CHAPTER 2

Molecular Biology of the OXPHOS System

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Abstract

The mitochondrion contains a circular DNA genome (mtDNA) that serves as the basis for its own genetic system. This system is semiautonomous because the coding capacity of mtDNA is limited to 13 subunits of the respiratory chain apparatus and the rRNAs and tRNAs necessary for their translation. The inheritance of mtDNA differs from that of nuclear DNA in that it segregates randomly during mitosis and meiosis and is transmitted exclusively through the female germ line. Nucleus-encoded enzymes and factors direct the transcription and replication of mtDNA within the mitochondrial matrix. Mitochondrial translation also relies upon nucleus-encoded ribosomal proteins, synthetases and translation factors. In recent years, molecular mechanisms for the bi-genomic control of mitochondrial biogenesis and function have been elucidated.

Introduction

A variety of mutations in mitochondrial DNA (mtDNA) have been associated with human pathology.[1] Pathogenic mutations in mtDNA all affect the OXPHOS system because the mitochondrial genome is essential to the biogenesis of the respiratory chain apparatus. These observations have sparked considerable interest in the molecular mechanisms of mtDNA expression, inheritance, maintenance and replication. Much progress has been made in understanding the molecular biology of the OXPHOS system and major inroads have been made in elucidating nucleo-mitochondrial interactions (see refs. 2, 3). This chapter will present a general treatment of the basic molecular genetics of OXPHOS with the major focus on mammalian systems, although analogies to the yeast system will occasionally be incorporated. Recent findings on the regulation of nuclear genes governing the OXPHOS system will also be presented.

mtDNA

Observations made in the late 1950s and early 1960s pointed to the existence of a mitochondrial genetic system that was separate from that of the nucleus (see ref. 4). Early work demonstrated that isolated mitochondria had a protein synthetic machinery that could synthesize a small number of proteins. Further studies established the existence of mitochondrial ribosomes, rRNA and tRNA. Surprisingly, the system displayed antibiotic sensitivities that were more akin to prokaryotic translation than to eukaryotes. A major advance was the discovery of mitochondrial DNA in yeast and in other organisms.[5] Since these early discoveries, much has been done to define the structure and gene organization of mtDNA. The first complete mtDNA sequence was obtained from humans and sequences of mitochondrial genomes from many organisms have now been catalogued (http://megasun.bch.umontreal.ca/). A striking result from this work is that a similar complement of genes is conserved in mtDNAs from

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all multicellular organisms. In vertebrates these include genes for 13 protein subunits of respiratory chain complexes I, III, IV and V, two rRNAs and 22 tRNAs (Fig. 1).

In stark contrast to the nuclear genome, mtDNA in mammals and other vertebrates exhibits extreme economy of sequence organization. Human mtDNA exists as a covalently closed circle of about 16.6 kb. Its genes are in a head to tail arrangement with little or no intergenic regions and are completely devoid of introns. Certain respiratory chain protein genes overlap and the adenine nucleotides of UAA termination codons are supplied by polyadenylation.[6] Protein coding and rRNA genes are interspersed with tRNA genes which punctuate the sites of RNA processing (Fig. 1). The only substantial noncoding region is the D-loop, which gets its name from the triple stranded structure or displacement loop that is formed by association of the nascent H-strand in this region. The D-loop is the site of transcription initiation from bi-directional promoters and also contains the origin of H-strand DNA replication.[4] It should be noted that the structural economy found in vertebrates does not exist in plants and fungi where the mitochondrial genomes are much larger and contain intergenic regions, introns and multiple promoters and transcriptional units.[7,8] Another interesting anomaly is that the mito-

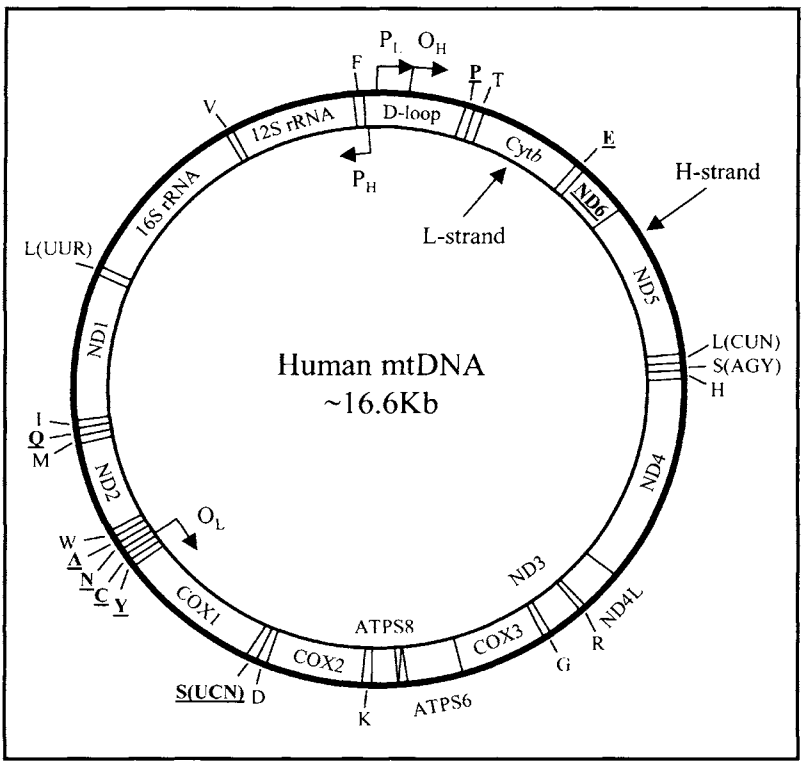

Figure 1. Human mitochondrial DNA (mtDNA). Genomic organization and structural features of human mtDNA are depicted in a circular genomic map. Protein coding and rRNA genes are interspersed with 22 tRNA genes (denoted by the single letter amino acid code). The D-loop regulatory region contains the L- and H-strand promoters (P_L and P_H, respectively) along with the origin of H-strand replication (O_H). The origin of L-strand replication (O_L) is displaced by approximately two-thirds of the genome within a cluster of five tRNA genes. Protein coding genes include: Cytochrome oxidase (COX) subunits 1, 2 and 3; NADH dehydrogenase (ND) subunits 1, 2, 3, 4, 4L, 5 and 6; ATP synthase (ATPS) subunits 6 and 8; cytochrome *b* (Cyt*b*). ND6 and the 8 tRNA genes encoded on the L-strand are in bold type and underlined, all other genes are encoded on the H-strand.

chondrial genetic system utilizes genetic codes that differ slightly from the universal nuclear code and that these differences are species specific. For example, in humans the universal AUA (isoleucine) and UGA (stop) codons specify methionine and tryptophan, respectively, in mitochondria (see ref. 9).

Mitochondrial Inheritance

The fact that mtDNA is a compartmentalized extrachromosomal element contributes to a mode of inheritance that differs from that of nuclear genes. Early work in yeast led to the observation that certain respiratory chain mutations displayed a cytoplasmic inheritance pattern that resulted from the random segregation of mtDNA molecules during mitosis. This provided genetic evidence that mitochondria had their own DNA before its existence was demonstrated physically. Somatic mammalian cells generally have 10^3-10^4 copies of mtDNA with approximately 2-10 genomes per organelle.[10] These genomes replicate in a relaxed fashion that is independent of the cell cycle that is defined by nuclear DNA replication (see refs. 2, 4, 9) Some mtDNA molecules undergo multiple rounds of replication while others do not replicate. This, along with random sampling during cell division, allows the segregation of sequence variants during mitosis.[11]

In mammals, mtDNA is strictly maternally inherited (see refs. 11, 12). The paternal lineage does not contribute mtDNA to the offspring nor is there known to be recombination between maternal and paternal sequence variants. The paternal mtDNA is lost during the first few embryonic cell divisions. In addition, because mtDNA is a multicopy genome, an individual may harbor more than a single sequence, a condition referred to as heteroplasmy. A sequence variant that is detrimental may be tolerated in low copy because the defective gene product(s) it encodes do not reach the threshold for disrupting cellular function. However, sequence variants are known to segregate rapidly from heteroplasmy to homoplasmy in passing from one generation to the next.[13] This can result in offspring in which the detrimental variant predominates, leading to a defective mitochondrial phenotype. The molecular basis for this rapid meiotic segregation has been explored and has been ascribed to a bottleneck or sampling error in the female germ line.

A massive amplification of mtDNA occurs during oogenesis from about 10^3 copies in the primary oocyte to approximately 10^5 copies in the mature oocyte (see ref. 11). Replication of mtDNA is halted in the mature oocyte and the existing population of mtDNA molecules is partitioned to the daughter cells during early cell divisions until the copy number is diluted to approximately that found in somatic cells (Fig. 2). Embryonic replication does not resume until the blastocyst stage of development.[14] For unknown reasons, the mitochondrial number is reduced to about 50 in primordial germ cells and increases to about 200 in the oogonia. Assuming the normal somatic cell number of genomes per organelle, the mtDNA copy number in germ cell progenitors is extremely small, on the order of 50 to 100 copies. This small number is consistent with a genetic bottleneck and, in fact, heteroplasmic mice constructed from two domestic strains were used to establish that nearly all of the mtDNA segregation occurs between the primary germ cells and the oogonia. Thus, the genetic bottleneck occurs early in the pathway of oogenesis and segregation of sequence variants is complete before the formation of primary oocytes. It is interesting to note that significant mitotic segregation does not appear to occur during embryogenesis. In addition, defective variants are not selectively eliminated or reduced during oogenesis or early embryonic development indicating that optimal mitochondrial respiratory chain function is not required for these processes.[11]

Replication, Transcription, RNA Processing

MtDNA Replication

Replication and transcription of mtDNA is completely dependent on nucleus-encoded gene products. In mammalian cells, replication is most intense near the nucleus and newly repli-

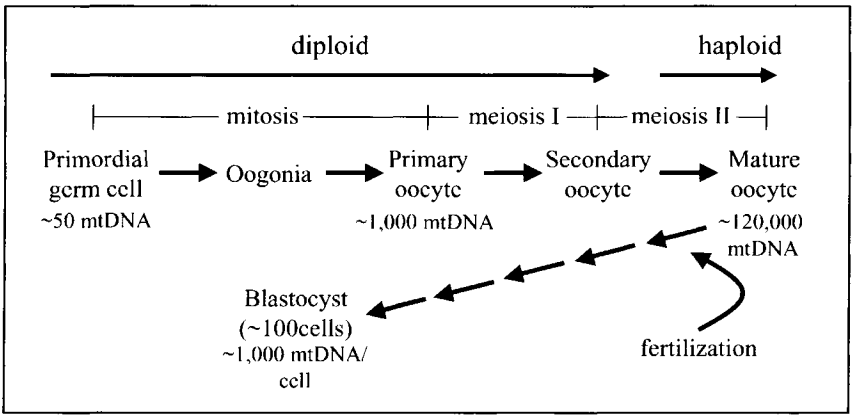

Figure 2. MtDNA copy number during oogenesis and early embryogenesis. Diagram depicts the amplification of maternal mtDNA from low copy in primordial germ cells to high copy in the mature oocyte and its dilution to approximately somatic cell levels during formation of the blastocyst.

cated molecules distribute outwardly through the mitochondrial network.[15] The overwhelming majority of evidence points to a mechanism of bi-directional replication where the replication origins for the two strands, termed heavy (H) and light (L) based on their buoyant densities, are displaced by about two-thirds of the genome.[16,17] This results in temporal as well as spatial separation of initiation events (Fig. 1). The D-loop regulatory region contains bi-directional promoters, (P_H) and (P_L), for transcribing H and L strands as well as the H-strand replication origin (O_H). As depicted in Figure 3, the RNA transcript initiated at P_L is cleaved in the vicinity of three evolutionarily conserved sequence blocks (CSB I, II, and III), and H-strand replication is initiated at the sites of these cleavages.[18] Thus, transcription is coupled to replication and the sites of RNA cleavage are transition sites between RNA and DNA synthesis. A decision must be made to continue transcription through the CSBs or to truncate the nascent RNA to initiate DNA replication. Although nothing is known of how the transition is regulated, a stable RNA-DNA hybrid that requires CSBs I and III has been demonstrated.[19] Even after DNA synthesis begins, the nascent strand is often terminated downstream from a conserved element referred to as a termination associated sequence (TAS).[20] This event may be important in controlling mtDNA levels and accounts for the triple-stranded D-loop structure.

Once the nascent H strand traverses two-thirds of the genome, L strand replication is initiated at O_L which is a short noncoding region within a cluster of tRNA genes.[16,17,21] Upon displacement of the parental H strand, O_L is thought to form a stem-loop structure.[22] This serves as the recognition site for a mitochondrial primase that produces a short RNA primer for the initiation of L strand replication. Initiation of DNA synthesis occurs near a G+C rich region at the base of the stem. The primase has been only partially purified and is thought to require RNA for catalytic activity.[23]

It should be noted that replication intermediates that are consistent with coupled leading and lagging strand replication from a single origin have also been detected.[24] This mode of replication was initially observed under conditions where cells were recovering from transient mtDNA depletion[24] but is now thought by the authors to represent the predominant mechanism of replication in dividing cells.[25] This conclusion has been challenged by the proponents of the classical strand-displacement model on the basis that the new model is supported mainly by the detection of replication intermediates using two-dimensional gel electrophoresis.[26] The precise structure of these intermediates has not been confirmed by other means and they may be the products of transcriptional events. By contrast, multiple lines of experimental evidence,

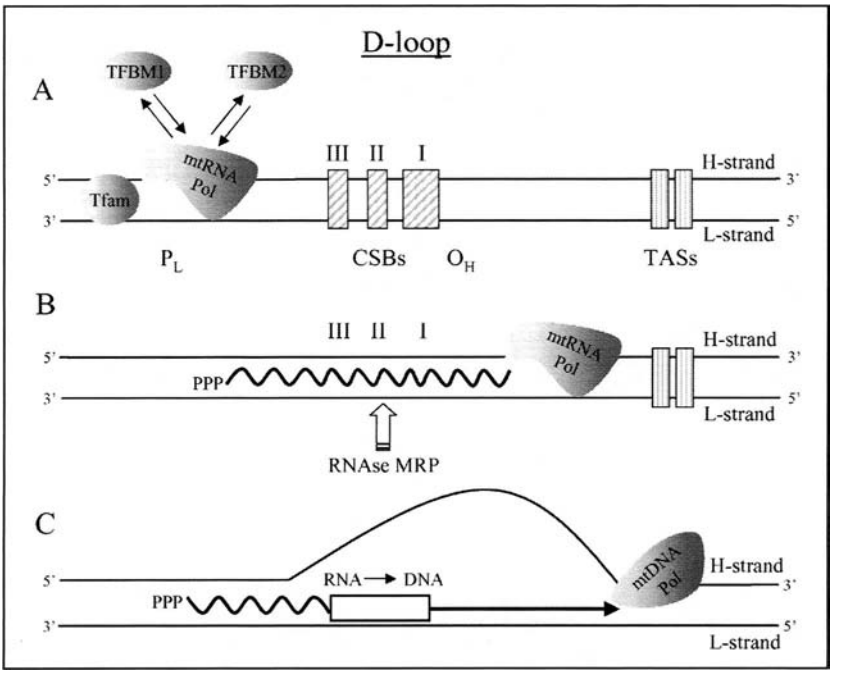

Figure 3. Schematic representation of the initiation of mtDNA transcription and replication within the D-loop regulatory region. A) Transcription is initiated at P_L by mtRNA polymerase (mtRNA Pol) in the presence of Tfam, a stimulatory factor that unwinds DNA, and one of the two isoforms of mtTFB (TFBM1 or TFBM2) which presumably act as dissociable specificity factors. The latter resemble rRNA dimethyltransferases and thus may also function in RNA modification or processing. B) The nascent RNA transcript is extended around the genome but with some frequency is cleaved at specific sites in the vicinity of the conserved sequence blocks (CSBs) by RNAse MRP. These truncated RNAs serve as primers for mtDNA replication initiated at O_H. C) The RNAse MRP cleavage sites correspond to the heterogeneous 5' ends of the newly synthesized H-strand and represent the transition sites between RNA and DNA synthesis. Nascent H-strands may terminate at termination associated sequences (TASs) giving rise to the D-loop structure.

including the precise mapping of replication origins and the detection of the predicted replication intermediates by electron microscopy, support the strand-displacement model.[16,26]

Many of the key players in mtDNA transcription and replication have been characterized in recent years and all of these are products of nuclear genes. DNA polymerase γ, the only known mitochondrial DNA polymerase, is a heterodimer of large (125-140kD) and small (35-54kD) subunits and is highly conserved from yeast to man.[27] A mutation of the large subunit in yeast that eliminates catalytic activity results in a loss of mitochondrial DNA without affecting cell viability.[28] Polymerase γ has both a 5' → 3' polymerase as well as a 3' → 5' exonuclease that eliminates mis-incorporated bases and facilitates the fidelity of mtDNA replication.[29] Both activities are associated with the large subunit. The function of the small subunit remains unknown, although it likely contributes to primer recognition and processivity.[30,31] The primer RNA for H-strand replication is generated by cleavage of the L-strand transcript by mitochondrial RNA processing (MRP) endonuclease (Fig. 3). This ribonucleoprotein contains a nucleus-encoded RNA that is essential for catalysis (MRP RNA) and is most abundant in the nucleolus where it participates in the processing of 5.8S rRNA precursors.[17] Although its association with mitochondria has been questioned,[32] both its cleavage specificity and in situ hybridization profile argue strongly for its function in mtDNA replication.[33-35] RNAse MRP

cleaves an R-loop containing the H-strand origin of replication at specific sites that match the in vivo priming sites.

During replication, exposed single-stranded regions are bound by mitochondrial single-stranded binding protein (mtSSB). In yeast, mtSSB is required for mtDNA maintenance, consistent with its role in mtDNA replication.[36] Genes for mammalian homologues have been characterized[37] and the crystal structure of human mtSSB has been solved. The mtSSB is structurally distinct from nuclear SSB but bears a strong structural similarity to the *E. coli* protein.[38] Topoisomerases and helicases have also been associated with mitochondria. Of particular note is a newly discovered mitochondrial protein designated as 'twinkle' because of its punctate cytoplasmic staining pattern.[39] Twinkle has a helicase domain resembling that of bacteriophage T7 gene 4 and mutations in the human nuclear gene encoding twinkle are associated with the autosomal dominant form of progressive external ophthalmoplegia. The inherited form of this disease is characterized by multiple deletions in mtDNA. Although a mechanism has yet to be elucidated, the genetic evidence suggests that twinkle function is essential for maintaining the integrity of the mitochondrial genome.

Transcription

Transcription initiation has also been well characterized. In yeast, transcription is initiated at approximately twenty transcriptional units throughout the genome (see ref. 7). In contrast, vertebrate transcription is initiated at two promoters, P_H and P_L for heavy and light strands respectively. These are spaced only 150 nucleotides apart within the D-loop regulatory region.[40,41] The H- and L-strand transcriptional units differ from most nuclear genes in that they are polygenic, specifying more than one RNA gene or mRNA. In addition to the RNA primer for H-strand replication, P_L also directs the synthesis of a transcript that is processed to one mRNA and eight of the 22 tRNAs (Fig. 1). The polygenic transcript directed by P_H is processed to 14 tRNAs, 12 mRNAs and the two rRNAs. The activities of both promoters require a 15 nucleotide conserved sequence motif that defines the core promoter. In addition, both promoters share an upstream enhancer that serves as the recognition site for Tfam (previously mtTF-1 and mtTFA) an HMG box protein that stimulates transcription through specific binding to the upstream enhancers (Fig. 3). Tfam also binds nonspecifically to apparently random sites on mtDNA.[42-44]

Enzymes and factors involved in mtDNA transcription have been identified and characterized. In yeast, transcription is directed by a 145kD core polymerase encoded by *RPO41* and a 43kD specificity factor, also known as sc-mtTFB, encoded by *MTF1* (see refs. 2, 4, 7). The polymerase shares sequence similarities with the T7 and T3 bacteriophage polymerases, which are also comprised of a single subunit. The primary structure of the sc-mtTFB specificity factor bears some resemblance to prokaryotic sigma factors[45] but a recent crystal structure reveals significant homology to rRNA methyltransferase.[46] The polymerase and specificity factor transiently interact and both are required for specific transcription initiation in vitro.[47] Genetic evidence supports a functional interaction between the two factors in vivo as well.

A vertebrate polymerase and a specificity factor that is required for specific initiation has been characterized biochemically in *Xenopus laevis*.[48] Although purification of the human polymerase has been elusive, a human cDNA that encodes a protein with sequence similarity to yeast mitochondrial and phage polymerases has been identified in database screenings.[49] The encoded protein localizes to mitochondria suggesting that it is a bona fide mitochondrial polymerase. A human mtTFB cDNA has also been isolated and the encoded protein has properties consistent with it being a functional homologue of sc-mtTFB.[50] The protein is localized to mitochondria, can bind DNA and stimulates transcription from an L- strand promoter in vitro. More recently, two isoforms of h-mtTFB, termed TFBM1 and 2, have been identified.[51] TFBM1 is identical to the original isolate but has about one-tenth the transcriptional activity of TFBM2. Both proteins work together with Tfam and mtRNA polymerase to direct proper initiation from H- and L-strand promoters (Fig. 3) and, like the yeast factor, both are related to

rRNA methyltransferases. It has yet to be determined whether the proteins are bi-functional or whether they evolved a single function from an ancestral methyltransferase. The availability of these human cDNAs should open the way for mechanistic studies on mammalian mitochondrial transcription.

Tfam is thus far the most well-characterized vertebrate factor involved in mitochondrial transcription initiation. As mentioned above, it was first identified as an HMG-box protein that recognizes enhancer elements in P_L and P_H.[17,47,52] Like other HMG proteins, Tfam can bend and unwind DNA, properties potentially linked to its ability to stimulate transcription upon binding DNA immediately upstream from the sites of transcription initiation.[43,53] In addition to specific promoter recognition, Tfam binds nonspecific DNA with high affinity. This property along with its abundance in mitochondria suggests that it plays a role in the stabilization and maintenance of the mitochondrial chromosome through its phased binding to nonpromoter sites. ABF2, a related HMG box factor from Yeast, resembles Tfam and is required for mtDNA maintenance and respiratory competence.[54] Expression of Tfam in ABF2-deficient Yeast cells can rescue both phenotypes suggesting that the two proteins are functionally homologous. Despite this functional complementation, ABF2 lacks an activation domain present in Tfam and does not stimulate transcription. A Tfam knockout mouse displays embryonic lethality and a depletion of mtDNA confirming an essential role for the protein in mtDNA maintenance in mammals.[55] In addition, Tfam levels correlate well with increased mtDNA in ragged-red muscle fibers and decreased mtDNA levels in mtDNA-depleted cells.[56] The correlation with mtDNA content is also observed for mtSSB in contrast to polymerase γ, which is expressed constitutively.[57] Despite these intriguing correlations, it is unclear which, if any of these, is the key limiting factor whose expression is regulated in controlling mtDNA copy number.[58]

Termination and RNA Processing

Transcription termination and RNA processing also play an important role in governing the steady-state levels of mitochondrial transcripts. Transcripts from P_H initiate either upstream from the $tRNA^{Phe}$ gene that precedes the 12S rRNA or near the 3'-end of $tRNA^{Phe}$ near its border with 12S rRNA. Initiation is more frequent at the upstream site and these transcripts terminate at a strong bi-directional terminator that is downstream from the 16S rRNA gene while a minority of transcripts traverse the entire H-strand.[59] Termination also occurs in the opposite direction thus attenuating L-strand transcription before a region where no L-strand genes are present. These termination events are thought to control the ratio of rRNA to mRNA in the mitochondrial matrix. The terminator consists of 28 nucleotides within the $tRNA^{Leu}$ gene immediately downstream of 16S rRNA. This sequence binds a trans-acting factor called mTERF, a 34kD protein that specifies site-specific transcription termination in vitro.[60] A structural prediction based on a mTERF cDNA revealed three leucine zippers that are involved in intramolecular interactions and facilitate binding of the protein to the target DNA. However, although the expressed recombinant protein displayed the expected binding specificity, it was not sufficient to direct termination in vitro suggesting that an additional component(s) may be required.[61]

Little is known about the regulation of RNA processing in mitochondria although the enzymatic machinery is at least partially characterized. One unique feature of mitochondrial genomic organization is that tRNA genes are dispersed around the mtDNA and flank the rRNA genes and nearly all of the protein coding genes (Fig. 1). Thus, most RNA processing sites occur at the junctions between tRNAs and other transcripts. This suggests that tRNA secondary structure may serve as the signal for enzymatic cleavage and release of individual RNA species.[6] In those cases where tRNA genes are not at the junctions, the adjacent RNAs may form structures that resemble the tRNA cleavage sites. Several enzymatic activities have been implicated in RNA maturation in human cells using an in vitro system, although there is some controversy as to the precise identity of the enzymes involved.[62] It is generally believed

that the 5'-end is processed by an RNAse P activity and the 3'-end by an unidentified endonuclease. Whether the human mitochondrial RNAse P has the same H1 RNA present in nuclear RNAse P and also the same substrate specificity remains controversial. In yeast, the mitochondrial RNAse P is comprised of a nucleus-encoded protein and a mitochondrially-encoded RNA that is necessary for catalysis.[63] Once the tRNAs are excised, CCA is added to their 3'-ends by an ATP(CTP)-tRNA-specific nucleotidyltransferase. The processed mRNAs are polyadenylated by a mitochondrial poly(A) polymerase but are lacking 5'-untranslated regions or a 7-methylguanylate cap structure. The rRNAs are modified by a short 3'-addition of adenyl residues.

Recombination and Repair

The mitochondrial genome acquires mutations at a rate 10 to 20 times faster than the nuclear genome resulting in a higher rate of molecular evolution and to the accumulation of disease mutations.[64] Several contributing factors to this phenomenon include the absence of mitochondrial histones, the existence of replicative intermediates with extensive regions of single-stranded structure and the lack of nucleotide excision repair pathways. In addition, although a high frequency of genetic recombination between mitochondrial genomes occurs in yeast, no recombination between paternal and maternal genomes or between heteroplasmic sequence variants has been observed in vertebrates. However, while deficient in nucleotide excision and mismatch repair, mitochondria are capable of base excision repair in response to oxidation and alkylation.[65] Damaged bases may be generated spontaneously or removed enzymatically by DNA glycosylases. For example, differential splicing of the transcript for uracil DNA glycosylase dictates whether the enzyme is localized to the nucleus or mitochondria and other glycosylases may be targeted to mitochondria as well. In addition to glycosylases, mitochondria possess other necessary activities for a base excision repair pathway including an endonuclease, ligase and polymerase and the reaction has been reconstituted in vitro using mitochondrial enzymes.[66] This mode of repair is significant considering that mtDNA is located near the respiratory chain which produces the bulk of reactive oxygen species that can oxidize DNA and other macromolecules. It has been postulated that oxidative damage to mtDNA may contribute to degenerative diseases and aging.

Mitochondrial Translation System

The mtDNA contribution to the mitochondrial translation system is restricted to the production of 2 ribosomal RNAs and 22 tRNAs. This number of tRNAs is smaller than the 32 required by the wobble hypothesis. One explanation for this difference is that for those amino acids with four possible codons, a U is present in the wobble position of a mitochondrial tRNA that allows recognition of all four codons (see refs. 2, 9). The protein components necessary for translation, including ribosomal proteins, tRNA synthetases, and the initiation and elongation factors, are all encoded by nuclear genes. Mitochondrial translation is bacteria-like both in its sensitivity to antibiotics that act on the ribosome, and in the use of N-formylmethionyl-tRNA for initiation. In addition, it was recognized soon after their discovery that mitochondrial ribosomes were smaller than those found in the cytosol with mammalian mitoribosomes having a sedimentation coefficient of 55S.[67] However, mitoribosomes are larger and more massive than bacterial ribosomes and they also have more protein subunits, shorter RNAs (16S and 12S) and are lacking 5S rRNA. Additionally, mitoribosomal proteins bear no close sequence similarity to either bacterial or eukaryotic ribosomal subunits suggesting that they are subject to different selective constraints.[68]

Many of the mechanistic details of mitochondrial translation in vertebrate systems have yet to be uncovered. In contrast to their cytosolic counterparts, mitochondrial mRNAs are lacking both a 5'-untranslated region and a 7-methylguanylate cap structure which facilitate ribosome binding and scanning to localize the initiation codon. The absence of these features may account for the production of larger amounts of mRNA with reduced translational efficiency.

Binding of the ribosome to mitochondrial mRNA is not sequence specific and apparently occurs in the absence of initiator tRNA. Initiation factors may be involved in initiator codon recognition but the only such factor identified in mammalian mitochondria is mtIF-2. Human and bovine homologues have been cloned and display sequence similarity with GTPases and IF-2 from *E. coli*.[69,70] The mitochondrial protein binds the small ribosomal subunit and facilitates binding of fMet-tRNA. MtIF-2 is subsequently released from the complex upon GTP hydrolysis followed by association of the large subunit to complete the initiation complex. In addition, mitochondrial elongation factors (mtEF-Tu, mtEF-Ts and mtEF-G) have been isolated and cDNAs obtained.[71,72] The structural and functional characteristics of these factors bear a strong resemblance to prokaryotic elongation factors.

Bi-Genomic Expression of the Respiratory Chain

Nuclear Activators and Coactivators

The limited coding capacity of mtDNA necessitates that the nuclear genome contributes the majority of gene products that are essential for mitochondrial function. Of the 100 or so respiratory chain proteins required for electron transport and oxidative phosphorylation, most are the products of nuclear genes. The nucleus also controls the biogenesis of the respiratory apparatus and the maintenance of mtDNA by providing all of the structural and enzymatic machinery required for mitochondrial transcription, translation and DNA replication. However, the nuclear contribution is not limited to the expression of the respiratory chain. Essential pathways for the oxidation of pyruvate and fatty acids, the biosynthesis of heme and certain amino acids are at least partially associated with the mitochondria.[73,74]

It has been well established in yeast that the regulated expression of nuclear genes in response to environmental signals is a key mechanism for mediating changes in respiratory metabolism. The availability of oxygen and nonfermentable carbon sources regulates the expression of many nuclear genes encoding respiratory chain proteins.[75] This occurs via the activation or induction of specific transcriptional activators and repressors that are targets of metabolic signaling pathways. Respiratory gene expression is also subject to regulation in mammals in response to diverse signals. These include hormonal stimulation, cyclic nucleotides,[76] oncogenic transformation, contractile activity,[77] temperature, and unidentified stimuli during pre[78] and post-natal[79] development. In many of these cases, respiratory chain subunits are induced at the mRNA or protein level but the specific regulatory mechanisms are not understood.

In recent years, a number of transcriptional regulators have been implicated in the expression of nucleus-encoded respiratory genes in mammals. Two such factors, NRF-1 and NRF-2, were identified as part of the characterization of cytochrome *c* and cytochrome oxidase promoters (see refs. 74, 80). It is now clear that one or both of these factors act on the majority of nuclear genes encoding subunits of the respiratory chain complexes. They are also involved in the expression of mitochondrial transcription and replication factors (Tfam, mtTFB and MRP RNA), heme biosynthetic enzymes and other proteins required for respiratory function. Thus, as summarized in Figure 4, these factors have the potential to integrate the expression of nuclear and mitochondrial genetic systems in response to cellular energy demands. NRF-1 is a transcriptional activator that binds a G + C rich pallindromic recognition site as a homodimer.[81] It exists as a phosphoprotein in vivo and phosphorylation enhances its DNA binding and transcriptional activities. NRF-1 phosphorylation also contributes to the growth-regulated induction of cytochrome *c*.[82] Targeted disruption of the NRF-1 gene in mice results in lethality at the blastocyst stage of development and depletion of mitochondrial DNA.[83] NRF-2 (originally defined in mice as GABP) has multiple subunits that either bind DNA or contribute activation or cooperative binding functions to a heterotetrameric complex.[84,85] This complex binds to tandemly arranged sites in many respiratory promoters (see refs. 3, 74, 80, 84, 86).

Although NRFs are key players, the nuclear genetic control of mitochondrial function cannot be explained by these factors alone. Several respiratory genes do not contain recognition

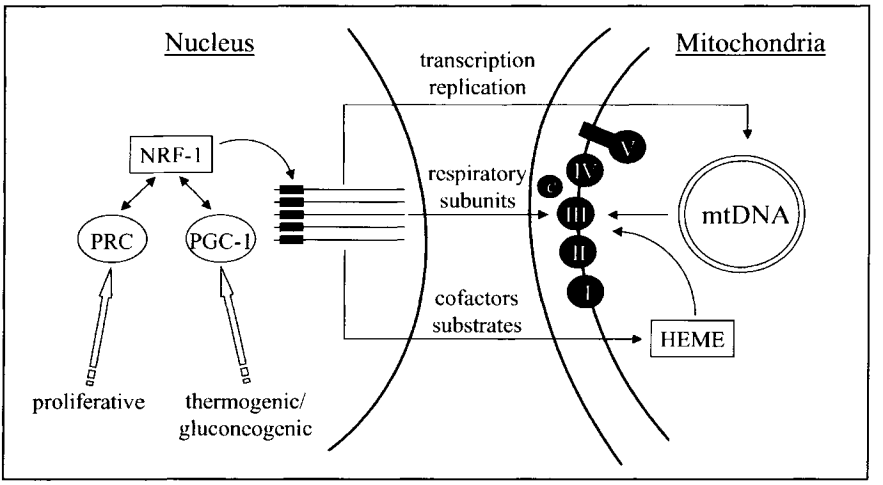

Figure 4. Activator-coactivator interactions in mitochondrial biogenesis. Nuclear respiratory factor 1 (NRF-1) is representative of transcription factors that act on nuclear genes whose products are required for expression and function of the mitochondrial respiratory apparatus. NRF-1 is a target for a small family of transcriptional coactivators whose members include PRC and PGC-1. The expression of these coactivators is regulated by proliferative, thermogenic and gluconeogenic signals thus placing NRF-1 and its target genes under the control of key signaling pathways. Activator-coactivator interactions may serve an integrative function in regulating cellular energetics.

sites for NRF-1 or NRF-2 and a number of other transcription factors have been implicated in respiratory gene expression. These include Sp1, CREB, YY1 and muscle-specific factors among others (see refs. 3, 86). In addition, nuclear genes for other mitochondrial functions (e.g., fatty acid oxidation) are controlled by transcription factors (e.g., PPARα) that do not act on respiratory promoters.[87] These observations beg the question of how multiple transcription factors can be integrated into a program of mitochondrial biogenesis. Part of the explanation came with the discovery of PGC-1, a transcriptional coactivator that induces mitochondrial biogenesis by interacting with NRF-1, PPARα and possibly other nuclear factors.[88,89] PGC-1 is markedly induced in brown fat during adaptive thermogenesis and has the remarkable property of being able to induce mitochondrial biogenesis when expressed ectopically in cultured cells or in transgenic mice.[3,89] In the presence of NRF-1, PGC-1 can trans-activate NRF-1 target genes that are necessary for the biogenesis of mitochondria and the expression of a functional respiratory chain (Fig. 4). PGC-1 interacts with NRF-1 in vitro and in vivo and a dominant negative allele of NRF-1 interferes with the ability of PGC-1 to induce mitochondrial proliferation.[88] PGC-1 also interacts with PPARα to induce the enzymes of fatty acid oxidation.[89] PPARα is a major activator of this pathway and is enriched in tissues with high oxidative energy demands. PGC-1 can bind PPARα and trans-activate PPARα-dependent promoters. Thus, the functional interplay between PGC-1 and certain nuclear transcription factors appears to define a major regulatory pathway for the biogenesis of mitochondria.

Recently, transcriptional coactivators related to PGC-1 have been identified. PGC-1 related coactivator (PRC) has several structural features in common with PGC-1 including an activation domain, an LXXLL coactivator signature, and an RNA recognition motif.[90] PRC is indistinguishable from PGC-1 in its ability to interact with NRF-1 and to activate NRF-1 target genes. However, it differs from PGC-1 in its mode of regulation. PRC is not significantly induced during adaptive thermogenesis but is induced when cells are stimulated to proliferate by serum growth factors (Fig. 4). PRC is down regulated when cells exit the cell cycle upon contact inhibition or withdrawal of serum. The results suggest that PRC may control mito-

chondrial biogenesis in response to proliferative signals. PGC-1β is a second PGC-1 family member that is closely related to PGC-1 and shares a similar tissue distribution.[91] Although it is not induced in brown fat upon cold exposure, it is induced in liver in response to fasting. This latter property is shared with PGC-1 which has recently been implicated in the induction of gluconeogenesis.[92] Thus, the differential regulation of members of this family of coactivators may help coordinate the biogenesis of mitochondria with pathways of cellular energy metabolism.

Retrograde Regulation

A second mode of bi-genomic regulation concerns the response of nuclear genes to changes in mitochondrial activity. This phenomenon is well-studied in yeast and has been termed retrograde regulation (see ref. 2). In yeast cells lacking mtDNA (ρ^0 cells) the nuclear *CIT2* gene, encoding peroxisomal citrate synthase, is markedly induced. This enzyme is part of the glyoxylate cycle and its induction in response to a defect in mitochondrial respiration allows cells to convert two carbon compounds such as acetate to carbohydrate.[93] The peroxisomal citrate synthase and other peroxisomal proteins are regulated by the basic helix-loop-helix transcription factors Rtg1p and Rtg3p.[94] A third protein Rtg2p facilitates the translocation of Rtg1p and Rtg3p to the nucleus in response to a mitochondrial deficiency. This retrograde pathway allows cells to adapt to defects in respiratory energy production.

Although the Rtg pathway has not been identified in vertebrates, there are a number of examples where nuclear gene expression appears to be altered by mitochondrial deficiency. In certain mitochondrial diseases, defective mitochondria proliferate in diseased muscle fibers giving rise to ragged red fibers.[95] Specific nuclear genes involved in ATP production also display elevated expression in cells with mtDNA mutations.[96] A change in the pattern of nuclear gene expression, involving proteins of the mitochondrial inner membrane as well as intermediate filaments and ribosomes, is observed in human cells upon depletion of mtDNA.[97] Chicken cells depleted of mtDNA or treated with the mitochondrial protein synthesis inhibitor chloramphenicol have increased levels of mRNAs for elongation factor 1α, β-actin, v-*myc* and GAPDH.[98] Presumably, these examples represent nuclear responses to deficiencies in ATP production. Although no unifying mechanisms have been advanced to explain these phenomena, recent studies suggest that retrograde signaling may be mediated by calcium.[99] Mitochondrial impairment, either by depletion of mtDNA or by metabolic inhibitors, mediates a stress response that coincides with elevated cytosolic calcium levels. The response includes increased expression of calcium-responsive transcription factors and cytochrome oxidase subunit Vb. Calcium has also recently been linked to a PGC-1-dependent pathway of mitochondrial biogenesis in skeletal muscle.[100] Transgenic mice, expressing a constitutively active calcium/calmodulin-dependent protein kinase in skeletal muscle, display increased mtDNA copy number and respiratory cahin enzymes as well as elevated PGC-1 levels. Thus, calcium may be an important link between the relay of extracellular signals to the nucleus and the bi-directional communication between nucleus and mitochondria. Much needs to be done before it is clear whether the various changes in gene expression that coincide with mitochondrial impairment represent a physiologically meaningful pathway of retrograde regulation in vertebrate cells.

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