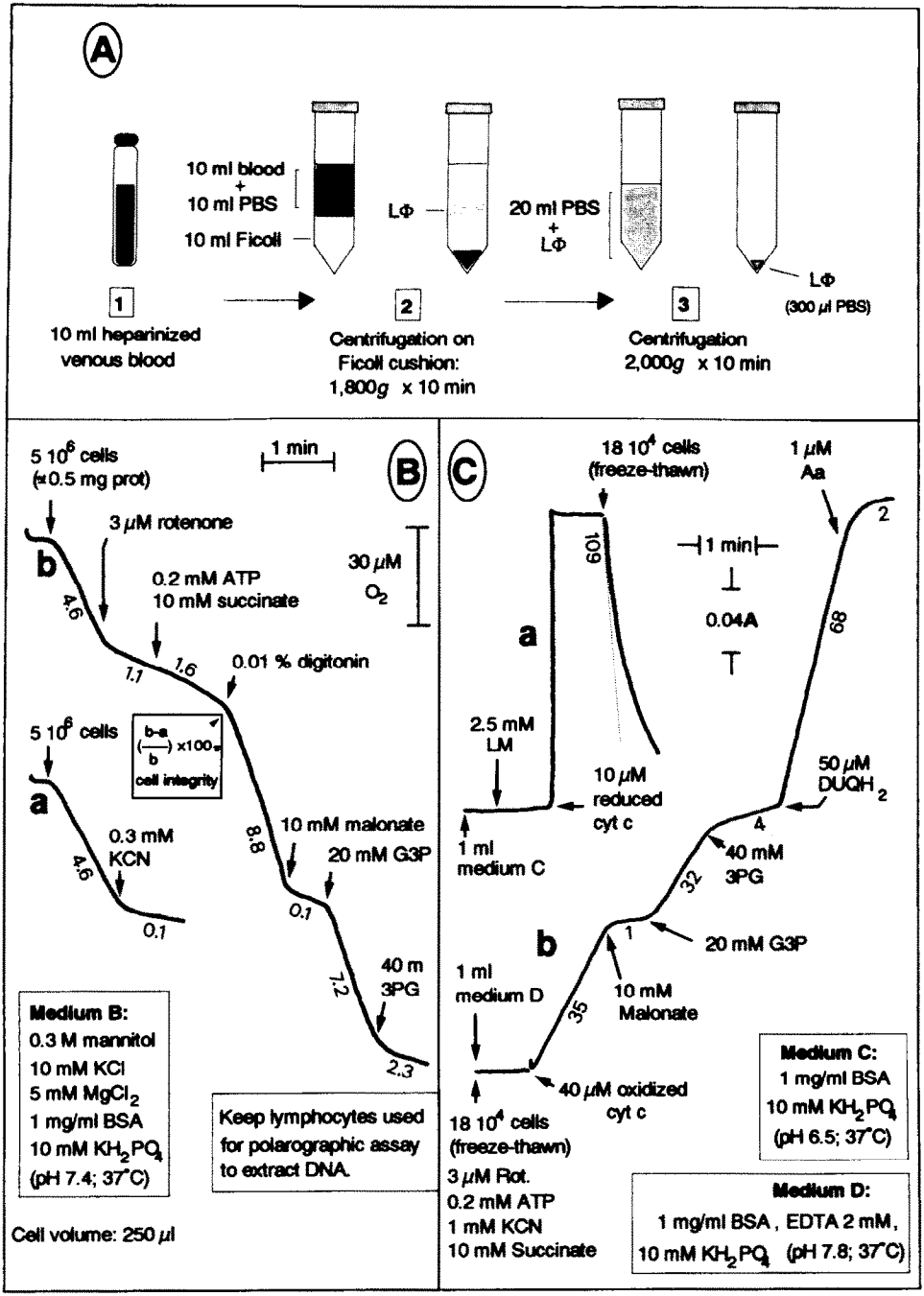
3. Screening for RC defects in circulating lymphocytes

Studies of RC activities in lymphocytes isolated from 10 ml blood (Fig. 3A) include the polarographic study of intact cell respiration and of mitochondrial substrate oxidation and the spectrophotometric measurement of RC complex activities (Figs. 3B and 3C). Details of a typical set of experiments routinely carried out in our laboratory for the screening of defective RC in lymphocytes are presented in Figs. 3B and 3C.

Polarographic studies are usually performed using two polarographic cells, one being reserved for studies requiring the use of the highly hydrophobic rotenone (a specific inhibitor of RC complex I which tends to bind to plastic parts of the polarographic cells). After measurement of intact cell respiration (Fig. 3B, trace a), succinate oxidation by detergent-permeabilized cells is measured (Fig. 3B, trace b). Comparison of the rates of succinate oxidation in the absence and in the presence of digitonine (0.01% w/v) gives a rough estimation of cell integrity (better estimated by the spectrophotometric measurement of the lactate dehydrogenase activity in the absence and presence of a detergent). After inhibition of succinate oxidation by malonate (a competitive inhibitor of the succinate dehydrogenase), addition of glycerol-3-phosphate triggers an increase of oxygen uptake that can be 60–70% inhibited by 3-phospho-glycerate or fully inhibited by KCN. This oxidation of glycerol-3-phosphate is catalyzed by a FAD-containing glycerol-3-phosphate dehydrogenase, an enzyme associated with the inner mitochondrial membrane that feeds electrons to complex III of the RC. These polarographic studies give an estimation of the overall respiratory activity of the lymphocytes.

The activity of the RC complexes is next spectrophotometrically measured under V_{max} conditions, at pH optima (Fig. 3C). Cytochrome *c* oxidase (complex IV) is measured using freeze-thawed cells (one cycle to avoid precipitation of the biological

Fig. 3. Biochemical screening of respiratory chain defects on circulating lymphocytes. (A) Isolation of lymphocytes on a Ficoll cushion. (B) Polarographic study of intact cell respiration (trace a) and of substrate oxidation by permeabilized cells (trace b). Numbers along the traces are average values (n = 11) of oxygen uptake in nmol/min per mg protein for controls. (C) Spectrophotometric assays of respiratory chain activities at 550 nm shown by the oxidation (trace a) and the reduction (trace b) of cytochrome *c*. Trace a: assay of complex IV (cytochrome *c* oxidase). Provided that low amounts of protein are used, the pseudo-linear initial rate gives a confident estimate of the cytochrome *c* reductase activity. Trace b: successive assays of the succinate (complexes II + III), glycerol-3-phosphate (glycerol phosphate dehydrogenase plus complex III) and quinol (complex III) cytochrome *c* reductase. Complex II and complex V can be measured in freeze-thawed cells under conditions described for isolated mitochondria (see Fig. 5). Cyt, cytochrome; $DUQH_2$, decyl ubiquinol; G3P, glycerol-3-phosphate; LM, lauryl maltoside; LΦ, lymphocytes; PBS, phosphate-buffered saline; 3PG, 3-phospho-glycerate; Rot, rotenone. Numbers along the traces are average values (n = 11) of control activities in nmol cytochrome *c* oxidized (trace a) or reduced (trace b)/min per mg protein.


A
10 ml blood
+
10 ml PBS
10 ml Ficoll
LΦ
20 ml PBS
+
LΦ
LΦ
(300 µl PBS)
1
10 ml heparinized venous blood
2
Centrifugation on Ficoll cushion: 1,800g x 10 min
3
Centrifugation 2,000g x 10 min
B
5 10⁶ cells (≈0.5 mg prot)
1 min
3 µM rotenone
0.2 mM ATP
10 mM succinate
30 µM O2
0.01 % digitonin
4.6
1.1
1.6
5 10⁶ cells
(b-a)/b x100 = cell integrity
a
0.3 mM KCN
4.6
0.1
8.8
10 mM malonate
20 mM G3P
0.1
7.2
40 m 3PG
2.3
Medium B:
0.3 M mannitol
10 mM KCl
5 mM MgCl2
1 mg/ml BSA
10 mM KH2PO4
(pH 7.4; 37°C)
Cell volume: 250 µl
Keep lymphocytes used for polarographic assay to extract DNA.
C
18 10⁴ cells (freeze-thawn)
1 µM Aa
2
100
1 min
0.04A
68
a
2.5 mM LM
10 µM reduced cyt c
1 ml medium C
50 µM DUQH2
4
40 mM 3PG
32
1
20 mM G3P
b
1 ml medium D
35
10 mM Malonate
40 µM oxidized cyt c
18 10⁴ cells (freeze-thawn)
3 µM Rot.
0.2 mM ATP
1 mM KCN
10 mM Succinate
Medium C:
1 mg/ml BSA
10 mM KH2PO4
(pH 6.5; 37°C)
Medium D:
1 mg/ml BSA, EDTA 2 mM,
10 mM KH2PO4 (pH 7.8; 37°C)

material) by following the oxidation of the reduced cytochrome *c* in a hypotonic medium (10 mM phosphate buffer, pH 6.5) and in the presence of lauryl-maltoside, a detergent allowing the measurement of a maximal rate of cytochrome *c* oxidase activity (Fig. 3C, trace a). As the enzyme is rapidly inhibited by oxidized cytochrome *c* (the K_i for oxidized cytochrome *c* in human muscle mitochondria is about 3 μM, roughly equivalent to the K_m for the reduced cytochrome *c*), the enzyme reaction tends to slow down as it proceeds and accumulates oxidized cytochrome *c*. However, the use of a reduced amount of cells allows measurement of a pseudo-linear initial rate of cytochrome *c* oxidation that can be use to estimate cytochrome *c* oxidase activity. When required, the enzyme activity can be more accurately calculated assuming a first-order rate for the cytochrome *c* oxidase reaction. The activities of several mitochondrial cytochrome *c* reductase activities are next measured (Fig. 3C, trace b). Reduction of cytochrome *c* is first brought about by the addition of succinate. In order to avoid the inhibitory effect of oxaloacetate on the succinate dehydrogenase, the assay takes place in the presence of rotenone (to reduce the potential formation of oxaloacetate from the malate produced from fumarate during succinate oxidation) and of ATP, which protects the succinate dehydrogenase from the inhibitory effect of oxaloacetate [8]. Under these conditions, rates are reasonably linear and allow a confident estimation of the succinate cytochrome *c* reductase activity (complexes II and III). After inhibition of the succinate cytochrome *c* reductase by malonate, the glycerol-3-phosphate cytochrome *c* reductase (glycerol-3-phosphate dehydrogenase plus complex III) and the antimycin-sensitive decyl ubiquinol ($DUQH_2$) cytochrome *c* reductase (complex III) are measured. An estimation of complex II and of glycerol-3-phosphate dehydrogenase activities can also be obtained by measuring the rates of dichlorophenol indophenol reduction triggered by succinate and glycerol-3-phosphate respectively (not shown, see Fig. 4C for details).

It should be stressed that no specific method for a confident measurement of complex I activity is currently available for lymphocytes [9]. The oxidation of NADH-generating substrates (or of exogenously added NADH) by detergent-treated or freeze-thawed cells appears too variable (at least in our hands) in the controls to be included as a screening test. On the other hand, the spectrophotometric measurement of rotenone NADH-cytochrome *c* reductase (measuring the activity of complexes I + III) is obscured by the presence of an extremely high rotenone-resistant activity, preventing the confident measurement of any potential defect. Finally, we found that short-chain ubiquinone homologues were poor electron acceptors for complex I in these cells. Therefore, rotenone-sensitive NADH quinone reductase could not be used in the screening procedure either. Consequently, the only indirect indication of a defect in complex I activity comes from the measurement of a low rotenone-sensitive respiration of the intact cells. Obviously, this might also be caused by a restriction of NADH-generating substrates in the mitochondrial matrix. On the other hand, a partial defect of complex I activity could remain undetected if it is non-rate-limiting for the overall respiration process. Nevertheless, we were able to identify a RC defect in 38 young patients by investigating RC activity in isolated lymphocytes. Beside these enzymological studies, molecular investigations by Southern blot analysis of major rearrangements of the mitochondrial genome are routinely per-

formed using lymphocytes already used for polarographic studies. In particular, patients presenting with Pearson syndrome (an often fatal childhood mitochondrial disorder associated with pancytopenia, exocrine pancreatic insufficiency and liver dysfunction; McKusick number 260560) have been shown to consistently harbor high amounts of deleted mtDNA molecules in their lymphocytes [10].

Taking into account the results obtained on lymphocytes, clinicians can direct further investigations to another tissue. Peripheral skeletal muscle tissues, with their high mitochondrial content and a relatively easy access, have been widely used and histological studies have become standardized in laboratories specializing in muscle pathology.

4. Investigations on isolated mitochondria from skeletal muscle tissue

Among the various approaches to studying RC activity in skeletal muscle tissue (e.g. studies on permeabilized fibers, on muscle homogenates, low-speed supernatants), isolation of a mitochondria-enriched fraction has been found preferable since it allows numerous detailed analyses to be performed (e.g. spectral studies, mitochondrial protein synthesis), as well as the validation of spectrophotometric measurements of isolated enzyme activity by polarography and vice versa. In particular, polarographic measurement of the overall activity of the mitochondrial RC, including respiratory controls (which in part reflect the integrity of the inner mitochondrial membrane), allows the quality of the preparation to be evaluated. Moreover, any potential defects can then be characterized by different and independent types of measurement of enzyme activities (polarographic or spectrophotometric), reducing possible experimental errors. Polarographic studies also permit detection of mitochondrial defects not attributable to the RC (e.g. defects of substrate, cation or adenylate carriers, Krebs cycle enzymes, and fatty acid oxidation). Finally, polarographic study is the only easy method for detecting potential defects in cytochrome *c* (apart from spectrophotometric quantitation of the cytochrome components of the RC, which requires a relatively large amount of mitochondria). In any case, the study of isolated mitochondria should be accompanied by the assay of cytochrome *c* oxidase activity in the initial homogenate. This allows for the detection of any abnormal mitochondrial content in the muscle and, based on an average yield of 30–40% for our standard isolation procedure, should reveal any major selective loss of particular mitochondria during the isolation procedure. Purification of mitochondria should be avoided since it increases the risk of undesirable selection of a particular fraction of the mitochondrial population, potentially leading to the loss of defective mitochondria.

Using a small amount of muscle tissue (100–200 mg), the isolation procedure can be performed in less than 1 h (Fig. 4). The slicing of the tissue is a crucial step that determines the yield of the procedure. The tissue, thoroughly washed, should be extensively minced with scissors, then immediately immersed into the extraction medium. While gram amounts of muscle tissue (1–5 g) have been used previously for the investigation of RC activity, precluding such investigation in young children, the described isolation procedure starting from 100–200 mg of muscle tissue routinely

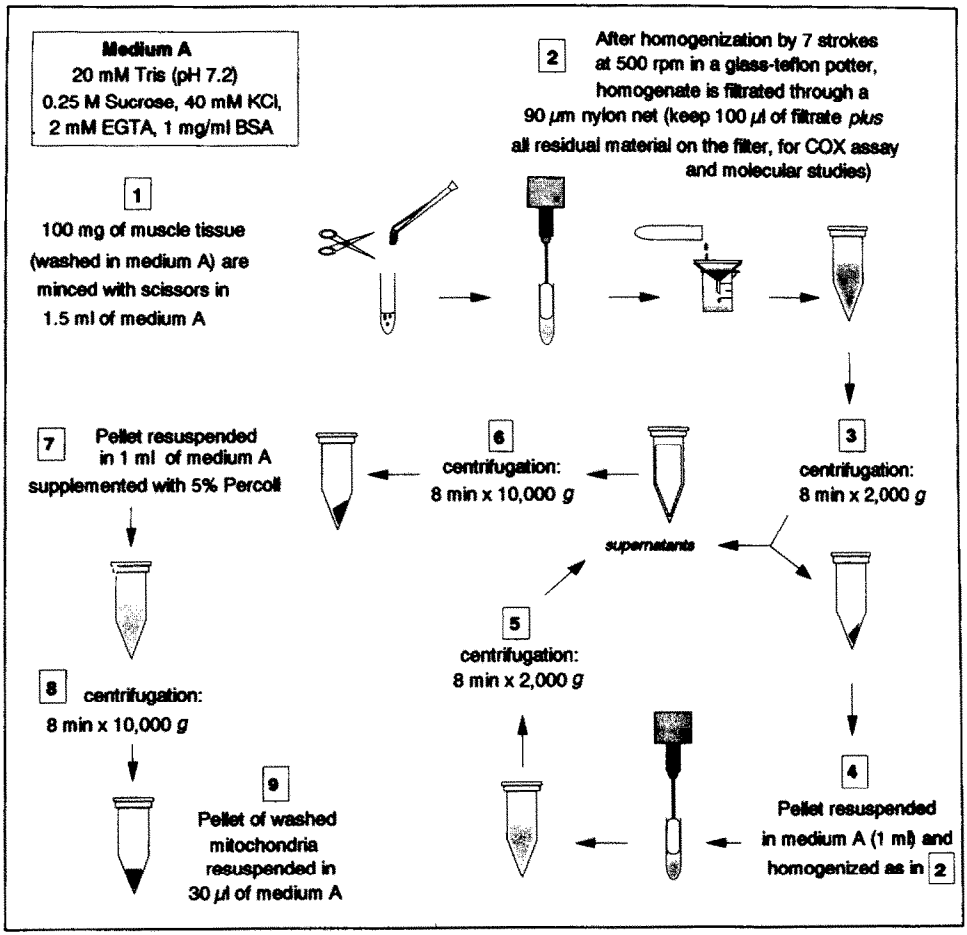


Fig. 4. Preparation of isolated mitochondria from small amounts of skeletal muscle tissue. The complete procedure takes about 1 h and 100 mg of muscle tissue leads to about 300 μg mitochondrial protein (based on the cytochrome *c* reductase activity pelleted in the mitochondrial fraction, yield was generally about 40% of total mitochondria).

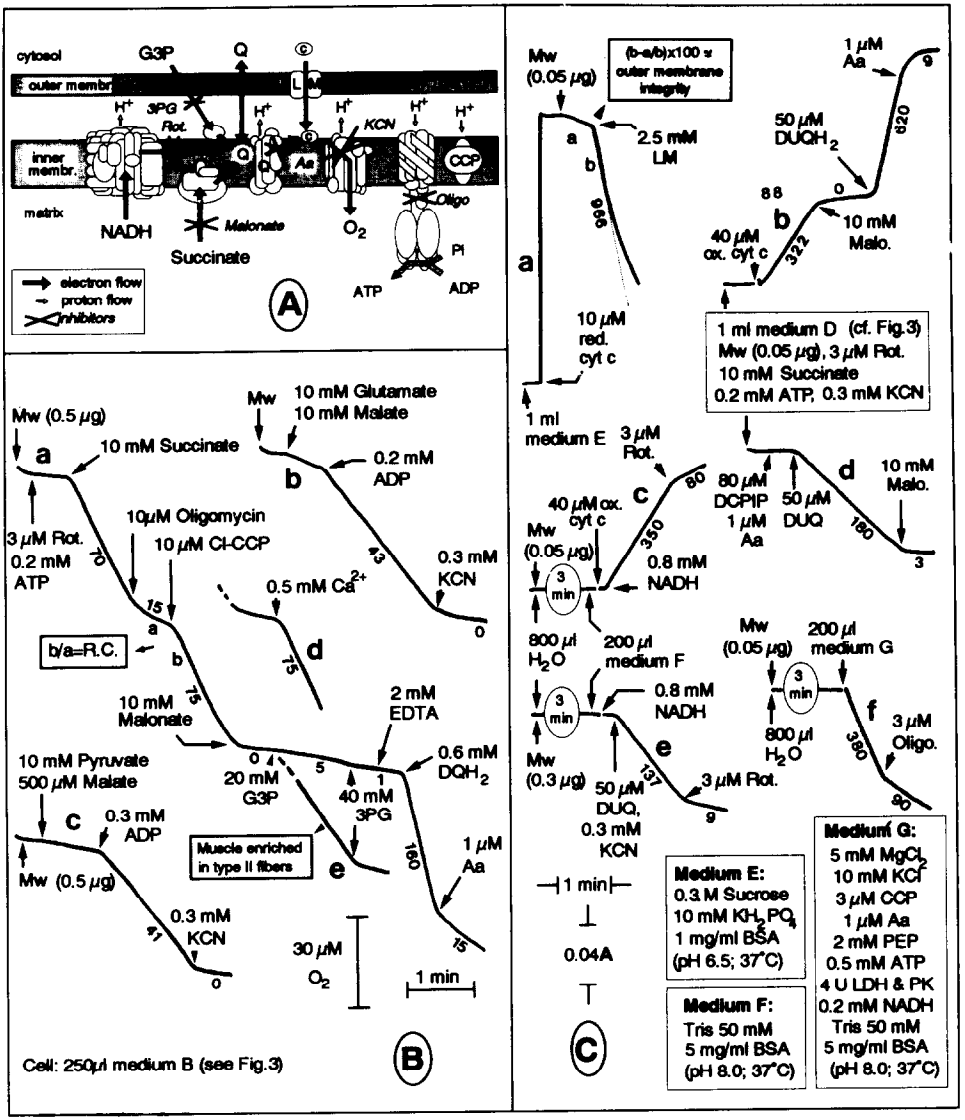
leads to a mitochondrial-enriched fraction containing about 400–500 μg protein, sufficient to characterize RC defects. A complete set of experiments allowing for the characterization of RC defect is presented in Fig. 5 which also includes a comprehensive scheme of the organization of the RC (Fig. 5A). Measured by polarographic method (Fig. 5B, trace a), an active oxidation of succinate is generally observed in the presence of rotenone and ATP, which can be strongly reduced by oligomycin (a specific inhibitor of the ATPase). A similar inhibition can be obtained by using atractyloside (a specific inhibitor of the adenylate carrier), while the presence of EDTA also tends to reduce this initial rate of succinate oxidation (not shown). This

pattern of sensitivity to inhibitors is indicative of an external hydrolysis of the added ATP into ADP through the action of the non-mitochondrial Mg^{2+}-dependent ATPase. The presence of this contaminating activity prohibits the use of oxidation rates measured in the presence of ADP and of ATP to calculate a value for the respiratory control. However, respiratory control can be conveniently determined by using the rates of oxygen uptake obtained in the presence of oligomycin and in the presence of an uncoupler, such as carbonyl cyanide *m*-chlorophenylhydrazone (*m*-Cl-CCP). The respiratory control measures the coupling between the electron flux, which establishes an electro-chemical gradient across the inner mitochondrial membrane, and the ATP synthesis, which uses this electro-chemical gradient. It therefore largely reflects the integrity of the inner mitochondrial membrane. After inhibition of succinate oxidation by malonate, rate of glycerol-3-phosphate oxidation can be measured, which is indicative of the content of the muscle in type II fibers. Last, an active duroquinol (DQH_2) oxidation is measured which is fully sensitive to KCN. Malate plus glutamate (Fig. 5B, trace b), or malate plus pyruvate (Fig. 5B, trace c) are also used as substrates, bringing about similar rates of oxygen uptake. Finally, an estimation of the activity of the calcium import system (an electrical uniport that uses the electrochemical gradient to function) can be gained through the measurement of the stimulation of the oligomycin-inhibited succinate oxidation rate by calcium (Fig. 5B, trace d).

The activity of the various RC complexes is then spectrophotometrically measured, separately or by group (Fig. 5C). Cytochrome *c* oxidase is measured in an isoosmotic medium (10 mM phosphate buffer plus 0.25 M sucrose, pH 6.5) allowing the intactness of the mitochondrial membranes to be preserved (Fig. 5C, trace a). Under these conditions, a low rate of cytochrome *c* oxidation should be measured, the external mitochondrial membrane being essentially impermeable to external cytochrome *c*. Upon addition of a detergent such as lauryl maltoside, this external membrane is made permeable and the reaction can freely proceed. This allows estimation of the integrity of the external mitochondrial membrane by comparing the rate of cytochrome *c* oxidation in the absence and in the presence of the detergent. When using a simple wavelength spectrophotometer care should be taken to not confuse the rate of cytochrome *c* oxidation (KCN sensitive) and the unspecific decrease of absorbance (unaffected by KCN) due to the effect of the detergent on the mitochondria. The activities of the succinate and of the decyl ubiquinone cytochrome *c* reductases (Fig. 5C, trace b) are next measured as above described for lymphocytes. An estimation of the rotenone-sensitive complex I activity can be obtained by measuring the rotenone-sensitive NADH-dependent cytochrome *c* reduction (Fig. 5C, trace c; complexes I + III), or the rotenone-sensitive NADH oxidation in the presence of decyl ubiquinone (Fig. 5C, trace e; complex I). These two assays are performed on mitochondria burst by 3 min incubation in distillated water. An estimation of complex II activity can be obtained by measuring the rate of succinate-dependent reduction of dichlorophenol indophenol in the presence of decyl ubiquinone. Under these conditions, decyl ubiquinone is reduced by electrons from complex II and further reacts with dichlorophenol indophenol. By using phenazine-methosulfate instead of the decyl ubiquinone, an estimation of the succinate

dehydrogenase activity can be obtained under similar conditions. Finally, the activity of the ATPase (complex V) can be measured by a coupling assay using lactate dehydrogenase and pyruvate kinase as coupling enzyme (Fig. 4C, trace f). Oligomycin-sensitive ATP hydrolysis catalysed by the ATPase is coupled to the pyruvate kinase reaction, which, in the presence of phosphoenolpyruvate as a substrate, phosphorylates ADP and produces pyruvate. The latter is then used by the lactate dehydrogenase to oxidize NADH with the concomittant production of lactate.

Comparison of the rates of RC complex activities measured separately and by



group by the different methods allows detection of defects as well as possible experimental problems. As discussed later, comparison between the rates of the various activities is a powerful method for characterizing and quantifying defects. More specialized investigations can also be carried out on isolated mitochondria. They include spectral studies of respiratory chain cytochromes (requiring at least 100 μg mitochondrial protein and a spectrophotometer equipped with micro-cuvettes), analysis of mitochondrial protein synthesis (requiring about 100 μg mitochondrial protein) and spectrophotometric measurement of membrane potential by studying changes of absorbance of a dye, such as safranine (requiring about 20 μg protein and a double wavelength spectrophotometer).

Again based on the clinical presentation and the results of the muscle investigation, clinicians can ask for further investigations on targeted organs, such as liver or heart.

5. Investigations in liver, heart and other tissue homogenates

For investigation of RC activities in liver or in heart, only very small samples (1–20 mg) can be taken, which precludes isolation of a mitochondrial fraction. As a consequence, investigations have to be performed on whole homogenates. Methods of obtaining liver tissue by needle biopsies (about 10 mg) and of cardiac tissue

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Fig. 5. Biochemical investigation of respiratory chain activity in isolated muscle mitochondria. (A) The organization of the mitochondrial respiratory chain. (B) Polarographic study of substrate oxidation. Trace a: succinate oxidation in the presence of rotenone and ATP. Due to the presence of contaminating ATPase, respiratory control (indirect estimation of the inner membrane integrity) is best performed by comparing the rates of oxidation in the presence of oligomycin (the specific inhibitor of mitochondrial ATPase) and in the presence of an uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (*m*-Cl-CCP). In the presence of EDTA (not shown), contaminating ATPase is essentially inactive and the ADP/O ratio as well as respiratory controls can be calculated according to Chance and Williams [22]. After inhibition of succinate dehydrogenase by its competitive inhibitor (malonate), addition of glycerol-3-phosphate allows for a rough estimation of type II fiber content in the muscle tissue. Antimycin-sensitive oxygen uptake triggered by duroquinol measures the activity of complex III plus IV. Trace b: oxidation of malate in the presence of glutamate. Trace c: oxidation of pyruvate in the presence of malate at low concentration. Trace d: effect of Ca^{2+} on the oligomycin-inhibited rate of succinate oxidation. The active transport of Ca^{2+}, using the electrochemical gradient of the inner mitochondrial membrane to enter the matrix space, allows an increase of the electron flux from succinate to oxygen. Trace e: oxidation of glycerol-3-phosphate by mitochondria from fiber II type-enriched muscle tissue. (C) Spectrophotometric assays of respiratory chain enzyme activities. Trace a: oxidation of cytochrome *c* at 550 nm (complex IV). Trace b: reduction of cytochrome *c* at 550 nm successively triggered by succinate (complexes II + III) and quinol (complex III). Trace c: rotenone-sensitive reduction of cytochrome *c* at 550 nm triggered by NADH (complexes I + III). Trace d: succinate-dependent reduction of dichorophenol indophenol at 600 nm (complex II). Trace e: rotenone-sensitive NADH oxidation in the presence of quinone (complex I) at 340 nm. Trace f: assay of ATPase (complex V) shown by the oxidation of NADH at 340 nm (standard coupled assay with lactate dehydrogenase and pyruvate kinase, [23]). Aa, antimycin a; CCP, carbonyl *m*-chlorophenylhydrazone cyanide. DCPIP, dichlorophenol indophenol; DQH_2, duroquinol; DUQ, $DUQH_2$, decyl ubiquinone and ubiquinol, respectively; LDH, lactate dehydrogenase; LM, lauryl maltoside; Malo, malonate; Mw, washed mitochondria; Oligo, oligomycin; PEP, phosphoenolpyruvate; 3PG, 3-phospho-glycerate; PK, pyruvate kinase; Rot, rotenone. Numbers along the traces are average values ($n = 17$) of control activities.

by endomyocardial biopsies (1–2 mg) have been previously described [11,12]. Samples should be immediately freeze-dried in liquid nitrogen. They should be kept in liquid nitrogen (or at −80°C). The next steps of the procedure are shown in Fig. 6A. Most RC enzyme activities can be measured in such homogenates, under the conditions described above for muscle mitochondria (Fig. 5). Use of frozen samples raises specific problems, mainly related to the differential loss of enzyme activities that results from the instability of the mitochondrial inner membrane structure. Incorrect handling of control heart and liver samples was found to lead to a progressive loss of succinate- and NADH-cytochrome *c* reductase activities, without parallel losses of complex I, II, III, or IV activities. Physical disorganization of the membrane lipids could lead to dysfunction of the ubiquinone pool, causing the observed loss of activities. In support of this, we found a high prevalence of succinate- and rotenone-sensitive NADH-cytochrome *c* reductase defects in tissue samples prepared for histological studies and used for biochemical investigations. We tested succinate- and NADH-cytochrome *c* reductase activities in both freshly frozen tissues and samples prepared for histological study in two patients and found artifactual succinate- and NADH-cytochrome *c* reductase deficiencies in the histological samples. It is noteworthy that further ageing of the sample leads to the loss of other activities as well, presumably through peroxidative chain reactions involving membrane lipids [13]. Whatever the mechanisms actually at work, these potential artifactual losses of enzyme activities suggest that great caution is required when dealing with incorrectly handled samples.

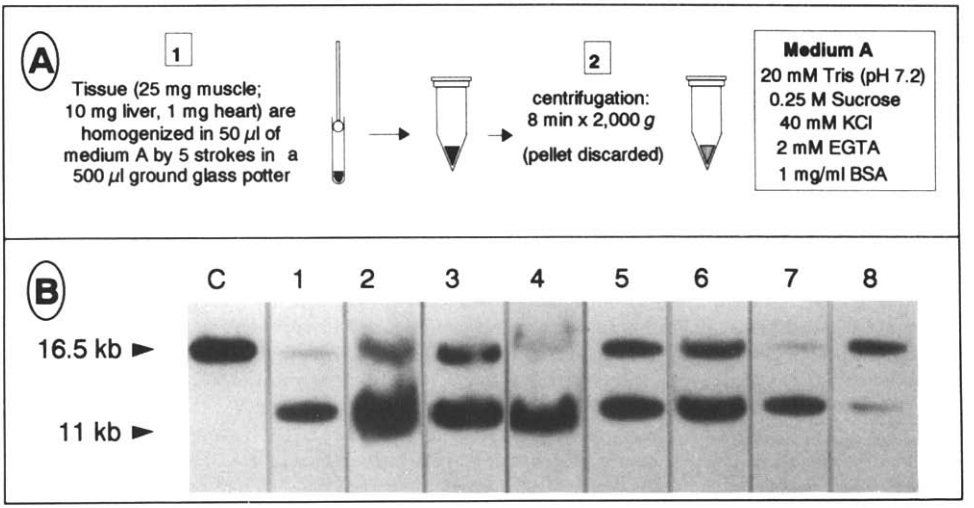

Fig. 6. Preparation and molecular investigation of tissue homogenates. (A) Preparation of homogenates from frozen tissues (−196°C or −80°C). (B) Southern blot of mtDNA from various tissues of a patient with Pearson syndrome and a control. Total DNA for different tissues (5 µg) was digested, separated by agarose (0.7%) gel electrophoresis and transferred onto nylon filters and hybridized with [^{32}P]dCTP-labeled single-strand mtDNA probes (2 × 10^6 counts/min per ml). C: control; 1–8: tissue derived from patient 1. Lane 1: bone marrow; lane 2: polymorphonuclears; lane 3: T lymphocytes; lane 4: non-T lymphocytes; lane 5: lymphoblastoid cell lines; lane 6: muscle; lane 7: small intestine; lane 8: cultured skin fibroblasts.

Enzymological investigation of microbiopsies of liver and heart tissues have proved to be of considerable value for the diagnosis of RC disorders in patients with isolated liver or heart involvement [12,14,15]. It also provides a clue to the study of differential processing or isozymic patterns of RC enzymes in humans, through potential organ-specific defects [14]. All these studies on biopsy samples can be prolonged by using cultured cells, mainly fibroblasts and transformed lymphocytes.

6. Investigations on cultured cells

RC activity can be studied in cultured fibroblasts and Epstein-Barr virus-transformed lymphocytes, as described for isolated lymphocytes (see Fig. 3). Alternatively, mitochondria isolated from both types of cell culture can be studied [16]. However, rather erratic expression of RC defects and the instability of the phenotypic expression, as reported for cytochrome *c* oxidase defects [17], hamper the usefulness of cultured cells in screening procedures. Nevertheless, we have observed that adding uridine to culture media allows normal growth of respiratory-deficient cells, thereby stabilizing RC defects during culture [18]. Uridine permits resumption of nucleic acid synthesis blocked in these cells by a defect of the RC-linked dihydroorotate dehydrogenase activity, itself secondary to the RC defect [19].

7. Data handling

A major problem encountered in the identification of defective RC enzyme activities lies in the very high scattering of control data over 1 to almost 2 orders of magnitude, depending on the tested enzyme and the tissues (Table 1). As a consequence, control data frequently overlap with those of patients, limiting the ability to reach a reliable diagnosis of respiratory chain deficiencies. However, we have observed that a constant ratio of RC enzyme activities is a consistent feature of oxidative phosphorylation [20]. This might be required for an optimal functioning of

Table 1
The very conserved ratios of respiratory chain enzyme activities among various human tissues. Cytochrome *c* oxidase and succinate cytochrome *c* reductase were measured in tissue homogenates as described in Fig. 3

Homogenates	COX[a] (nmol cyt *c*/min per mg prot.)	SCCR	COX/SCCR ratio
Deltoid muscle ($n = 12$)	56–654	18–184	3.2 ± 0.4
Liver ($n = 8$)	56–283	21–91	2.5 ± 0.4
Heart ($n = 16$)	290–1340	43–368	3.1 ± 0.3
Brain ($n = 3$)	217–386	75–61	3.5 ± 0.4
Bone marrow ($n = 3$)	16–61	6–18	3.0 ± 0.3
Kidney ($n = 3$)	131–237	36–67	3.5 ± 0.4
Lymphocytes ($n = 11$)	69–146	23–46	3.1 ± 0.2
Lymphoblastoids ($n = 12$)	56–142	14–47	3.3 ± 0.4
Fibroblasts ($n = 8$)	90–134	24–45	3.1 ± 0.5

[a]COX, cytochrome *c* oxidase; SCCR, succinate cytochrome *c* reductase.

the RC. We found that most of the ratios between the activities of the different RC complexes or segments show a normal distribution with narrow ranges. This is illustrated for cytochrome *c* oxidase deficiency in ten patients (Fig. 7). Absolute cytochrome *c* oxidase activities of patients were measured, showing a clear overlap with control data. In addition, cytochrome *c* oxidase activities of the patients were related to the activity of the succinate cytochrome *c* reductase and the ratios compared with control ratios. Under these conditions, patients whose absolute cytochrome *c* oxidase values were still in the low normal range could be unambiguously diagnosed as cytochrome *c* oxidase deficient. Obviously, this approach does not hold true for generalized RC defects. In this latter instance, low absolute values of RC enzyme activities can be tentatively compared with Krebs cycle enzymes (citrate synthase, fumarase), although the ratios between level of Krebs cycle and RC enzyme activities are not necessarily as conserved as ratios within the RC [21]. As illustrated for cytochrome *c* oxidase and succinate cytochrome *c* reductase (Table 1), ratios between enzymes of the RC are not only consistent in one given tissue, they appear to be roughly similar in all human tissues we have investigated so far, with the noticeable exception of the high succinate-quinone reductase activity measurable in liver and kidney. This allows for the rapid and confident characterization of RC defects among tissues despite wide variations in absolute activities found in different tissues. A second exception is RC-associated glycerol-3-phosphate dehydrogenase, which is known to vary greatly between tissues, or even between components of one tissue (it can be useful as a marker to estimate abnormal content of type II fiber in muscle preparations, see Fig. 5B, trace e). We found insignificant glycerol-3-phosphate dehydrogenase activity in heart, kidney, and liver, whereas this glycolysis-associated enzyme was highly active in brain, fibroblasts and lymphocytes.

Calculation of enzyme rates, oxygen consumption kinetics, percentage of inhibition and protein content are a lengthy process; additional calculation of the ratios

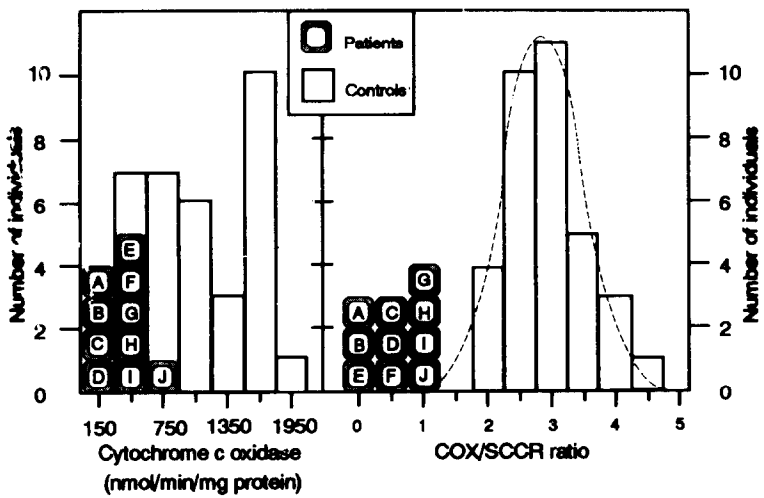

Fig. 7. Cytochrome *c* oxidase activities of patients and controls, both as absolute rates and relative ratios to the succinate cytochrome *c* reductase. Enzymes were assayed as described in Fig. 5.

between all the activities that are required for one investigation (as many as 20 for a study on isolated mitochondria) are also time consuming. Relatively simple programs written for microcomputers allow all these calculations (from slopes of experiments performed under standard conditions) to be made and all values and ratios to be compared with reference tables, as well as defects and discrepancies between data to be pinpointed. It should be noticed that all activities dependent on a single substrate should present coherent quantitative relationships if measured under non-rate-limiting conditions. Thus succinate cytochrome *c* reductase should be roughly 2- and 4-fold higher, respectively, than the succinate-dichloroindophenol reductase and the succinate-dependent oxygen uptake (1 e^- being required to reduce cytochrome *c*, whereas 2 and 4 e^-s are required to reduce dichloroindophenol and oxygen, respectively). While some variations cannot be avoided due to different experimental assay conditions (e.g. osmolarity, cation concentrations), large discrepancies observed in controls denote problems in one or more of the assay conditions (e.g. rate limiting conditions, contaminating activity). In experiments with isolated mitochondria from muscle tissue, the complete process (from the beginning of the isolation procedure to the printing of data) takes less than 3 h, allowing the clinician to undertake further investigations rapidly, thus reducing hospitalization time.

8. Molecular analysis of respiratory chain defects

Because very few of the numerous nuclear genes encoding for RC components have been localized and sequenced in humans, screening for mutations in these genes is not feasible at present. On the other hand, molecular study of major mtDNA rearrangements (deletion, duplication) or of point mutations already described in several mitochondrial disorders [2] can be easily performed.

Total DNA extracted using the standard procedure [22] is precipitated with ethanol and pelleted (15 min at 13,000 × *g*) in order to quantitatively recover all the extracted mtDNA. Standard Southern blotting and amplification by polymerase chain reaction are then performed [23].

In order to avoid repeated tissue biopsies, we generally extract DNA from material discarded during mitochondrial preparation (see Fig. 5), from samples of the initial homogenates and/or from fractions (e.g. cells, mitochondria) previously used for enzyme assays; if required, all the fractions can be pooled and frozen at −20°C for further DNA extraction. This technique yields sufficient DNA for Southern blotting. Thus, starting with a 2 mg endomyocardial biopsy and after RC enzyme assays, we can obtain about 500 ng total DNA, sufficient to allow detection of mtDNA rearrangements by Southern blotting and hybridization with mtDNA probes. A similar technique can be successfully applied to all tissues when looking for mtDNA rearrangements (Fig. 6B).

9. Concluding remarks

As mitochondrial RC disorders can affect any organ or tissue, we have developed a set of scaled-down biochemical methods that allow a multi-tissue screening for RC defects even in young children. Unfortunately, the large number of genes (more than

100) involved in the biogenesis of the mitochondrial RC currently precludes efficient molecular screening. Using the above described screening procedure, we have investigated 606 patients in 3 years — mostly children (from a few days to a few years of age) selected on clinical and/or metabolic grounds (usually lactic acidosis) — and found a biochemical defect affecting one RC complex (or more) in 131 (22%). A genetic defect was characterized in 27 (about 5%). All but one of the patients harboring mutated mitochondrial genomes in their tissues were found to have a deficient RC in the clinically affected tissue. Among the identified RC defects, 31% affected complex I, 2% complex II, 15% complex III, 27% complex IV, 17% complexes I and IV and 8% were generalized.

Presenting symptoms included neurological and muscular diseases (weakness, myoclonic epilepsy and ragged red fibers (MERRF) and Leigh syndrome), but these accounted for less than 20% of our patients. For the vast majority of the patients (80%) non-neuromuscular organs were initially involved (failure to thrive, pancytopenia, proximal tubulopathy, dwarfism, diabetes mellitus, cardiomyopathy, hepatic failure). Altogether, no correlation between the nature of the defect (generalized or localized, specifically affecting a given RC complex) and the clinical presentation could be made. Finally, whilst most of our patients were selected after demonstration of blood lactic acidosis, we found that two patients affected with isolated hypertrophic cardiomyopathy of mitochondrial origin [14] had normal redox ratios in their fluids.

In the absence of identified underlying mutations for most mitochondrial disorders, biochemical assays of RC complexes on chorion villi and cultured amniocytes can be considered for prenatal diagnosis of these conditions when a stable expression of the defect in the fibroblasts of the proband has been demonstrated. However, such a procedure has major drawbacks. Both the random distribution of defective mitochondria between cells and tissues during embryogenesis and the currently unpredictable evolution of the different (normal and defective) mitochondrial populations severely compromise the efficiency of such prenatal diagnosis.

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