

Review article

# A century of mitochondrial research: achievements and perspectives

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## 1. Introduction

A recent issue of *Science* (Vol. 283, March 5, 1999) had a cover showing a mitochondrion and a headline inside asserting that ‘Mitochondria Make A Comeback’. Why the revival of interest in this fascinating organelle? It is probably not fair to say that interest in mitochondria had waned. For those with an interest in the field, noteworthy progress was made steadily since their definite identification and isolation almost 50 years ago, and major milestones can be found throughout the subsequent decades. Molecular biologists may have ignored mitochondria because they did not immediately recognize the far-reaching implications and applications of the discovery of the mitochondrial genome (mtDNA). It took time to accumulate a database of sufficient scope and content to address many challenging questions related to anthropology, biogenesis, disease, evolution, and more.

In looking back, one realizes that research on mitochondria is subject to the same developmental restraints so wittily defined by S. Brenner for much of modern biological research: ‘In molecular biology there are technical advances, discoveries, and ideas – usually in that order’. Thus, the electron microscope and the ‘exploration of cells with a centrifuge’ (De Duve, 1975) defined them as subcellular organelles

and allowed the discovery of their major biochemical functions. More recently, electron microscopic tomography is providing new insights about the morphology of the mitochondrial inner membrane. Molecular genetics techniques contributed in a major way to our understanding of the biogenesis of mitochondria. An increase in computational power permitted the tackling of crystal structures for the multisubunit membrane complexes II, III, IV, and V. This gives new impetus for structure-function studies aimed at understanding electron transport and proton pumping. The ‘staining’ of mitochondria in living cells by a combination of techniques (including the use of green fluorescent protein and aquorin), and confocal microscopy and computer-enhanced image analysis have become vital for cell biological studies and our understanding of the integration of mitochondria into cells of various types.

Two relatively recent developments have brought mitochondria back into the limelight for a broader audience. First, the elucidation of the genetic basis for a large number and variety of ‘mitochondrial diseases’ starting in the late 1980s, has made studies of mitochondria more relevant to the medical community. There are also indications that Parkinson’s disease, Diabetes mellitus, possibly Alzheimer’s disease, and even aging in general are influenced in time of onset and severity by mitochondrial deficiencies or dysfunction. Second, within a broader context, mitochondria have been shown to play a central role in apoptosis, or programmed cell death, with far-

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reaching implications for developmental biology, cancer research, senescence and death of an organism (Tables 1 and A1).

## 2. Scope of this review

An effort to cover the major findings in one single book was made by this author not so long ago (Scheffler, 1999). The question becomes: what can yet another review contribute to the general enlightenment on this subject? It is perhaps fitting that the launching of a new journal devoted to mitochondria should begin with a brief account of where the field is at this time. What follows is a brief summary of our present knowledge and understanding, illustrated with a few examples, and references will be restricted to a sampling of the most recent papers and reviews. This essay will also attempt to define some of the outstanding challenges and questions still to be solved in the field.

## 3. Mitochondria and evolution

### 3.1. Current view

The fundamental distinction between prokaryotic and eukaryotic organisms is based on two salient characteristics: eukaryotic cells have a genome contained within a membranous nuclear envelope with pores, and the vast majority of eukaryotes have mitochondria. Sometimes the latter are barely recognizable, as in the case of hydrogenosomes (Andersson and Kurland, 1999). The research of the past 30 years has largely consolidated the hypothesis of mitochondria as endosymbionts of a primitive eukaryote (Margulis, 1981). The *serial endosymbiont* theory postulates that a proto-eukaryotic cell without mitochondria evolved first, and this organism then captured a proteobacterium by endocytosis. After establishing a symbiotic relationship, the loss of redundant genes and the transfer of genes from the bacterium to the nucleus lead to the currently observed distribution of genes between the

Table 1  
History of mitochondrial research

Date	Milestone
Pre-1900	First descriptions of mitochondria in cells; speculations about them as bacteria
1930s	Formulation of urea and TCA cycle
1950s	Identification of mitochondria by electron microscopy Mitochondria as site of fatty acid oxidation Mitochondria as site of respiration and oxidative phosphorylation O. Warburg speculates that a major distinction between normal cells and cancer cells is a reduced rate of respiration in cancer cells Discovery of cytoplasmic inheritance in yeast Definitive respiratory chain analysis
1960s	Discovery of DNA in mitochondria Fractionation and characterization of major complexes of electron transport chain and ATP synthase. Definition of mobile carriers ubiquinone and cytochrome <i>c</i>
1962–1970s	Formulation of Chemiosmotic Hypothesis and slow acceptance by the scientific community
1980s	Complete sequence of a mammalian mitochondrial DNA Identification of all genes on mammalian mtDNA Elucidation of poly-cistronic transcripts, RNA processing, mRNAs Identification of translation products In vitro mitochondrial protein import and definition of requirements Basics of mtDNA replication First molecular identification of cause of mitochondrial diseases
1990s	First complete sequences of plant mtDNAs Transformation of mtDNA in yeast (introduction of DNA by microprojectiles) Mitochondria and apoptosis: bcl2, cytochrome <i>c</i> ; the permeability transition Crystal structures of complex III, complex IV, F <sub>1</sub> ATPsynthase, Direct demonstration of molecular rotation associated with complex V activity

two genomes (Gray, 1993; Gray et al., 1999). Major questions are:

1. What was the bacterial ancestor, and which living bacteria are most closely related to this ancestor? The rickettsial subdivision of the  $\alpha$ -proteobacteria (*Rickettsia*, *Anaplasma*, *Ehrlichia*) are obligate intracellular parasites that satisfy a number of criteria defining the closest relatives.
2. What was the ancestor of the proto-eukaryote? The *Archeozoa* comprise a group of amitochondriate eukaryotes believed to constitute the branches of the eukaryotic phylogenetic tree that emerged before the establishment of the endosymbiotic relationship (Cavalier-Smith, 1981). However, the finding of mitochondrial heat shock protein genes in the nuclear genome of *Giardia lamblia* has been interpreted as evidence that at one time mitochondria-like endosymbionts existed in the ancestors of these modern amitochondriate eukaryotes (Roger et al., 1998).
3. When did the endosymbiosis take place and was it a unique event in the history of evolution of life on earth?

A 'revisionist view of eukaryotic evolution' is rapidly gaining acceptance (Gray et al., 1999; Vellai and Vida, 1999). The new scenario envisions a 'big bang' involving the 'fusion' of two types of bacteria (anaerobic archeobacteria (host) and respiration-competent proteobacteria (symbiont)) to form a primitive eukaryote from which all present eukaryotes evolved. In other words, there was no distinct amitochondriate eukaryote, but a defining moment for eukaryotes was intimately linked with the acquisition of a proteobacterium destined to become the mitochondrion.

A very large number of mtDNAs from diverse organisms have now been completely sequenced. Most mitochondrial genomes encode a small number of proteins of the complexes I, III and IV of the electron transport chain, and of complex V, the ATP synthase. In mammals and most metazoans the number is 13, but in the human malaria parasite *Plasmodium falciparum* this number is reduced to three. mtDNAs also encode two ribosomal RNAs and a set of tRNAs sufficient for translation of mitochondrial mRNAs (in metazoans). In many fungi, plants, and kinetoplastid protozoa one or more tRNAs have to be imported from the cytosol. Most animals have a

small mtDNA of  $\sim 16.5$  kb. Although similar in size ( $\sim 16.5$  kb) and gene content, the order of the genes is subject to rare, relatively unique rearrangements (Boore, 1999). Other organisms have larger mtDNAs, with the largest found in plants (up to  $\sim 350$  kb). Nevertheless, the number of genes encoded is not correspondingly greater. A record (and atypical) number of 97 genes is found on the mtDNA (69 kb) of the freshwater protozoan *Reclinomas americana* (Gray et al., 1999) including those encoding four subunits of a multisubunit RNA polymerase with resemblance to a eubacterial-type polymerase. The *R. americana* mitochondrion has been called 'the mitochondrion that time forgot' (Lang et al., 1997; Palmer, 1997), because its genome is believed to be the least reduced in gene number relative to a presumed eubacterial precursor.

A second fruitful approach for evolutionary studies is to focus on the nucleotide sequences of specific genes. Valuable and convincing deductions can certainly be made, but absolute proofs, for example to distinguish hypotheses about the monophyletic vs. multiphyletic origins of mitochondria, or for a definitive ancestry and relationship to present day bacteria, are not possible, because of the absence of a common root. The choice of algorithms in computer analysis and other methodological subtleties can give rise to the generation of artifactual trees.

### 3.2. Future directions

Current sequencing efforts are adding many more mtDNA sequences from many different species to the database in order to remove existing uncertainties about phylogenies. It would also be exciting to find more 'mitochondria that time forgot', that is mtDNA retaining ancestral eubacterial genes, to clarify which genes were contributed by each partner in the earliest symbiotic relationship, and how much redundancy there was. One could add the discussion of mtDNAs whose transcripts require extensive editing, as in the kinetoplast protozoa, or one could discuss the inclusion of large amounts of 'junk DNA', i.e. non-coding, repetitive sequences, etc., that could be molecular fossils of transposon-like elements.

There is an aspect of evolution related to mitochondria that may deserve more attention. Some of the earliest studies of mammalian hybrid and cybrid cell

lines had revealed that mitochondria from one mammalian species are generally incompatible with the nuclear genome of another. One can speculate that subunits of electron transport complexes encoded by the nuclear genome must be compatible with those encoded by mtDNA. Recent studies have shown that chimpanzee and gorilla mitochondria can be functional when introduced into human  $\rho^0$  cells (cells lacking mtDNA), but orangutan mitochondria are not fully functional (Barrientos et al., 1998; Kenyon and Moraes, 1997). Thus it becomes necessary to consider that nuclear and mitochondrial coding sequences have to evolve in a manner that assures that the respective subunits of ETC complexes retain their functional interactions. The above considerations are also important in another context. Much publicity has been generated by advances in stem cell research and the cloning of animals. The claim of a human-cow embryo was indeed ‘greeted with skepticism’ (Marshall, 1998), but perhaps not entirely for exclusively scientific reasons. In another recent publication it was reported that bovine oocyte cytoplasm supports development of embryos produced by nuclear transfer of somatic nuclei from various mammalian species (Dominko et al., 1999). It should definitely be a consideration that a mammalian oocyte has ~100,000 mtDNAs, and that the contribution of mtDNA introduced from the somatic cell nuclear donor is two orders of magnitude smaller. The fact that no pregnancies have been carried to term should therefore not surprise.

## 4. Mitochondria and cell biology

### 4.1. Structure and morphology

The earliest electron microscopic observations of mitochondria defined their basic morphology: a matrix enclosed by the inner membrane, and an intermembrane space (IMS) between the inner and outer membrane. The folding of the inner membrane serves to increase the surface area of this membrane. The folds as visualized by transmission electron microscopy have been termed cristae. However, the original interpretations of cristae as ‘baffle-like’ (Palade) and as ‘septa-like’ (Sjostrand) already indicated a disparity, which was resolved in favor of the first model for

the following decades. A revisionist view of mitochondrial membrane topology is now beginning to emerge in this area, too, based on newer methods such as electron microscope tomography (Mannella, 2000; Perkins and Frey, 2000).

The topologically continuous surface area of the inner membrane is now divided into two distinct domains (Fig. 1). One area is juxtaposed closely to the outer membrane (‘inner boundary membrane’), and it makes close contacts with the outer membrane in numerous positions. The second membrane domain forms the cristae, which are now interpreted to form tubular or lamellar structures, connected to and continuous with the inner boundary membrane by small tubular structures named *crista junctions* by Perkins et al. (1997) and *pediculi crista* by Daems and Wisse (1966). One interpretation is that the whole system is possibly quite dynamic depending on the physiological state of the mitochondria, and somewhat variable in different cell types. For example, the unusual mitochondrial morphology of Leydig cells (and other steroid producing cells) has attracted attention for decades. A recent study (Prince, 1999) has added to the detailed description of ‘cristae morphology’ in such cells, but the author also concludes that the observations reemphasize our lack of understanding of structure, morphology and function.

Questions raised by these findings and interpretations are interesting: (1) Do these crista junctions represent some real boundary that restricts the diffusion of ions from the space between the outer membrane and the inner boundary membrane (*intermembrane*) to the space inside the tubular or lamellar cristae (*intracristal*)? (2) Are proteins differentially distributed between these two spaces, or between the two membrane domains? (3) Can the membrane potential become a localized property, i.e. is it possible that the  $\Delta\Psi$  is not uniform along the entire surface of the inner membrane? (4) It is apparent that this unorthodox view of the inner membrane also has implications for understanding mitochondrial biogenesis and the targeted insertion of proteins into the inner membrane. What maintains the different inner membrane domains?

### 4.2. Fusion and fission

A major challenge is posed by the observation that

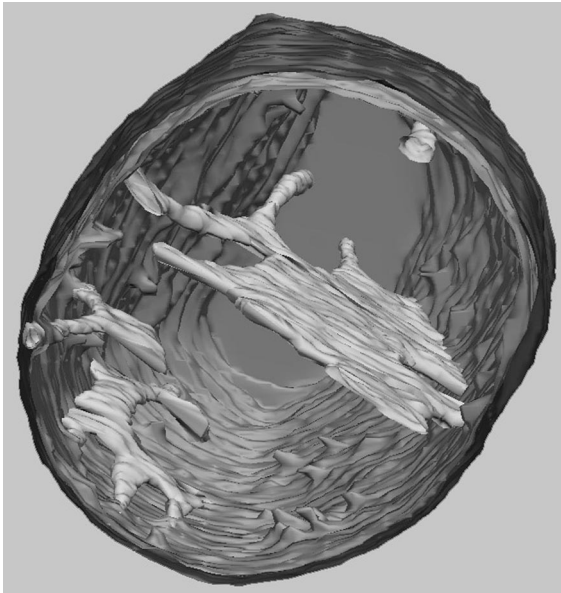


Fig. 1. Partial reconstruction of a mitochondrion as viewed by electron tomography. The outer membrane is very dark and the inner boundary membrane is shown in a lighter shade. Only a few cristae are shown, with the emphasis on a prominent lamellar shape in the center. It is connected to the inner boundary membrane by several tubular structures (crista junction). (Photograph courtesy of Drs Terry Frey and Guy Perkins, San Diego State University and UCSD).

mitochondria can undergo fission and fusion (Bereiter-Hahn and Voth, 1994). A priori one might have expected fission to occur as part of the mechanism of mitochondrial multiplication and cell division. The need for fusion is less obvious. Static images of ‘dividing’ mitochondria have been seen by electron microscopy, showing clearly the formation of a septum. It was concluded, initially from serial sections of yeast cells, and more recently from observations with live fungi (*Saccharomyces cerevisiae*, *Aspergillus nidulans*) and HeLa cells by confocal microscopy (Nunnari et al., 1997; Rizzuto et al., 1998; Suelmann and Fischer, 2000), that mitochondria form a highly dynamic continuous reticulum or network that was constantly undergoing fissions and fusions. The prediction that proteins would be continuously mixed up and exchanged between mitochondria or scrambled within the reticulum has been verified by such experiments. However, there are indications that the same cannot be said about the population of mtDNAs, with implications for the genetic conse-

quences of heteroplasmy, mtDNA segregation during cell division, and segregation of mtDNA polymorphisms from one generation to the next (see Section 8.3).

Genetic approaches in a few favorable organisms are beginning to uncover some clues about the mechanisms and proteins required for fusion/fission. The protein FtsZ has been implicated in the formation of a ring-like scaffold at the site of constriction in all dividing bacteria. Beech et al. (2000) have identified a protein named Ftz-mt in a unicellular algae, *Mallomonas splendens*. It is nuclear-encoded, but has been localized to mitochondria by immunofluorescence microscopy, and shown to contain a mitochondrial targeting signal. It is obviously tempting to ascribe to this protein a function in mitochondrial division (Beech et al., 2000; Martin, 2000). However, no such protein has been found in mitochondria from yeast or other higher eukaryotes. Another clue came from mutations in *Drosophila* that block a developmentally regulated fusion of mitochondria during spermatogenesis. The gene *fuzzy onions* (*fzo*) encodes a membrane-associated GTPase. In the absence of the *fzo* gene product mitochondria still aggregate, but fail to fuse (Fuller, 1993; Hales and Fuller, 1997). A homology cloning approach has identified an orthologous protein, Fzo1p, in yeast. In a conditional *fzo1* mutant mitochondrial fusion appears to be blocked, the mitochondrial reticulum becomes fragmented, and mitochondrial fusion is absent after mating (Hermann et al., 1998; Rapaport et al., 1998). Fzo1p is a protein spanning the outer mitochondrial membrane, with the GTPase activity in a cytoplasmic domain. However, the protein is also tightly associated with the inner membrane, a structural arrangement that may assure that both membranes participate in the fusion event in a coordinated fashion.

It is likely that fission is an event requiring internal components and structures, while fusion is the result of the interaction of surface elements in the initial phase, but made more complex by the participation of the inner membrane. Since fusion involves identical organelles, it is not immediately obvious to draw parallels to the well-studied problem of vesicle traffic between the endoplasmic reticulum and Golgi.

The maintenance of the mitochondrial reticulum in yeast depends on continuous fusions and fissions (Nunnari et al., 1997). Mutant screens for cells with abnormal mitochondrial distributions in *S. cerevisiae*

have uncovered a protein (Dnm1p) belonging to the dynamin family. Dnm1p co-fractionates with mitochondrial membranes (Bleazard et al., 1999; Otsuga et al., 1998). Dynamin was originally discovered as a protein involved in endocytosis in animal cells. A whole family of such proteins was subsequently identified and characterized as GTPases. The activity of Dnm1p is epistatic to that of Fzo1p, that is, *dnm1* mutations prevent the mitochondrial fragmentation in *fzo1* mutant strains (Bleazard et al., 1999). A closely related human protein is DRP1. Mutant DRP1 proteins, when transiently expressed in cultured cells, cause an abnormal distribution of mitochondria in the cell (Smirnova et al., 1998). A similar approach has implicated the dynamin-like DLP1 protein in maintenance of mitochondrial distribution and morphology in mouse cells (Pitts et al., 1999).

#### 4.3. Distribution of mitochondria in the cell

The dynamic behavior of mitochondria in a cell, their movement and their morphology, is now recognized to be the result of intricate interactions of proteins on the outer surface and various components of the cytoskeleton that include actin filaments, microtubules and intermediate filaments (Scheffler, 1999). Such interactions can lead to actual movement of an organelle, or, if the entire structure forms an extended reticulum, the overall shape and distribution of the reticulum within the cell can be determined in a highly dynamic fashion by such repeated and possibly short-lived interactions (Yaffe, 1999).

Our understanding of the fundamental structural properties of microtubules, microfilaments and intermediate filaments is relatively well-advanced. Numerous proteins are associated with these diverse filaments, and among those a group of proteins referred to as molecular motors has achieved a prominent position. Type I myosins can move vesicles along actin filaments. F-actin filaments have been shown to support axonal transport of mitochondria. Retrograde and anterograde transport have been observed. Several studies suggest that mitochondrial movement in neurites may involve a diverse set of motors acting on microtubules or microfilaments at different intrinsic speeds and directions (Lee and Hollenbeck, 1995; Morris and Hollenbeck, 1993, 1995; Overly et al., 1996). During axonal outgrowth

mitochondria have to be moved primarily from the soma towards the growth cone. What happens in mature neurons? Do mitochondria turn over at the nerve terminal, to be replaced, or are mitochondria shuttling back and forth during their lifetime?

Dyneins and kinesins are families of microtubule-associated motors distinguishable by the direction of movement. With reference to mitochondria, several kinesin-like proteins were identified and implicated in mitochondrial behavior (Conforti et al., 1999; Gong et al., 1999; Khodjakov et al., 1998; Pereira et al., 1997; Tanaka et al., 1998). It should be noted that kinesins typically have two heavy and two light chains; the C-terminus of the heavy chains in combination with the light chains determines specificity and binding to organelles/vesicles. Thus, heavy and light chain genes for mitochondria-associated kinesins must be identified

A problem closely related to the mitochondrial distribution within a cell is the (equal) segregation of mitochondria during cell division. This may seem less of a problem in animal cells dividing by cytokinesis, but it becomes very apparent in budding yeast. The Yaffe lab has pioneered in the study of mutants (*mdm* mutants) that are defective in mitochondrial inheritance (Berger and Yaffe, 1996; Yaffe, 1999). Some of the Mdm proteins (Mdm10p, Mnn1p, Mdm12p, and Mdm17p alias Mgm1p) have been shown to be components of the outer membrane and thus are undoubtedly responsible for the interaction of mitochondria with cytoskeletal structures and motors. Mdm17p (alias Mgm1p) is yet another dynamin-like protein. Other Mdm proteins (Mdm1p, Mdm14p, Mdm20p) are found in the cytoplasm. Mdm1p is a component of a novel cytoskeletal structure. In vitro the Mdm1 protein can form structures reminiscent of intermediate filaments (not previously known to exist in *S. cerevisiae*), but the precise in vivo structure is not completely clear.

While microtubules play apparently no role in the mitochondrial distribution in *S. cerevisiae* (Huffaker et al., 1988), the situation is different in *Schizosaccharomyces pombe* (Yaffe et al., 1996; Yaffe, 1999). Outer membrane components identified in *S. cerevisiae* are conserved in *S. pombe*, suggesting that a similar set of components in the two types of mitochondria can interact with diverse cytoskeletal structures in different organisms (Yaffe, 1999).

#### 4.4. Future prospects

It is clear that only a small number of genes/proteins responsible for mitochondrial morphology and distribution have been identified so far, and this number can be expected to increase from genetic experiments in yeast and other organisms, combined with a growing data base of sequence information. Homology searches may help in expanding insights from one organism to another, but as some of the examples have illustrated, some caution is in order with extrapolations.

A new direction is indicated by the finding that protein ubiquitination, mediated by the ubiquitin ligase Rsp5p, is essential for mitochondrial inheritance and normal morphology in *S. cerevisiae* (Fisk and Yaffe, 1999). Defining targets for this protein modification is obviously one significant goal, and understanding the control of the overall process is another.

### 5. Mitochondria and molecular genetics

Finding mtDNA in mitochondria was clearly a milestone (Nass and Nass, 1963). It follows that mtDNA must be replicated and transcribed for mitochondrial multiplication. Mechanisms for recombination and DNA repair must also be considered.

Almost all the relevant genes are nuclear genes. One group includes genes specifying proteins such as the DNA polymerase, and proteins required for recombination and repair, and RNA polymerase, RNA processing enzymes, and all the proteins required for an autonomous protein synthesis in the matrix. Nevertheless, yeast and mammalian cells have been described with mitochondria that have no mtDNA (so-called  $\rho^0$  cells). Similarly, hamster cells have been described with severe defects in mitochondrial protein synthesis (Scheffler, 1999). Therefore, DNA replication and protein synthesis may not be absolutely essential for mitochondrial multiplication. A large group of genes encode proteins required for protein import into mitochondria. Since there are multiple pathways for diverse proteins (e.g. matrix vs. inner membrane proteins, for example), a deficiency in one such gene may not eliminate mitochondria completely, but the functional consequences may

also be severe. Finally, there are  $\sim 1000$  proteins in mitochondria participating in a wide variety of biochemical pathways. Defects in any one of these genes will not affect mitochondrial biogenesis, but at the level of the whole organism such mutations can be the cause of serious diseases.

#### 5.1. DNA replication

DNA replication in animal cells is quite well understood, thanks to pioneering work by Clayton and his colleagues (Shadel and Clayton, 1997). Replication starts at one origin ( $O_H$ ) with an RNA primer transcribed from the light-strand promoter (LSP). To make the first primer requires the mtRNA polymerase and the transcription factor mtTFA (Shadel and Clayton, 1997). The synthesis of a short transcript opens an R-loop. The transcript is then processed by the mitochondrial RNA processing enzyme to form a primer, which is elongated by a DNA polymerase. The result is a larger D-loop ( $\sim 1$  kb) that is of distinct size and was first recognized in the electron microscope because DNA replication pauses at this point. Eventually, elongation proceeds to make the H-strand. Half way around the circle, when replication has passed the origin ( $O_L$ ) for L-strand synthesis, the resulting secondary structure of the displaced single stranded DNA attracts the mtDNA primase for the synthesis of a second primer from which the L-strand is elongated. Removal of the RNA primers and ligation of the completed DNA circles finishes the replication process (for details see Shadel and Clayton (1997)). The origin for the heavy strand synthesis and the two promoters for transcription from the heavy and light strands are located within a 'control region' of  $\sim 1$  kb in size that is flanked by the tRNA<sup>Phe</sup> and tRNA<sup>Pro</sup> genes. Within it a few short sequence boxes have been characterized to be species specific and required for R-loop formation and RNA processing, but much of this sequence is thought to be genetically neutral and hence full of sequence polymorphisms.

DNA replication is relaxed in cells in culture, meaning that it is not strictly coupled to nuclear DNA replication during S-phase. Not all mtDNAs are replicated in synchrony, and some may be replicated twice during a cell cycle, while others are not replicated at all. It has been shown that (a) mtDNA can be replicated in differentiated quiescent cells, and

(b) mitochondria in the vicinity of the nucleus are more likely to replicate their mtDNAs than mitochondria located in peripheral regions of the cell (Davis and Clayton, 1996). These observations raise several interesting questions. There must also be a mechanism to control the copy number.

Replication of mtDNA in fungi and plants is less well understood with regard to origins and replication intermediates, but the enzymes involved are likely to be similar. The pol  $\gamma$  gene (*MIP1*), genes for two transcription factors (sc-mtTFA and sc-mtTFB), and the gene for the processing RNase (*NME1*) have been cloned in yeast.

#### 5.1.1. DNA repair

It had been surmised for some time that damage to mtDNA from reactive oxygen species (ROS) might lead to the accumulation of abnormal bases in mtDNA, and such cumulative damage is hypothesized to be a factor in aging, neurological degeneration, and possibly cancer. At the same time it had been technically challenging to demonstrate the existence of repair mechanisms, and it became a widely held assumption that mitochondrial DNA repair mechanisms were poorly developed or inefficient. This view is beginning to change as the result of the use of more sensitive assays, and from the finding of mitochondrial enzyme activities associated with DNA repair (Croteau and Bohr, 1997; Croteau et al., 1999; LeDoux et al., 1999; Sawyer and Van Houten, 1999).

#### 5.2. Transcription

Two transcription start sites (LSP and HSP) for transcription of the heavy and light strands are located in close proximity in the control region of mammalian mtDNA. Short transcripts from the LSP can be converted into primers for DNA replication, or they can be elongated to include the entire mtDNA circle. Similarly, the transcript in the other direction is a single, long, polycistronic transcript. Micol et al. have described a mitochondrial transcription termination factor (Micol et al., 1996). Endonucleolytic cleavage of both primary transcripts produces 22 tRNAs, 12 S and 16 S rRNAs, and 13 mRNAs. (Attardi, 1993; Attardi and Schatz, 1988). The whole mechanism has been aptly described as ‘the tRNA punctuation model of RNA processing’ (Ojala et al., 1981). All mRNAs

and even rRNAs in mammalian mitochondria are polyadenylated; the mammalian mRNAs have extremely short 5' and 3' untranslated regions. In other organisms with larger mtDNAs there are many more promoters, and polycistronic transcripts are not the rule. Thus, multiple promoters can be identified, such as the 17 nucleotide promoter in monocot plant mitochondria containing the characteristic CRTA motif at the transcription initiation site (Binder et al., 1996). For short transcripts from very large templates one also requires a mechanism for transcriptional termination. Introns have been found in transcripts in fungi and plants. The subject has been expertly reviewed for yeast (Costanzo and Fox, 1990) and plants (Bonen, 1991), and a recent book on the *Molecular Biology of Plant Mitochondria* (Levings III and Vasil, 1995) is a particularly rich source of information.

#### 5.3. RNA editing

A fascinating phenomenon that has complicated the identification of specific open reading frames in mtDNA from various organisms is RNA editing. Editing is inferred from the observation of a different primary sequence in the DNA template and the RNA transcript. In the simplest case, common in plant mitochondria and chloroplasts, a deamination reaction converts a cytidine to a uridine (C  $\rightarrow$  U), and rarely a uridine to a cytidine. To cite one recent comprehensive study, 456 C to U conversions were identified in the *Arabidopsis thaliana* mt transcripts, of which 441 were in open reading frames (ORFs), eight in introns, seven in UTRs, and none in rRNA or tRNAs (Giege and Brennicke, 1999). Editing affected various ORFs to different degrees (between 0 and 18.9% of the codons). No significant sequence patterns surrounding the editing sites were discerned.

A much greater challenge was the unraveling of RNA editing in kinetoplastid protozoa (Alfonzo et al., 1997; Benne, 1996). The insertion (or deletion) of one or more uridines at many locations can in some cases almost double the length from the primary transcript to the final mature mRNA. The primary transcript is untranslatable literally and figuratively. The editing proceeds in a systematic manner from the 3' end to the 5' end and requires the participation of so-called guide RNAs. Protein coding transcripts are derived from a maxicircle mtDNA, while guide

RNAs are transcribed from either the maxicircle or from many different minicircles. A complete in vitro system with purified components remains a goal for the future.

Another aspect of this phenomenon is to place it into an evolutionary perspective and to explain when it evolved and why (Simpson and Thiemann, 1995). Kinetoplastic protozoa are representatives of the earliest divergence of eukaryotic cells, and they are the only organisms with extensive ‘pan-editing’. A postulated relationship of pan-editing to splicing has been rejected based on our current understanding of the different mechanisms.

#### 5.4. Translation

It is noteworthy that more than 20 years after the recognition of an autonomous translation system in mitochondria there has been no success in establishing an in vitro translation system with components derived exclusively from mitochondria. Today it is not so obvious why there is a problem, except for the practical challenge of preparing sufficient quantities of mitochondria to prepare mRNAs, tRNAs, ribosomes and ‘factors’. Elongation factors with a strong resemblance to bacterial factors have been purified and their genes cloned (Clark et al., 1996; Worlax et al., 1997, 1996; Zhang and Spremulli, 1998). A major gap exists in our understanding of the initiation step. Only one initiation factor (IF-2<sub>m</sub>) has been characterized. Mammalian mt mRNAs have exceptionally short or no 5′ UTRs, and they have no 5′ cap. There are also no Shine–Dalgarno sequences for positioning the small ribosome on the mRNA.

In contrast, in yeast, *S. cerevisiae*, and in plant mitochondrial mRNAs, significant 5′ and 3′ UTRs exist. Yeast mutants defective in mitochondrial translation factors have been found (Fox, 1996). The unexpected discovery was made that specific nuclear-encoded factors exist for the translation of individual transcripts such as the *COX3* mRNA. These unique translational activators appear to bind to sequence elements in the 5′ UTR. They are also associated with the inner membrane, and one can speculate about their role in initiating co-translational membrane insertion of integral membrane proteins made in the matrix (Sanchez et al., 1998; Scheffler, 1999). Another recent paper (Green-Willms et al.,

1998) provides evidence for recognition of a feature of the 5′ UTR by the small ribosomal subunit, based on mutations in *MRP21* and *MRP51* genes that encode proteins associated with this ribosomal subunit. In *mrp21* or *mrp51* cells mitochondrial protein synthesis is completely blocked. These proteins therefore play a general role, in contrast to the mRNA-specific translational activators.

#### 5.5. Mitochondrial protein import

Since the vast majority of mitochondrial proteins are encoded by nuclear genes and synthesized in the cytosol, their import into mitochondria, and their correct targeting to either the matrix, or the intermembrane space, or the inner or outer membranes became a problem that was recognized some time ago. Much progress has been made (Bauer et al., 2000; Koehler et al., 1999a; Neupert, 1997; Pfanner, 1998; Schatz, 1998).

The most significant features of this process can be summarized as follows:

1. Protein import can be post-translational in vitro, but in vivo there is evidence that co-translational import is possible; it is unclear whether it is the rule.
2. Imported proteins typically have N-terminal leader sequences that are cleaved off during import. In some cases this proteolytic processing occurs in two steps (Hendrick et al., 1989).
3. Mitochondrial leader sequences (also referred to as targeting sequences or signal sequences) have no recognizable amino acid sequence motif, but common features include a net positive charge and the ability to form amphiphilic alpha helices (Hammen and Weiner, 1998).
4. A small number of proteins (e.g. some integral membrane proteins of complex I) are imported without further processing (Walker et al., 1992). Some proteins such as integral membrane carrier proteins contain multiple internal import signals.
5. Protein import into the matrix and intermembrane space and insertion into the inner membrane requires a membrane potential.
6. Proteins to be imported are unfolded on the outside with the help of chaperones (cytoplasmic hsp70), and matrix proteins are also first received by a matrix hsp70 chaperone and then delivered to a

- large complex (chaperonin) composed of hsp60 and cpn10 peptides. The latter assists in proper folding and assembly to the native functional structure(s).
7. Integral membrane multi-subunit complexes exist, or are assembled in both the outer and inner membranes to provide channels for the peptide chains to be translocated across these membranes. These complexes have been referred to as TOM and TIM complexes for the outer and inner membrane, respectively. In one model, TOM and TIM complexes are transiently (?) associated to form a continuous channel for the translocation of a soluble/hydrophilic protein from the cytoplasm to the matrix. Individual subunits in the outer complex are referred to as Tom70, Tom40, Tom22, Tom20, Tom5, Tom6, and Tom7 (where the number indicates the molecular mass in kDa).
  8. It is becoming clear that there are two distinct TIM complexes, one (TIM23) for import of soluble matrix proteins, and the other (TIM22) for the import and membrane insertion of integral inner membrane proteins with multiple transmembrane helices (Bauer et al., 2000). TIM23 is composed of subunits Tim23, Tim17 and Tim44, while TIM22 has subunits Tim22 and Tim54.
  9. A more recent insight is the recognition of the role of hetero-oligomeric protein complexes localized in the intermembrane space. The [Tim9]<sub>3</sub>[Tim10]<sub>3</sub> and the [Tim8]<sub>3</sub>[Tim13]<sub>3</sub> complexes appear to be soluble, while the [Tim9]<sub>3</sub>[Tim10]<sub>2</sub>[Tim12] complex is more firmly associated with the TIM22 integral membrane complex. Thus, TOM and TIM23 may form a continuous channel from the cytosol to the matrix, and the imported protein is guided directly from TOM to TIM23. Carrier proteins such as the adenine nucleotide transporter (ANT) are thought to be transiently associated with the intermembrane complexes before being delivered to TIM22 for insertion into the membrane. Interest in the Tim8 protein and structurally related members (Tim9, Tim10, Tim12, Tim13) has been heightened by the discovery that a rare, X-linked neurodegenerative disorder, the Mohr–Tranebjaerg syndrome is due to a defective Tim8a subunit that had also been identified as the deafness/dystonia peptide DDP1 mapped at Xq22 (Jin et al., 1996; Koehler et al., 1999b; Tranebjaerg et al., 1995).

Special attention must be paid to the insertion into the inner membrane of proteins made in the mitochondrial matrix. The protein Oxa1p was initially identified as an inner membrane protein required for cytochrome oxidase assembly in yeast. More recently (see Hell et al. (1998)) for additional references) it was shown to be essential for the proper membrane integration and orientation for all membrane proteins made in the matrix; they are exported with their N-terminus leading and then extending into the IMS. Even nuclear-encoded proteins have been found with their N-terminus in the IMS, and it is postulated that they are first imported into the matrix and then exported. The resemblance to bacterial N-tail export has been noted, and energetic considerations of the process are consistent with the ‘positive-inside rule’ (Stuart and Neupert, 1996).

An area where progress has been relatively slow is the elucidation of the mechanisms required for the assembly and functional maturation of the complexes of the electron transport chain. In the simplest case of complex II, flavin has to be covalently linked to the Fp protein (Robinson et al., 1994), three iron-sulfur clusters have to be formed in the Ip subunit (Lill et al., 1999; Schilke et al., 1999), and a b-type heme group has to be incorporated into the membrane anchor proteins. What is the order of assembly? The problem becomes considerably more complicated for the other complexes, most of all for complex I (NADH-ubiquinone oxidoreductase) with ~42 different subunits, seven of which are encoded by mtDNA.

Mutational analysis is coming to the rescue again in organisms like yeast, where increasing numbers of genes are being identified as necessary for the assembly of cytochrome oxidase (complex IV) or ATP synthase (complex V) (Church et al., 1996; Glerum et al., 1997; Hell et al., 2000; Tzagoloff and Dieckmann, 1990). However, complex I is absent in *S. cerevisiae*, and other organisms or approaches must be exploited.

## 6. Mitochondria and energy metabolism

### 6.1. The electron transport chain

Major milestones in biochemistry, cell biology and bioenergetics were achieved in the study of mitochondria (Scheffler, 1999). The significant findings related to the Krebs’ cycle, electron transport and oxidative phos-

phorylation will be familiar to most readers. The chemiosmotic hypothesis has triumphed over alternatives. An expert review of 'oxidative phosphorylation at the fin de siècle' has been presented by Saraste (1999).

Within the last 5 years the X-ray structures of complexes III and IV have been solved (Iwata et al., 1995; Tsukihara et al., 1995; 1996; Xia et al., 1997; Yoshikawa et al., 1998; Yu et al., 1998), while the structure of complex II is so far available indirectly and incompletely from the structure of fumarate reductase from *Escherichia coli* (Iverson et al., 1999).

#### 6.1.1. What remains to be solved?

One problem that has been greatly stimulated by the available structures, but not solved unambiguously, is the path of the protons through the complex when proton pumping is coupled to electron transport (complexes I and IV). This problem is intimately linked to the coupling mechanism itself. Complex I, with 42 distinct subunits, remains a major challenge for the structural biochemist and crystallographer. Medium resolution three-dimensional models of isolated and negatively stained complex I particles from *E. coli* and *Neurospora crassa* were calculated from electron microscopy using the random conical tilt reconstruction technique (Guenebaut et al., 1998). The complex contains ~28 'accessory' proteins in search of a function. In a few cases it has been shown that these proteins are not simply accessory, but essential for assembly or activity (Runswick et al., 1991; Schneider et al., 1995; Au et al., 1999).

Complex II, the simplest of all the complexes, is not involved in proton pumping (Scheffler, 1998). An interesting property of the complex is its diode like-behavior (Sucheta et al., 1992). In certain protozoa, e.g. parasitic helminths, a developmentally controlled switch in isozymes for one or more subunits (and a switch from ubiquinone to rholoquinone) may bring about the conversion from succinate dehydrogenase to fumarate reductase (Tielens and Van Hellemond, 1998).

Complex III in mammals has ten subunits (eight in yeast), of which one (cyt b) is encoded by the mt genome. The functionally important subunits are the cytochrome *b* with two heme groups ( $b_{562}$  and  $b_{566}$ ), the iron-sulfur protein (Rieske factor), and the cytochrome  $c_1$ , and those are the only subunits found in bacteria. Curiously, in *Neurospora* and potato two large peripheral subunits are processing proteases and

they play a role in protein import. Proton translocation in complex III has been discussed most prominently since the time when P. Mitchell proposed the so-called Q-cycle. Its modified, currently accepted form has been formulated by Trumppower (Brandt and Trumppower, 1994; Trumppower, 1990). In the Trumppower model, in effect, two  $QH_2$  molecules are oxidized sequentially to form two reduced cytochrome *c* molecules, and two quinones, one of which is reduced again to quinol. The crystal structure has verified two sites for Q binding,  $Q_P$  and  $Q_N$ , but details of the mechanism at each Q-site are still controversial (Crofts and Berry, 1998; Matsuno-Yagi and Hatefi, 1997a,b). How can two electrons from quinol be directed into two separate pathways?

The crystal structure of bovine complex IV (cytochrome oxidase) was presented in two landmark papers in 1995 and 1996 (Tsukihara et al., 1995, 1996), with further refinements and interpretations in 1998 (Yoshikawa et al., 1998), while the structure of the simpler *Paracoccus denitrificans* complex was described at about the same time (Iwata et al., 1995). The crystal structure clarified the two metal centers with Mg and Zn, and the precise path for the electrons. Detailed aspects of the last reaction at the binuclear heme  $a_3$ - $Cu_B$  center are still subject to some heated debate. Cytochrome oxidase has been the object of many studies aimed at understanding its assembly (with genetic approaches primarily in yeast), and the control of its activity. The debate continues about whether small molecules like ATP, ADP, etc. can exert a direct control over the activity of specific complexes (Arnold and Kadenbach, 1999; Kadenbach et al., 1997). Similarly, complex IV contains tissue-specific isozymes/subunits that invite speculations about regulatory mechanisms (Kadenbach et al., 1990). Finally, the issue has been raised that the pump may not work with a fixed H/O ratio, and this slippage mechanism may even have some regulatory significance.

Finally, there are indications that complexes do not float in the lipid layer of the inner membrane totally independent of each other. The formation of supramolecular complexes forming a respirasome has been proposed recently (Schagger and Pfeiffer, 2000).

#### 6.2. Control of oxidative phosphorylation

A thoughtful and informative review with many

primary references on the subject concludes with 'There is no simple answer to the question 'what controls respiration?'' (Brown, 1992). For decades biochemists have investigated enzymes with the view to determining binding constants for substrates, products and inhibitors,  $v_{\max}$  or turnover rates, feedback control, allosteric mechanisms and the relevant ligands, regulation of activity by protein modifications (e.g. phosphorylation). Ideas such as rate limiting enzymes in a pathway (or 'bottlenecks') evolved. A new look at the bigger picture including whole pathways integrated into a cell has led to the more sophisticated theoretical framework referred to as metabolic control analysis (Kacser, 1995; Kacser and Burns, 1997). Formal and explicit applications of flux control theory to mitochondria can be found in the papers by Brand and colleagues (Brand and Kessler, 1995), Soboll et al. (1998) and others (Boumans et al., 1998; Letellier et al., 1998; Mazat et al., 1997; Wiedemann and Kunz, 1998).

A distinction should be made between long term control (related to differentiation, for example) and short term control. Short-term control mechanisms allow a cell to adjust its energy metabolism to demands that can fluctuate rapidly (Fig. 2). Such demands include not only ATP production, but also various biosynthetic activities of mitochondria, regulation of cellular calcium levels, etc. To give just one example, high levels of NADH in mitochondria cause feedback inhibition of pyruvate dehydrogenase and  $\alpha$ -ketoglutarate dehydrogenase and hence can shut down the Krebs' cycle. Assuming that NADH and  $O_2$  are present in abundance, is therefore the electron transport chain the rate-limiting component in respiration, and is there a specific complex within the ETC that dominantly controls its activity? How? It could be present in limiting amounts, or its activity could be subject to feedback mechanisms. The obvious factor is the  $\Delta\mu_H$ , since electron transport is coupled to proton pumping against this gradient. In the absence of a dissipating reaction respiration will come to a stop, as observed long ago when it was found that ADP and  $P_i$  were required for 'state 3' respiration in intact mitochondria. Under physiological conditions the availability of ADP may become rate-limiting if biological work does not turn over the ATP, or if the adenine nucleotide transporter (ADP/ATP antiporter) is too slow. Substrate level phosphorylation from

glycolysis should perhaps also be included in the big picture. The activity of complex V can control  $\Delta\mu_H$  and therefore respiration. Both ATP and ADP have been shown to be allosteric effectors of cytochrome oxidase (Arnold and Kadenbach, 1999; Rohdich and Kadenbach, 1993; Anthony et al., 1993). Less well explored is the possibility that subunits of electron transport complexes may be phosphorylated by specific kinases as part of a regulatory mechanism. In the absence of ADP and  $P_i$ ,  $\Delta\mu_H$  can be dissipated by artificial uncouplers such as dinitrophenol or FCCP, by the natural uncoupling proteins, UCP1, UCP2, UCP3, or by other transport processes for nucleotides, ions, and other small molecules.

### 6.3. ATP synthase: structure and function

An illustrious series of studies on ATP synthase (complex V) by many laboratories were crowned in 1994 by the publication of a 2.8 Å resolution structure of the  $F_1$  ATPase (Abrahams et al., 1994). The subunit composition of the  $F_1$  subcomplex and its three-fold rotational symmetry (ultimately verified by the crystal structure) with respect to the three  $\alpha$  and  $\beta$  subunits supported increasingly detailed speculations about the mechanism of ATP synthesis and hydrolysis (Fig. 3). A rotating three site model was first proposed by Boyer (Gresser et al., 1982), with refinements added over the years (Boyer, 1997; Weber and Senior, 1997). A spectacular confirmation combining insights from biochemical studies and the crystal structure was provided by Noji and colleagues (Noji et al., 1997) who were able to visualize directly the rotation of the  $\gamma$ -subunit driven by ATP hydrolysis. Elegant molecular modeling and structural changes responsible for energy transduction have been described by Oster's laboratory (Elston et al., 1998; Wang and Oster, 1998), and several recent reviews have advanced and refined the interpretation of structure and mechanism (Bianchet et al., 1998; Kagawa, 1999; Nakamoto et al., 1999; Sambongi et al., 1999; Stock et al., 1999). In an elaboration of the experimental approach of Noji et al., Sambongi and colleagues have now demonstrated directly that the  $\gamma$ -subunit and the c oligomer ring rotate together during ATP hydrolysis (Sambongi et al., 1999).

A mystery yielding only slowly to biochemical and structural analyses is the precise arrangement

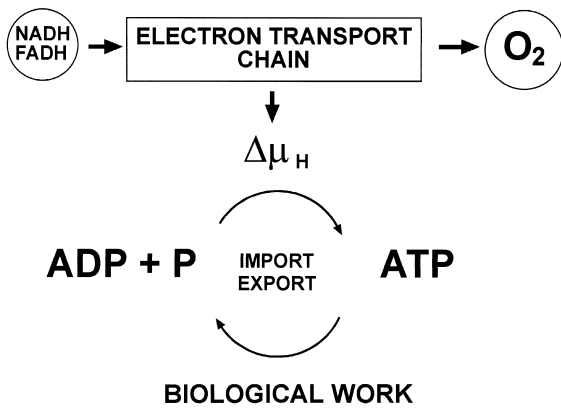


Fig. 2. Control of electron transport and oxidative phosphorylation. For a detailed discussion see the text.

of the c-subunits in the integral membrane  $F_0$  subcomplex, and the precise positioning of the a-subunit, two b-subunits, the  $\delta$ -, and  $\epsilon$ -subunits, and others<sup>1</sup> (Hausrath et al., 1999; Wilkens and Capaldi, 1998). Stock et al. (1999) have recently described the structure of the purified  $F_1F_0$  complex from yeast (13 subunits). After crystallization, only the  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -,  $\epsilon$ - and c-subunits were detected in the crystal. The most surprising finding was that the complex contained only ten c-subunits. Previous arguments had favored nine or 12 to satisfy symmetry considerations and to accommodate the estimated  $H^+/ATP$  ratio of 3 or 4. A principle of symmetry mismatch may be a common feature of molecular rotary engines, since it has also been found in other systems. The experimental number of ten c-subunits per ring suggests that the  $H^+/ATP$  ratio may be non-integer. A further interpretation of a non-integer number requires an understanding of the mechano-elastic properties of the whole ensemble and further proof that the entire c ring rotates in ten steps relative to the three steps of the  $\alpha_3\beta_3 F_1$  subcomplex (Hausrath et al., 1999; Junge et al., 1997; Wilkens and Capaldi, 1998).

#### 6.4. Ion transport across the inner membrane

With the major focus on protons, the transport of other small molecules and ions across the inner membrane often escapes attention. Alternate routes for protons, and the uptake of the cations  $Na^+$ ,  $K^+$ ,

$Ca^{2+}$ ,  $Mg^{2+}$ , and  $Fe^{2+}$  deserve attention, and special considerations also apply to the transport of  $P_i$ , ADP, and ATP (Scheffler, 1999). Protons can cross the inner membrane as participants in antiport transport mechanisms, for example  $Na^+/H^+$  antiport,  $K^+/H^+$  antiport,  $1Ca^{2+}/2H^+$  antiport, in the import of  $P_i$ , and by the activity of the nicotinamide nucleotide transhydrogenase (Hatefi and Yamaguchi, 1996).

There are by now at least three uncoupling proteins (UCP-1, UCP-2, and UCP-3) (Fleury and Sanchis, 1999; Garlid and Jaburek, 1998; Gonzalez-Barroso et al., 1998; Jaburek et al., 1999; Jezek and Garlid, 1998; Ricquier, 1998). UCP-1 or thermogenin was discovered in brown adipose tissue where it serves as a proton leak to uncouple electron transport from ATP synthesis and hence in thermoregulation in newborns and hibernating animals (Matthias et al., 1999; Nedergard and Cannon, 1992; Nicholls and Locke, 1984; Nicholls and Rial, 1999; Silva and Rabelo, 1997). Interest in the uncoupling proteins arises also from a highly plausible connection to problems relating diets and weight control (Lowell and Spiegelman, 2000; Matthias et al., 1999). The structure of these proteins places them in a larger mitochondrial carrier family including the ANT (Klingenberg, 1976, 1993; Klingenberg et al., 1995; Garlid et al., 1998; Jaburek et al., 1999; Jezek and Garlid, 1998).

$Na^+$  ions can enter the mitochondria via a simple, unregulated  $Na^+/H^+$  antiport or via a more complicated  $Na^+/Ca^{2+}$  antiporter (Brierley et al., 1994; Garlid, 1994). There are at least three mechanisms for  $K^+$  uptake: an electroneutral  $K^+/H^+$  antiport, an ATP-regulated  $K^+$  channel, and another 'leakage channel' driven by the  $\Delta\Psi$ . The purified  $K^+$  channel (mitoK<sub>ATP</sub><sup>+</sup>) has been studied by Garlid's laboratory (Paucek et al., 1996; Yarov-Yarovoy et al., 1997). These potassium channels most likely play a dominant role in mitochondrial volume control and in the maintenance of the architecture of the inner membrane and intermembrane space.

<sup>1</sup> There is some risk of confusion here. Studies have been carried out with bacterial, yeast, mammalian and plant enzymes. They all have the  $\alpha$ -,  $\beta$ -,  $\gamma$ -, a-, b-, c-subunits, but they may differ in the number of additional subunits. The nomenclature is also not always consistent: bacterial and chloroplast  $\epsilon$ -subunits are equivalent to the mitochondrial  $\delta$ -subunits.

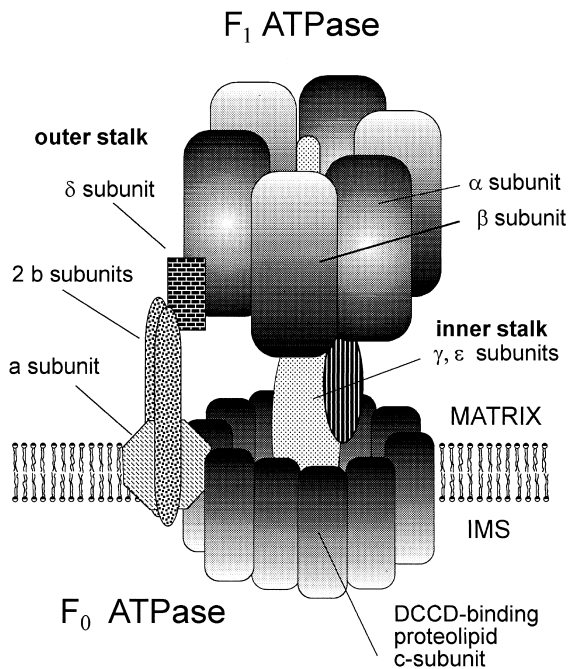


Fig. 3. Schematic view of the ATP synthase in the inner mitochondrial membrane. The F<sub>1</sub> subcomplex is made up of the α-, β-, and γ-subunits, and possibly others, depending on the method of isolation. The F<sub>0</sub> subcomplex contains the a-, b- and c-subunits. See text for further discussion.

Another ion with a significant relationship to mitochondria is calcium (Hansford, 1994). First of all, mitochondria constitute a buffering system for modulating cytosolic calcium, and under certain conditions they can become overwhelmed, for example, in glutamate-mediated excitotoxicity in neurons (Brustovetsky and Dubinsky, 2000; Castilho et al., 1999; Cheng et al., 1999; Dubinsky and Levi, 1998; Nicholls et al., 1999; Nicholls and Budd, 2000; Reynolds, 1999). In addition, free Ca<sup>2+</sup> ions in mitochondria are important regulators of various metabolic enzymes, among them the dehydrogenases of the Krebs' cycle. Gunter and Gunter have presented an expert review of the energetics of Ca<sup>2+</sup> transport in relation to ΔΨ (Gunter and Gunter, 1994). Elegant methods for measuring cellular and mitochondrial calcium concentrations have been developed in recent years (Tsien and Poenie, 1986; Jouaville et al., 1999; Rizzuto et al., 1992, 1998; Rutter et al., 1996).

The role of iron in the proteins of the electron transport chain has long been emphasized with reference to the cytochromes and the iron-sulfur centers. The elucidation of iron transport into mitochondria and its incorporation into hemes and [Fe-S] clusters is of more recent origin from the use of the yeast as a model system for genetic and biochemical studies (Askwith and Kaplan, 1998; Eide, 1998; Hentze and Kuhn, 1999; Kaplan, 1999; Li et al., 1999; Link et al., 1998; Radisky et al., 1999; Schilke et al., 1999). One of the noteworthy outcomes of such studies was the recognition of Friedrich's ataxia as a mitochondrial disorder (Kaplan, 1999) and the cloning of the yeast homologue frataxin. An apparatus for [Fe-S] cluster biosynthesis in yeast resembles the bacterial complex. It contains at least ten subunits including a cysteine desulfurase to produce elemental sulfur, a ferredoxin involved in reduction, and two chaperones (Lill et al., 1999). It is also noteworthy that [Fe-S] proteins are found in the cytosol and in the nucleus. The combination of iron and sulfur for such proteins is initiated in mitochondria from which they are exported by the ABC transporter (Atm1p) of the inner membrane. Earlier investigations had already clarified the role of cytosolic aconitase as the iron-responsive protein (IRP) and its role in regulating stability or translatability of specific mRNAs (Casey et al., 1988; Harford and Klausner, 1990; Hentze and Kuhn, 1999; Hentze et al., 1988; Klausner et al., 1993; Philpott et al., 1994).

Finally, the ANT has been of obvious interest for a long time as an essential function for the exchange of ATP/ADP between the mitochondrial matrix and the cytosol. More recently attention has been accentuated because of its central role in forming the permeability transition pore (see below). Its structural relatedness to a family of mitochondrial carrier proteins (UCP-1, -2 and -3) has already been noted (Klingenberg, 1993). At least three isoforms of the nucleotide transporter (ANT-1, -2 and -3) in mammals are expressed differentially in various tissues. It is one of the more abundant proteins in the inner mitochondrial membrane. A homodimer with 12 transmembrane segments constitutes the functional carrier (antiporter). Two well known inhibitors, bongkreikic acid and atractyloside, bind to opposite sites of the carrier (bongkreikic acid binds to the matrix side), but not both at the same time (Klingenberg et al., 1995).

### 6.5. The mitochondrial permeability transition (MPT)

The mitochondrial permeability transition has been operationally defined as the opening of a ‘megapore’ that permits solutes of less than  $\sim 1500$  Da to cross the inner membrane freely, causing the breakdown of the membrane potential. Fluorescence of dyes whose uptake is dependent on  $\Delta\Psi$  is frequently used to follow the MPT, particularly in connection with the study of apoptosis (Bernardi et al., 1998; Crompton, 1999; Scheffler, 1999) and excitotoxicity. While it seems clear that the MPT in intact cells is a key event in the apoptotic pathway, the precise signaling mechanism that induces it is not yet worked out. A continuing challenge is to define the components of the pore precisely, because the transient nature of the pore has made it difficult to isolate. It is also necessary to recognize clearly that  $\Delta\Psi$  is a property of the inner membrane and that the PT occurs there. However, proteins located in the outer membrane or binding to the outer membrane have also been implicated, and this relationship requires further clarification. A major component is thought to be the ANT, because the MPT is inhibited by bongkreikic acid and favored by atractyloside. How is this homodimer with a normally restricted ‘channel’ for ATP or ADP converted to a megapore? (Ruck et al., 1998). Complexes between the voltage-dependent anion channel (VDAC) in the outer membrane, hexokinase (cytosol), mitochondrial creatine kinase and ANT have been proposed to constitute the permeability transition pore (Beutner et al., 1998a,b). Another suggestive observation requiring further clarification is the inhibition of the MPT by cyclosporin A (Doyle et al., 1999). This inhibitor is targeted to cyclophilin (a peptidyl-proline *cis-trans* isomerase, PPIase); however, multiple cyclosporin A-binding proteins have been fractionated from mitochondria (Woodfield et al., 1998). One of these, cyclophilin D, has also been shown to bind strongly to the VDAC (Crompton et al., 1998).

A further insight is that mitochondria release cytochrome *c* and an apoptosis inducing factor (AIF) (alias Apaf-1) (Susin et al., 1999), causing caspase 9 activation and the subsequent cascade of proteolytic reactions. There is still some discrepancy between findings that suggest that cytochrome *c* can be released prior to the MPT, and others that suggest the MPT precedes the release of cytochrome *c* (for

example Heiskanen et al. (1999); Zhuang et al. (1998)). It should be kept in mind that AIF and cytochrome *c* are localized in the IMS, and their release requires an opening in the outer membrane. A possible scenario is that the outer membrane is physically ruptured by swelling of the matrix volume. Finally, it should be mentioned that, for example, Fas-induced apoptosis does not depend on mitochondrial changes, and Bcl-2 fails to protect cells against such a stimulus (Huang et al., 1999).

Members of the Bcl-2 family of proteins are protective, while Bax and other related proteins are pro-apoptotic (Kluck et al., 1997; Yang et al., 1997; Reed et al., 1998; Tsujimoto and Shimizu, 2000). While these proteins alone can form ion channels when incorporated into lipid bilayers (see Schendel et al. (1997), for example), one current model suggests that these proteins can interact with the ANT. Bax and ANT cooperate in forming a channel that is electrophysiologically different from channels formed by either protein alone; Bcl-2 and ANT mutually inhibit each other in channel formation (Brenner et al., 2000). Thus, these proteins can influence ion fluxes into and out of mitochondria (e.g.  $\text{Ca}^{2+}$  (Zhu et al., 1999)), and in some conditions either prevent (Bcl-2) or promote (Bax) the MPT.

## 7. Mitochondria and intermediary metabolism

A cell is clearly no longer viewed simply as a bag of enzymes, and we understand that the diverse reactions comprising metabolism are significantly compartmentalized. Mitochondria and the reactions taking place *within* the organelle occupy a central position on any metabolic chart. The discovery of the Krebs’ cycle and the urea cycle were milestones in the history of biochemistry. Another classical discovery was the role of mitochondria in fatty acid oxidation by Lehninger and Kennedy. The biosynthesis of heme is also well understood in many organisms. Continuing challenges in this and many other metabolic pathways arise from attempts to understand the overall flux through the pathways, the regulation of these fluxes by a variety of mechanisms, and the integration of each pathway into the overall metabolism of a given cell or tissue. Thus, while we understand fatty acid synthesis and degradation very well, dozens of

books on weight control at any given time testify to the confusion that still reigns even among the ‘experts’.

Hydroxymethylglutaryl CoA (HMG-CoA) is a key intermediate in the formation of cholesterol, farnesyl and geranyl side chains for protein modification, and the biosynthesis of dolichol and ubiquinol. The latter is particularly relevant for mitochondria. The jury is still out on whether it is actually helpful as a dietary supplement. In its reduced form ubiquinol may have an important role as an antioxidant (Grunler et al., 1994). It is advertised on the Web as useful in alleviating impotence, AIDS, cancer, heart disease, Charcot–Marie–Tooth disease, enhancing athletic performance, and more.

Cholesterol biosynthesis takes place in the ER or in peroxisomes. Mitochondria re-appear in the discussion of steroidogenesis, for example, testosterone synthesis in Leydig cells of the testis (Arakane et al., 1998a,b; Hales et al., 1999; King and Stocco, 1996; Manna et al., 1999). Cholesterol uptake by mitochondria is facilitated by the steroidogenic acute regulatory protein (StAR). Expression of this protein is subject to control by thyroid hormone (Manna et al., 1999). The protein has an N-terminal mitochondrial targeting sequence (Arakane et al., 1998a), and its functioning requires ATP and a membrane potential (King and Stocco, 1996). Nevertheless, from a series of experiments with recombinant protein constructs lacking the targeting sequence, Arakane et al. argued that StAR acts outside of mitochondria, independent of mitochondrial import (Arakane et al., 1998a,b). To reconcile some of these conflicting observations, these authors proposed that mitochondrial import is responsible for terminating StAR action and for the observed short functional half-life.

## 8. Mitochondria and disease

### 8.1. Introduction

Mitochondrial diseases or mitochondrial medicine started in 1988 with the molecular-genetic characterization of patients with Leber’s hereditary optic neuropathy (LHON), and familial mitochondrial encephalomyopathy (MERRF) by Wallace and

colleagues (Wallace et al., 1988a,b), and the identification of mtDNA deletions in patients with mitochondrial myopathies by Holt and colleagues (Holt et al., 1988). Myopathies, neuropathies, encephalomyopathies had been described over the years, and perhaps even linked with some mitochondrial abnormalities, but the proof of maternal inheritance of LHON definitely focused attention on a mutation in mtDNA, and on the expression of such mutations in individuals with ~1000 mtDNAs per cell. The subject has exploded, with hundreds of papers appearing every year. The manifestations of mitochondrial diseases are extremely diverse, include numerous symptoms of variable severity, affect many different organs, and mitochondrial deficiencies have been implicated in aging in general, in Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, diabetes mellitus, etc. The emphasis on mitochondria has brought some order and focus to the understanding of these diseases.

There are an estimated 1000 different proteins in mitochondria, and defects in many such proteins can be characterized and described under the heading ‘metabolic diseases’, or inborn errors of metabolism. One could also include defects in various transport mechanisms and ion channels. In the following the emphasis will be on mutations affecting the electron transport chain and oxidative phosphorylation.

### 8.2. The nature of the mutations

It is obvious that an individual cannot live without respiration and oxidative phosphorylation, although fibroblasts in tissue culture can proliferate with severe mutations or even in the absence of mtDNA (Scheffler, 1999). Therefore, mitochondrial diseases are diagnosed in living individuals (although in some cases death may occur soon after birth) with *partial* enzyme deficiencies. The discovery of mutations in mtDNA caused the initial excitement and rekindled medical interest in oxidative phosphorylation, but a priori nuclear mutations are not excluded. It is clear from the earlier discussion that nuclear mutations could affect mtDNA replication and repair, transcription, protein synthesis in the matrix, protein import, and other properties of mitochondria. For example, a reduction in the mitochondrial transcription factor (mtTFA) might affect mtDNA replication as well as

transcription and hence lower the level of mtDNA, and of transcripts, or both. Homozygotes ( $-/-$ ) with complete deficiencies would not be expected to be viable, while heterozygotes ( $+/-$ ) may be asymptomatic

Mitochondrial mutations can be subdivided into several classes: (1) major deletions in mtDNA that eliminate more than one gene (coding sequences, tRNA genes); and (2) point mutations in either a coding sequence or in a tRNA or rRNA gene.

A large deletion in mtDNA can be tolerated in an individual only when the individual is heteroplasmic. It appears straightforward to correlate the severity of the disease with the proportion of mutated mtDNA. Such a deletion in a heteroplasmic individual is expected to lower the activity of all complexes except complex II: first, because of the lowered gene dose for mitochondrially encoded subunits, and second, because of the reduced availability for one or more tRNAs for mitochondrial protein synthesis.

Missense mutations in a single coding sequence can also be present in heteroplasmic individuals, but the amino acid substitution may yield a protein with some residual function, and it is therefore possible to have homoplasmic individuals. Similarly, a point mutation in a tRNA gene may cause the tRNA to be less than 100% active, or such a mutation may cause a reduction but not failure in processing of the polycistronic transcript and the maturation of the tRNA. A mutation in a coding sequence will affect a specific subunit of one complex in the electron transport chain or ATP synthase, lowering the activity of this complex. Here is where additional questions arise: how does this defect affect respiration in general? What is rate limiting, or what is the flux coefficient (in the new terminology) for the particular step? Does it make any difference when ATP production is limited by a complex V mutation or by a mutation in the electron transport chain? What physiological consequences and distinct symptoms would be expected?

### 8.3. *Non-mendelian inheritance and mtDNA segregation*

Maternal inheritance raises several fundamental questions related to the segregation of mitochondrial genomes in heteroplasmic individuals (Lightowlers et al., 1997). Here a further distinction must be made

between segregation in oogenesis and hence from one generation to the next, and potential segregation of such genomes during embryogenesis, during growth to an adult, and even in adult somatic cells. Put as a simple question: how does a new mutation in one mtDNA molecule in a germline cell (or early embryo) become amplified relative to the wild type mtDNA population in somatic cells? How many generations does it take before symptoms appear in an individual who is found to be heteroplasmic with  $>50\%$  mutant mtDNA, or even homoplasmic with  $\sim 100\%$  mutant mtDNA? Is there any selective pressure operating, or is the entire progression subject only to stochastic events? Is there any selection occurring at any stage in oogenesis? And, is additional selective pressure operative in somatic cells in specific tissues?

A surprising discovery was that in mammals the ratio of mutated to wild type mtDNAs can change dramatically in a single generation, and a given heteroplasmic female can have offspring with widely differing ratios. Experiments in mice suggest a scenario that can account for the observed statistics (Jenuith et al., 1996). During a series of mitotic divisions producing oogonia in the developing ovary only a small subset of mtDNA molecules is involved in replication. Such small subsets of replicating mtDNAs give rise to the large number of mtDNAs found in the mature oocyte. The small subset is chosen at random in a heteroplasmic precursor germline cell, and is thus subject to relatively broad fluctuations in the ratio of the two genomes. The phenomenon has been referred to in some publications as a ‘bottleneck’, a term that has met opposition from some reviewers (Lightowlers et al., 1997).

Consider a single egg being fertilized. The resulting zygote will have a certain percentage of mutated mtDNA. Early cleavage divisions will distribute these mitochondria into two, four or eight cells, and at some stage mtDNA replication and mitochondrial biogenesis will resume in the embryo. In humans it is clear that heteroplasmic individuals with large deletions have a highly variable distribution of deleted mtDNA in different tissues, with high levels of deletions in muscle and the brain, and low in blood. Furthermore, such deletions are not usually maternally inherited, but appear spontaneously. When during oogenesis or embryogenesis did the mutation arise and what selective mechanisms are

responsible for the observed tissue distribution in the affected children?

Point mutations in the ATP synthase subunit 6 have been observed (neuropathy, ataxia, retinitis pigmentosa (NARP) and Leigh syndromes), and such individuals are always heteroplasmic, the severity of the syndrome being correlated with the fraction of mutated mtDNA. Individuals with LHON are usually homoplasmic. Comparable mouse models will be necessary to follow such fluctuations developmentally and in different tissues and over generations in related individuals.

In discussing heteroplasmy, it may have been assumed that in a given cell with hundreds of mitochondria and ~1000 mtDNAs, and with constant fusions and fissions occurring, the mtDNA molecules would be more or less mixed up, i.e. mitochondria represent a homogeneous population of subcellular organelles. If mitochondria were a heterogeneous population, and if their DNA replication depended on their position relative to the nucleus, heteroplasmy could become a variable by a stochastic mechanism.

A very provocative recent report was the extensive documentation of mtDNA mutations/sequence polymorphisms in tumors compared to matched blood samples or normal tissue (Fliss et al., 2000). Mitochondria have a history of being implicated in tumorigenesis since the time of Warburg, and there have been earlier reports of a high incidence of mutations in colon cancer. The new study examined tumors in human bladder, head and neck, and lung, and it comes to the surprising and puzzling conclusion that in many tumors the mutations were homoplasmic. How does a particular mtDNA mutation become dominant during neoplastic transformation and expansion of a clonal tumor cell population? Is there a cause and effect relationship? How is the rapid proliferation of tumor cells linked to selective replication/amplification of the mutated mtDNA? As the authors suggest, the finding may be important for detection of cancer, but it also raises interesting new questions about the replication of mitochondria in somatic and transformed cells.

One issue thought to be settled almost 20 years ago concerns the maternal inheritance of mtDNA and the complete absence of a contribution from the male. The debate was recently rekindled. First, evidence has been presented that mammalian mitochondria have the enzymatic machinery for recombination (Thyagarajan et al., 1996). From an analysis of link-

age disequilibrium in hominid mtDNA Awadalla et al. also have derived evidence for genetic recombination in mitochondria (Awadalla et al., 1999). This finding triggered thoughts about the possibility of recombination between (1) the maternal and the paternal mtDNA, (2) recombination between mtDNA in heteroplasmic individuals, and (3) recombination with DNA sequences transferred back to mitochondria from the nucleus. These may be rare events, and there is no doubt that over a few generations inheritance is maternal. The question is whether over an evolutionary time scale some contribution from a male could have been made.

It is accepted that mitochondria from the sperm enter the oocyte during fertilization. Do they persist and contribute to the population of mtDNAs in the developing organism? First of all, one can note that an oocyte has an estimated 100,000 mtDNAs, while the number of genomes contributed from the sperm is significantly lower (a few hundred mitochondria per fertilized human egg). In a series of inter-species crosses of mice and cattle with suitable mitochondrial markers biparental inheritance was demonstrated (Gyllenstein et al., 1991; Kaneda et al., 1995; Sutovsky et al., 1999). It has since been argued that paternal mitochondria (mtDNA) are specifically targeted for destruction in intra-species crosses by a mechanism that fails in inter-species crosses (Kaneda et al., 1995; Sutovsky et al., 1999). The recognition of the sperm mitochondria in the zygote is probably facilitated by the morphological differentiation that accompanies spermatogenesis, and a recent paper provides evidence for the ubiquitination of sperm mitochondria after entry into the egg, marking them for degradation by the embryo's proteasomes and lysosomes before or during the third embryonic cleavage (Sutovsky et al., 1999). As discussed by these authors, these considerations have serious implications for the practice of cloning and in vitro fertilization using sperm microinjection, and oocyte cytoplasm 'donation therapy'.

#### 8.4. Energy requirements and thresholds

An idea proposed by Wallace and developed over the years is that different tissues have different threshold requirements for oxidative phosphorylation. Below the threshold the tissue begins to behave abnormally, and symptoms appear. The brain, cardiac and

skeletal muscle are generally considered to have the highest energy demands, and perhaps not surprisingly, defects in oxidative phosphorylation are most commonly associated with neuropathies, myopathies and cardiomyopathies, often in combination, and frequently with problems in other organs. A few select examples will serve to illustrate clinical features of specific mitochondrial disorders and permit the formulation of questions that need to be answered for a full understanding of the disease.

### 8.5. Clinical features

- NARP includes symptoms such as sensory neuropathy, ataxia, retinitis pigmentosa, dementia, seizures, developmental defects. A point mutation (T8993G/C) in the ATPase 6 subunit is responsible when present in 70–80% of the mtDNAs. Lower levels of heteroplasmy are usually observed in NARP, while higher levels (often >60–80%) are typically associated with the Leigh syndrome presentation.
- MELAS (mitochondrial encephalomyopathy; lactic acidosis; stroke-like episodes) has a delayed onset and symptoms include migraine headaches, vomiting, seizures, mental retardation, strokes, hearing loss, exercise intolerance, and others. The most common mutation is the A3243G mutation in the tRNA<sup>Leu</sup> gene, but mutations in other tRNA genes (Val, Ser, Phe), and in the COX3 gene can lead to the same symptoms.
- MERRF (myoclonic epilepsy; ragged red fibers) has been found in heteroplasmic individuals with mutations in tRNA<sup>Lys</sup>, tRNA<sup>Ser</sup>, and tRNA<sup>Leu</sup> (C3256T). Not surprisingly, there are overlaps with the MELAS syndrome
- LHON is characterized by bilateral central vision loss that occurs with a delayed onset, is predominant in males (but no X-linked genes have been implicated so far), and may be followed by a recovery after many months. Ninety percent of all cases involve mutations in mitochondrial complex I genes (ND4, ND1, ND6), but mutations affecting complex III, complex IV, and complex V have also been reported. Homoplasmy with respect to the mutation is frequently observed.
- Kearns–Sayre syndrome (ophthalmoplegia, ataxia, retinitis pigmentosa, cardiac conduction defect and

elevated cerebrospinal fluid protein) is rarely familial, i.e. there is no maternal inheritance, and is most frequently caused by single, large mtDNA deletions. To maintain such mtDNAs, origins for DNA replication and promoter regions for transcription are preserved. Heteroplasmy depends on the tissue (high in muscle, low in blood cells), and it may change with age. It starts with a delay, but before 20 years of age, and it is progressive, leading to death between the age of 30 and 50. Identical deletions can be found in the Pearson syndrome (sideroblastic anemia, exocrine pancreas deficiency, gastrointestinal dysmotility, and renal tubulopathy).

Many more examples of clinical descriptions could be added, but the point to be made is that it would have been difficult to predict a priori the range of symptoms, the severity of symptoms, and the tissue(s) affected by a given mutation. Is a partial defect in complex I (NADH-ubiquinone oxidoreductase) due to a point mutation in ND4 different than a defect in the same complex due to the reduced availability of a tRNA required to translate subunits for this complex? Does it matter how much respiration and oxidative phosphorylation are reduced, regardless of the mechanism? Is the consequence of an altered tRNA structure on efficiency of translation different in different cell types? A complex IV deficiency may be tolerated more readily in some tissues and not in others. LHON is the most puzzling disease because of the highly restricted set of neurons affected. And why the delayed onset of the disease?

### 8.6. Reactive oxygen species

The production of reactive oxygen species (ROS), including H<sub>2</sub>O<sub>2</sub>, OH·, and O<sub>2</sub>·<sup>-</sup> (Chance et al., 1979; Halliwell, 1993; Kwong and Sohal, 1998; Miquel, 1998; Papa and Skulachev, 1997) may be a normal event from electrons leaking from the electron transport chain to oxygen at high potential sites, and aerobic organisms have developed a number of antioxidant defense mechanisms that include superoxide dismutases (Cu, Zn-SOD in the cytosol, Mn-SOD in mitochondria), glutathione peroxidase, and catalase (Gutteridge, 1994). Defects in electron trans-

port complexes or other perturbations of mitochondria can be responsible for excess production of ROS. Their reactivity with DNA, proteins and lipids makes it easy to blame them for a variety of pathologies, and aging in particular (Ames et al., 1995; Beckman and Ames, 1998; Shigenaga et al., 1994). One could argue, therefore, that a problem does not arise initially from a reduced respiration, but it becomes acute only after a period of damage accumulation from ROS-induced alterations in mtDNA, mitochondrial proteins or lipids. An ‘error catastrophe’ can be envisaged during which a mitochondrial mutation causes increased production of ROS, which in turn induce further mutations aggravating the problem.

### 8.7. Conclusion

The optimistic perspective is clearly that many diverse symptoms of variable severity, occurring alone or in combination have now been linked to defective energy metabolism in mitochondria. Thus, physicians are alerted to examine the state of mitochondria and their capacity for respiration, and in many cases it is routine to look for major deletions in mtDNA or in individual genes by PCR-based methods. The diagnosis and the specific cause for the symptoms become more definitive, better prognoses can be made about the progression and management of the disease, and in many cases genetic counselors will be able to give more reliable estimates of risk. It may ultimately become feasible to ameliorate the diseases with pharmacological interventions, or even gene therapy (for nuclear genes).

## 9. Mitochondria in anthropology and forensic science

Mitochondrial DNA has been referred to as the 24th chromosome in humans, and it was the first to be completely sequenced. Since there are up to a thousand mtDNAs per cell, it is the most abundant human chromosome. When the sample size is limited, for example in forensic investigations, or from very old archeological specimen (Krings et al., 1997; Ovchinnikov et al., 2000), mtDNA is often the only DNA present in sufficient quantity to be amplified by the polymerase chain reaction. The ~1.2 kb control region devoid of coding sequences is easily amplified

and sequenced, and a significant number of polymorphisms have since been catalogued in this region. Extensive sequencing of coding regions also has revealed many polymorphisms. Another major advantage is that mtDNA is passed only through the maternal lineage, and hence there is no recombination to complicate analyses of pedigrees and phylogenies.

Examples of forensic applications of unusual interest have been the matching of grandchildren and grandmothers in Argentina, where the parents were among the ‘disappeared’ victims of the military junta (King, 1991), and the identification of the skeletal remains of the murdered Tsarist family in Yekaterinburg (Gill et al., 1994; Ivanov et al., 1996). A large database of mtDNA sequence polymorphisms has been assembled by the FBI and by the Armed Forces DNA Identification Laboratory in Rockville, MD. The surprisingly large number of haplotypes in the control region has raised new questions about the mutation rate, about hot spots, and about whether mutations in this region are truly neutral or subject to selection pressure.

If DNA sequences in a phylogenetic tree represent a biological clock, one can say that the mitochondrial clock runs faster than the nuclear clock, which has been ascribed to an error-prone polymerase or the relative paucity of repair mechanisms (however, see above). Arguments about the rate at which the ‘clock’ runs continue (Gibbons, 1998).

Fascinating and stimulating results from mtDNA sequence analyses have been obtained during the past 15 years that have great relevance to understanding primate and human evolution, and the spread of the present day humans over the globe. The results and interpretations have not been unambiguous, generating some controversy. One problem is that the computer programs (PAUP – phylogenetic analysis using parsimony) have not yielded an absolutely unique solution, and hence there is still some uncertainty about a unique phylogenetic tree for all present human ethnic groups. Another problem is the definitive identification of the root of the tree. However, mtDNA sequence analyses, Y chromosome sequence data, and independent evidence place the root of the tree in Africa. The journalistically attractive notion of an ‘African Mitochondrial Eve’ is probably correct, if one does not think literally in terms of a single mother of us all (O’Connell, 1999; Owens and King, 1999; Paabo, 1999).

In a recent paper with a broader focus on primate

evolution Gagneux et al. (1998) combined a large number of data from many modern humans, the Neanderthal sequence (Krings et al., 1997), chimpanzees and gorillas from all parts of Africa, and orangutans from Borneo and Sumatra. The interpretation of the generated tree re-enforced the hypothesis that all present humans are descended quite recently from a small group and provides further arguments against the multiregional hypothesis (Cann et al., 1987; Vigilant et al., 1991).

Arguments about the rooting of hominoid trees can be resolved by knowing that chimpanzees are our closest ‘relatives’, gorillas diverged somewhat sooner, and orangutans before that. Independent evidence supporting this view comes from several sources, but the recent study of xenomitochondrial cybrids (Barrientos et al., 1998; Kenyon and Moraes, 1997) represents a particularly impressive example. A more contentious problem is to estimate the absolute timing of these events. It depends on a precise estimate of the rate of the mitochondrial clock, that is the rate at which mutations appear in the mitochondrial genome. Mutation rates, reversion rates, neutral mutations in the control region, different clock speeds at different locations on the genome, and even different clock speeds in different organisms (almost certainly true for widely diverged species) must be considered. There must also be a calibration of the time scale, based on an independent estimate of the time of divergence of humans from chimpanzees, or of the orangutan from the large African primates.

Analyses along the same lines with a focus on humans have succeeded in giving us a fairly detailed picture of the spread of humans throughout the world and even within a single continent (Owens and King, 1999). It is satisfying that other approaches, such as linguistic analyses and paleontological evidence have yielded similar conclusions. Arguments remain over details. How many crossings over the Bering Strait did occur? Are the data influenced by unknown bottlenecks, extinctions, back and forth migrations and even limitations of the computer programs? The answers may not be final, but the process is an exciting one that has brought geneticists, anthropologists, linguists, historians, and evolutionary biologists together in the exploration of our common roots.

## 10. Summary

If there is one conclusion from all of the above discussion it is surely that the study of mitochondria is far from exhausted. Their central role in metabolism and bioenergetics is well defined, and many, but not all, of the relevant macromolecular structures have been determined at atomic levels of resolution. Mitochondria represent a prime example of our understanding of biological structure and organization at a level above that of single proteins, simple membranes, and multiple compartments. But it is clear that a generic mitochondrion with constant properties from cell to cell (and from organism to organism) does not exist. The cell-specific and tissue-specific biochemical subtleties of mitochondria remain largely unexplored: specific cells differ not only in the number of mitochondria, but the mitochondria themselves may be partially differentiated to serve the needs of the specific cell type. Even within a given cell, not all mitochondria may be exactly the same, depending on their location. Such thoughts may be somewhat speculative, but it seems clear that we cannot understand mitochondrial diseases and the variable symptoms in detail from understanding electron transport and oxidative phosphorylation in liver cells.

When a fertilized egg is equipped with a certain population of mitochondria (and mitochondrial genomes), we must now anticipate not only developmentally normal differentiation of somatic cells *and* mitochondria, but there may be additional stochastic processes and somatic mutations in the mtDNA that may have significant consequences. Heteroplasmy is perhaps just an extreme manifestation of the evolution of mitochondrial genomes in a given organism from birth to death. Only some of that variation is passed through the germ line and through the maternal lineage from generation to generation. On any time scale, thousands of years of evolution, a century covering a pedigree, and a few decades of life of a single individual there are interesting problems related to the variability of the mitochondrial genome. In that sense mitochondria continue to be symbionts that adapt and evolve in every organism even in a single life time.

## Appendix

Table A1

Databases with mtDNA sequence information and other information on mitochondria

	Web site
Mitochondrial disorders I (MitoMap)	<a href="http://infinity.gen.emory.edu/mitomap.html">http://infinity.gen.emory.edu/mitomap.html</a>
Mitochondrial disorders II	<a href="http://www.neuro.wustl.edu/neuromuscular/mitosyn.html">http://www.neuro.wustl.edu/neuromuscular/mitosyn.html</a>
Review in <i>Nucleic Acids Res.</i>	<a href="http://www3.oup.co.uk/nar/Volume_28/Issue_01/introduction/">http://www3.oup.co.uk/nar/Volume_28/Issue_01/introduction/</a>
Bari Bioinformatic Group	<a href="http://bio-www.ba.cnr.it:8000/BioWWW/#AMMTDB">http://bio-www.ba.cnr.it:8000/BioWWW/#AMMTDB</a>
MitoPick	<a href="http://www-dsv.cea.fr/thema/MitoPick/Default.html">http://www-dsv.cea.fr/thema/MitoPick/Default.html</a>
The Center for Inherited Disorders of Energy Metabolism (CIDEM) at Case Western Reserve University (CWRU) School of Medicine, Cleveland, OH	<a href="http://www.cwru.edu/med/CIDEM/cidem.htm">http://www.cwru.edu/med/CIDEM/cidem.htm</a>
Fungal Mitochondrial Genome Project	<a href="http://megasun.bch.umontreal.ca/People/lang/FMGP/">http://megasun.bch.umontreal.ca/People/lang/FMGP/</a>
Organelle Genome Megasequencing Project	<a href="http://megasun.bch.umontreal.ca/ogmproj.html">http://megasun.bch.umontreal.ca/ogmproj.html</a>
Complex I Home Page	<a href="http://www.scripps.edu/mem/biochem/CI/">http://www.scripps.edu/mem/biochem/CI/</a>
Complex III Home Page	<a href="http://www.life.uiuc.edu/crofts/bc-complex_site/index.html">http://www.life.uiuc.edu/crofts/bc-complex_site/index.html</a>
Complex IV Home Page	<a href="http://www-bioc.rice.edu/~graham/CcO.html">http://www-bioc.rice.edu/~graham/CcO.html</a>
Direct observation of rotation of ATP synthase	<a href="http://www.res.titech.ac.jp/seibutu/nat">http://www.res.titech.ac.jp/seibutu/nat</a>
ATP synthetase (Wang and Oster)	<a href="http://nature.Berkeley.EDU/~hongwang/Pr">http://nature.Berkeley.EDU/~hongwang/Pr</a>

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