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Review

The mitochondrial genome: structure, transcription, translation and replication

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Abstract

Mitochondria play a central role in cellular energy provision. The organelles contain their own genome with a modified genetic code. The mammalian mitochondrial genome is transmitted exclusively through the female germ line. The human mitochondrial DNA (mtDNA) is a double-stranded, circular molecule of 16 569 bp and contains 37 genes coding for two rRNAs, 22 tRNAs and 13 polypeptides. The mtDNA-encoded polypeptides are all subunits of enzyme complexes of the oxidative phosphorylation system. Mitochondria are not self-supporting entities but rely heavily for their functions on imported nuclear gene products. The basic mechanisms of mitochondrial gene expression have been solved. *Cis*-acting mtDNA sequences have been characterised by sequence comparisons, mapping studies and mutation analysis both in vitro and in patients harbouring mtDNA mutations. Characterisation of *trans*-acting factors has proven more difficult but several key enzymes involved in mtDNA replication, transcription and protein synthesis have now been biochemically identified and some have been cloned. These studies revealed that, although some factors may have an additional function elsewhere in the cell, most are unique to mitochondria. It is expected that cell cultures of patients with mitochondrial diseases will increasingly be used to address fundamental questions about mtDNA expression.

Keywords: Mitochondrial DNA; Replication; Transcription; Translation; Protein synthesis; Mitochondrial encephalomyopathy; (Human)

Contents

Abbreviations: CSB, conserved sequence block; EST, expressed sequence tags; HMG, high mobility group; HSP, H-strand promoter; H-strand, heavy strand; IT, initiation of transcription site; LSP, L-strand promoter; L-strand, light strand; mtDNA, mitochondrial DNA; mtIF, mitochondrial initiation factor; mtEF, mitochondrial elongation factor; mtRNase P, mitochondrial ribonuclease P; mtSSB, mitochondrial single-stranded binding protein; mtTERM, mitochondrial transcription terminator; mtTFA, mitochondrial transcription factor A; O_H, origin of H-strand replication; O_L, origin of L-strand replication; RNase MRP, mitochondrial RNA processing endoribonuclease; TAS, termination-associated sequence

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1. Introduction

Mitochondria are the energy-transducing organelles of eukaryotic cells in which fuels to drive cellular metabolism are converted into ATP through the process of oxidative phosphorylation. Mitochondria have a double membrane. The outer membrane separates the mitochondrion from the cytosol. The inner membrane is invaginated to form the cristae which protrude into and define the matrix of the organelle. The five enzyme complexes of the oxidative phosphorylation system [1] are embedded in the mitochondrial inner membrane. Mitochondria contain their own genome, the mitochondrial DNA (mtDNA), which is located in the mitochondrial matrix. In mammalian cells, each organelle generally contains several identical copies of mtDNA [2–5].

Mitochondria are thought to have originated from incorporated α-purple bacteria [6]. During its evolution into the present-day powerhouses of the eukaryotic cell, the endosymbiont transferred many of its essential genes to the nuclear chromosomes. Nevertheless, the mitochondrion still carries hallmarks of its bacterial ancestor. For instance, mitochondria use an *N*-formylmethionyl-tRNA (fMet-tRNA) as initiator of protein synthesis [7,8].

For over 20 years, it has been recognised that, in mammals, mtDNA is only transmitted through the female germ line [9–12]. In mammalian sperm cells, the copy number of mtDNA is low (50–75 [13]), whereas in mammalian oocytes the copy number is extremely high ($\geq 10^5$ [2,14]). Therefore, the maternal inheritance of mtDNA observed in early studies could simply have been the result of dilution of the paternal contribution beyond the detection limit of the restriction enzyme analysis on which these studies relied. In a more recent investigation, in which a more sensitive, PCR-based technique was used, low levels of paternal mtDNA were detected in *inter*specific hybrids of the mice species *Mus musculus* and *Mus spretus* throughout development from pronucleus stage to neonates [15]. In *intra*specific offspring of *M. musculus*, however, paternal mtDNA was only detected in the early pronucleus stage [15]. In the majority of mammals, including humans, sperm mitochondria are transferred to the oocyte during fertilisation [16], but detailed morphological studies in rodents [15,17] and cows [18] have indicated that sperm-derived mitochondria are lost early in embryogenesis. The mechanism underlying the elimination of sperm-derived mitochondria is unknown but the leakage of paternal mtDNA in progenies of *inter*specific mice crosses suggest that this process is species specific. Although it is not clear to which extent the

observations in rodents and cows can be extrapolated to the human oocyte [16], Manfredi and colleagues [19] recently showed that re-population of experimental human somatic cells lacking endogenous mtDNA (ρ^0 cells) by mtDNA can occur after entry of human sperm but is an extremely rare event.

This review focuses on human mtDNA. Some aspects of the expression of human mtDNA, however, are still poorly characterised. In those cases, our current understanding of the situation in other eukaryotes will be discussed.

2. Structure of mtDNA

As mtDNA is a relatively small, abundant and easy to isolate DNA molecule, it has been the favourite target of early genome sequencing projects and the nucleotide sequence of mtDNA from a large number of species has now been determined [20]. The nucleotide sequence of the human mtDNA was the first documented complete sequence of a mitochondrial genome [21]. Structure and gene organisation of mtDNA is highly conserved among mammals [22]. The mammalian mitochondrial genome is a closed-circular, double-stranded DNA molecule of about 16.6 kb. The strands of the DNA duplex can be distinguished on the basis of G+T base composition which results in different buoyant densities of each strand ('heavy' and 'light') in denaturing caesium chloride gradients [23]. Most information is encoded on the heavy (H) strand, with genes for two rRNAs, 14 tRNAs, and 12 polypeptides. The light (L) strand codes for eight tRNAs and a single polypeptide. All 13 protein products are constituents of the enzyme complexes of the oxidative phosphorylation system [21,24–26]. Mammalian mtDNA displays exceptional economy of organisation. The genes lack introns and, except for one regulatory region, intergenetic sequences are absent or limited to a few bases. Both rRNA and tRNA molecules are unusually small [22]. Some of the protein genes are overlapping and, in many cases, part of the termination codons are not encoded but are generated post-transcriptionally by polyadenylation of the mRNAs [27]. A map of the human mtDNA is given in Fig. 1.

Soon after mtDNA sequences became available, comparisons with mitochondrial protein sequences

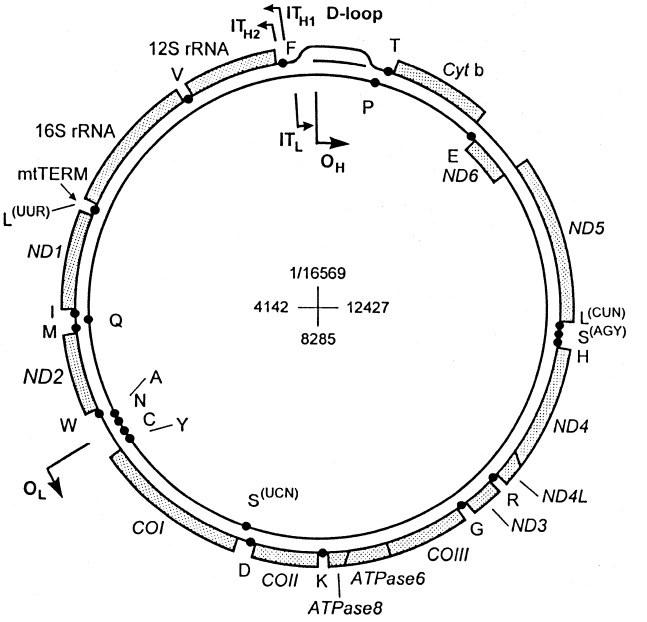

Fig. 1. Map of the human mitochondrial genome (16 569 bp). The outer circle represents the H-strand, containing the majority of the genes; the inner circle represents the L-strand. The D-loop is shown as a three-stranded structure. The origins of H-strand (O_H) and L-strand (O_L) replication and the direction of DNA synthesis are indicated by long bent arrows; the initiation of transcription sites (IT_L, IT_{H1}, IT_{H2}) and the direction of RNA synthesis are denoted by short bent arrows. The binding site for the mitochondrial transcription terminator (mtTERM) is indicated. The 22 tRNA genes are depicted by dots and the single letter code of the amino acid (isoacceptors for serine and leucine are distinguished by their codon sequence). The genes coding for the two rRNA species (12S and 16S) and the 13 protein coding genes are depicted by shaded boxes. *ND*, *CO* and *ATPase* refer to genes coding for subunits of NADH: ubiquinone oxidoreductase, ferrocytochrome *c*:oxygen oxidoreductase (cytochrome *c* oxidase) and F_1F_0-ATP synthase, respectively, whereas *Cyt b* encodes apocytochrome *b* of ubiquinol: ferricytochrome *c* oxidoreductase.

revealed deviations from the standard genetic code and later even variations in codon usage were found in mitochondria from different species (summarised in [28]). For instance, in mtDNA of most phylogenetic groups, TGA is used as a tryptophan codon, rather than as a termination codon. On the other hand, AGR (R = A, G) specifies a stop in mtDNA of vertebrates, codes for serine in mtDNA of echinoderms and codes for arginine in mtDNA of yeast, as in the standard genetic code.

Another surprising feature of the mitochondrial genetic system is its use of a simplified decoding

mechanism, allowing translation of all codons with less than the 32 tRNA species required according to Crick's wobble hypothesis. This reduction in the number of tRNA species is achieved by the use of a single tRNA with U in the first anticodon (wobble) position to recognise all codons of a four-codon family [29,30]. Fungal mitochondria use a modified U in the wobble position to read two codon families with a purine in the third position of the codon [31,32]. This mechanism prevents misreading of two codon families with a pyrimidine in the third position and is considered to be conserved in vertebrate mitochondria. This would imply that 24 tRNA species are required to decode mtDNA, however, as mentioned above, in vertebrates AGR codons indicate a stop and the corresponding tRNA gene is absent. Furthermore, a single tRNA species, with a modified C in the first anticodon position followed by AU, functions for initiation ($tRNA^{fMet}$) as well as elongation ($tRNA^{Met}$) and pairs with codon AUA in addition to AUG [28,33]. Hence, the 22 tRNA species encoded by human mtDNA are sufficient to translate all 13 mitochondrial protein genes.

In vertebrate cells that are metabolically active, a large proportion of the mtDNA duplexes contain a short three-stranded structure, called the displacement loop or D-loop [23], in which a short nucleic acid strand, complementary to the L-strand, displaces the H-strand. The D-loop region is bounded by the genes for $tRNA^{Phe}$ and $tRNA^{Pro}$ [34] and has evolved as the major control site for mtDNA expression, containing the leading-strand origin of replication and the major promoters for transcription (Fig. 1).

Mitochondria are not self-supporting entities in the cell. Replication and transcription depend upon *trans*-acting nuclear-encoded factors. Mitochondrial tRNAs are charged by imported aminoacyl-tRNA synthases and, in vertebrates, all mitochondrial ribosomal proteins are coded and synthesised outside the organelle. Enzymes of the various catabolytic pathways located in the mitochondria, as well as the components of the mitochondrial import machinery, are encoded by nuclear DNA. Even the enzyme complexes of the oxidative phosphorylation system are of hybrid genetic origin. All nuclear-encoded polypeptides destined for mitochondria are synthesised on cytosolic ribosomes, usually with a cleavable, N-terminal presequence for mitochondrial targeting and are subsequently imported into mitochondria (reviewed in [35]).

3. Transcription of mtDNA

3.1. Initiation of transcription

The basic mechanism of mitochondrial transcription has been solved in representative species of several phylogenetic groups (reviewed in [36–38]). Human mtDNA transcription initiation sites and promoter regions have been determined using a variety of techniques, including 5′-end mapping of primary mitochondrial transcripts by S1 nuclease protection experiments [39–41] and deletion [42–44], site-directed mutagenesis [45] and linker substitution analyses [46] of cloned mtDNA fragments in run-off transcription assays. All available data are consistent with the conclusion that there are two major transcription initiation sites in the D-loop (IT_{H1} and IT_L) situated within 150 bp of one another (Fig. 2). A promoter element with a 15-bp consensus sequence motif, 5′-CANACC(G)CC(A)AAAGAYA, surrounds the transcription initiation sites (underlined) and is critical for transcription [43,45]. H-strand transcription starts at nucleotide position 561 (IT_{H1}; numbering according to [21]) located within the H-strand promoter (HSP) and immediately adjacent to the $tRNA^{Phe}$ gene, whereas the L-strand transcription starts at nucleotide position 407 (IT_L) located within the L-strand promoter (LSP). Additional upstream enhancer elements are required for optimal transcription (Fig. 2). These elements, which were later shown to include binding sites for a transcription factor (mtTFA, see below), exhibit sequence similarity but only if one element is inverted relative to the other [43,45,47].

Despite the close proximity of the HSP and LSP, the initial in vitro transcription studies demonstrated that these elements are functionally independent [42,43,45,46]. This functional autonomy was later corroborated in patients with progressive external ophthalmoplegia, harbouring a large-scale deletion, including the HSP, in a sub-population of their mtDNA molecules. In situ hybridisation experiments revealed focal accumulations of deleted mtDNA

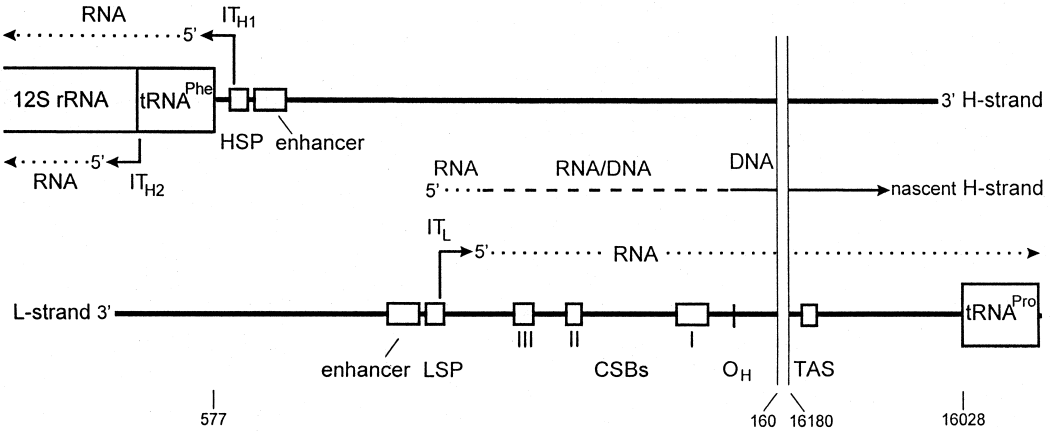

Fig. 2. Schematic representation of the initiation of transcription and replication of human mtDNA. The genes encoding 12S rRNA, $tRNA^{Phe}$ and $tRNA^{Pro}$ are indicated with boxes on the H- and L-strands. Transcription initiation sites and direction of synthesis are indicated by bent arrows, dotted lines represent synthesised RNA. In the D-loop region (the 1118-bp sequence between nucleotides 577 and 16028), two major transcription initiation sites are present. Transcription initiation site IT_{H1}, encompassed by the H-strand promoter (HSP), directs the transcription of the H-strand, whereas transcription initiation site IT_L, encompassed by the L-strand promoter (LSP), directs the transcription of the L-strand. A second, minor transcription initiation site (IT_{H2}) for H-strand transcription is located in the gene for $tRNA^{Phe}$ near the boundary with the 12S rRNA gene. Enhancer elements upstream of the HSP and LSP that are known to bind the mitochondrial transcription factor mtTFA are indicated. A short (RNA) transcript originating at IT_L serves as a primer for replication of the (leading) H-strand. Transitions from RNA to DNA occur within the dashed line, in the region around the conserved sequence blocks (CSBs) I, II and III. O_H is the origin of H-strand synthesis. Short DNA strands that are part of the triplex D-loop structure terminate near the termination-associated sequence (TAS).

and L-strand transcripts with concomitant depletion of H-strand transcripts in muscle fibres of the patients [48,49]. These results confirm the functional independence of the transcriptional promoters in vivo.

A second putative initiation site for H-strand transcription is located around nucleotide position 638 (IT_{H2}) in the $tRNA^{Phe}$ gene, immediately adjacent to the gene for 12S rRNA (Fig. 2). Its promoter region only shows limited similarity with the 15-bp consensus sequence and this site is used less frequently than IT_{H1} for transcription of the H-strand [39–41,43].

Although there is a fairly detailed picture of the *cis*-acting elements involved in mtDNA transcription, knowledge of the *trans*-acting nuclear-encoded factors is still incomplete. Biophysical fractionation of human mitochondrial transcription extracts have revealed the requirement of at least two *trans*-acting proteins: a relatively non-selective core RNA polymerase and a dissociable transcription factor which confers promoter selectivity on the polymerase [47,50]. The core enzyme is expected to interact with the HSP and LSP as mutations in these domains obliterate even the lowest level of transcription [46]. Mitochondrial RNA polymerases have not been purified to homogeneity. Nevertheless, human cDNA specifying mitochondrial RNA polymerase was recently identified by screening of an expressed sequence tags (EST) database with the yeast sequence [51]. Interestingly, the C-terminal half of the predicted polypeptide shares significant amino acid sequence identity with the single subunit RNA polymerases of T3, T7 and SP6 bacteriophages.

The human dissociable transcription factor acting in concert with the core mitochondrial RNA polymerase has been purified [52,53], its cDNA has been cloned and sequenced [54] and its gene has been characterised [55–57]. The factor, now termed mtTFA [58], is an abundant 25-kDa mitochondrial protein and is largely comprised of two high mobility group (HMG) domains separated by a 27-amino acid residue linker and followed by a 25-amino acid residue basic C-terminal tail. HMG domains are considered to be involved in DNA binding and are found in a rather diverse family of proteins whose members have been implicated in processes such as transcription enhancement and chromatin packaging

[59]. Mutation analysis of the human mtTFA has demonstrated that its C-terminal tail is important for specific DNA recognition and is essential for sponsoring of high levels of specific initiation of transcription [60].

In vitro DNase I protection studies in combination with run-off transcription assays have demonstrated that binding of mtTFA to regions immediately upstream of the HSP or LSP (10–40 bp upstream of each start site) is required for specific initiation of transcription [46,47,52,53]. Binding of mtTFA at these regions was recently supported by in organello footprinting experiments [61,62]. Both major transcription promoters in human mitochondria can function bidirectionally, in vitro as well as in vivo [63]. The asymmetric binding of mtTFA relative to the transcription start site may ensure that transcription proceeds primarily in an unidirectional fashion (Fig. 2). There is a strict requirement for the natural 10-bp spacing (one helical turn) between the mtTFA binding site and the start site of transcription [64]. The transcription factor has the ability to wrap and unwind DNA in vitro in an essentially non-specific protein-DNA complex [65,66] and scanning transmission electron microscopy recently revealed that the *Xenopus* homologue causes sharp bending of the DNA duplex at the promoter activation site [67]. These mtTFA-induced conformational changes of mtDNA may be required to allow the core RNA polymerase access to the template for initiation of the transcription process.

DNase footprinting and transcription studies have indicated that the affinity of mtTFA for its binding site immediately upstream of the LSP is relatively strong and that mtTFA markedly enhances L-strand transcription. Conversely, the affinity for its binding site upstream of the HSP is several fold weaker and mtTFA only moderately stimulates H-strand transcription [47,52,53,61]. This suggests that additional factors, not operating in the in vitro system may be necessary for effective transcription initiation at the HSP in vivo. As mammalian mitochondrial RNA polymerase has not been purified to homogeneity, other accessory proteins may be present in the active mitochondrial RNA polymerase fractions. Although there is no direct evidence for any mammalian supplementary components at the present time, an additional 40-kDa protein, obligatory for promoter-directed transcription selectivity of the core mitochondrial RNA polymerase, has been identified in several yeast species [68–70] and *Xenopus laevis* [71,72]. This factor, designated mtTFB, exhibits sequence homology to the dissociable σ subunit of bacterial RNA polymerases [69] which is responsible for promoter recognition of the bacterial holo-enzyme.

3.2. Elongation and termination of transcription

Once initiated at the LSP, the L-strand is transcribed as a single polycistronic precursor RNA, encompassing most, if not all, genetic information potentially encoded on the strand [73,74]. Although the HSP may direct transcription of the entire H-strand in a similar fashion, a more complicated model has been postulated by Attardi and colleagues [40]. In exponentially growing HeLa cells, the rRNAs are synthesised at a much higher rate than the individual mRNAs encoded on the H-strand [75]. This difference has been explained in part by the existence in vivo of two distinct initiation sites (IT_{H1} and IT_{H2}; Fig. 2) for H-strand transcription [39]. According to the dual H-strand transcription model, transcription starts relatively frequent at the IT_{H1} and then terminates at the downstream end of the 16S rRNA gene. This transcription process is responsible for synthesis of the vast majority of the two rRNA species. In contrast, transcription starting at IT_{H2} is less frequent but results in polycistronic molecules corresponding to almost the entire H-strand, yielding all the mRNAs and most of the tRNAs encoded on the H-strand. Evidence for two independently controlled, overlapping transcription units is supported by the observation, both in vivo and in isolated mitochondria, of two types of transcripts of the ribosomal genes with different kinetic properties [40]. Consistent with this model, Attardi's group found that the relative transcription rates of rRNA and mRNA can be modulated independently by the intercalating compound ethidium bromide [76] and by ATP [77]. The recently observed ethidium bromide and ATP-dependent modifications in protein-DNA footprints upstream of IT_{H1}, which could be correlated with changes of the rate of rRNA synthesis but not of mRNA synthesis, and the indication of a protein-DNA interaction site upstream of IT_{H2} have giv-

en further credence to the model [62]. Nevertheless, it is difficult to imagine how two initiation events taking place less than 100 bp apart can determine the fate of RNA synthesis at the distal end of the 16S rRNA gene, more than 2500 nucleotides downstream.

In addition to the dual H-strand transcription model, the difference in synthesis rate of rRNA and mRNA has been explained by an attenuation event at the border of the 16S rRNA and $tRNA^{Leu(UUR)}$ genes (Fig. 1). The first indication of early termination of the polycistronic H-strand transcript came from structural analysis of the 3′-ends of 16S rRNA molecules which revealed that a large proportion of the molecules have ragged 3′-termini that are coded for by the immediately adjacent gene for $tRNA^{Leu(UUR)}$ [78,79]. This suggests that mature 16S rRNA species are generated by imprecise transcript termination at the $tRNA^{Leu(UUR)}$ gene as well as by accurate endonucleolytic cleavage of the longer precursor RNA. Later, a crude protein fraction was isolated from mitochondrial lysates of HeLa cells which, in DNase I footprinting studies, protected the region immediately downstream of the mtDNA region corresponding to in vivo produced 3′-ends of 16S rRNA molecules and promoted specific termination of transcription [80]. The footprint encompasses a conserved tridecamer sequence block within the $tRNA^{Leu(UUR)}$ gene (nucleotide positions 3237–3249 of the human mtDNA; Fig. 1), which has been shown by in vitro deletion mutagenesis experiments to be essential and sufficient for directing termination [81].

The factor mediating attenuation of transcription has been termed mTERF [80] or mtTERM [82] and is known to induce bending of the DNA helix [83]. In vitro transcription studies have shown that mtTERM bound to its mtDNA target site functions bidirectionally and shows an even greater efficiency of termination in the reverse orientation relative to the promoter site [84]. Thus, in addition to an attenuation function for H-strand transcription, mtTERM may halt L-strand transcription at a site where no L-strand encoded genes are found downstream (see Fig. 1). Bound mtTERM probably stops elongation of transcription by constituting a physical barrier, rather than by a specific interaction with the mitochondrial RNA polymerase, because mtTERM also mediates termination of transcription by heterologous RNA polymerases [83].

Both Clayton's and Attardi's groups have shown that a polypeptide fraction of a mitochondrial lysate containing polypeptides with a molecular weight of around 34 kDa has the ability to produce the specific DNase I footprint at the termination site associated with mtTERM function [82,85]. The cDNA of the predominant polypeptide from this fraction was recently cloned and sequenced [86]. The polypeptide contains two widely separated basic regions and three leucine zipper motifs which were shown to be necessary for its specific DNA-binding capacity [86]. The footprint produced by the recombinant protein was similar but not identical to that produced by the 34-kDa polypeptide fraction. The recombinant protein was also shown to be unable to promote transcription termination in an in vitro system [86]. These observations suggest that an additional component of the 34-kDa polypeptide fraction is required for the termination activity. This apparent complexity of mtTERM is not surprising, given that it should be able to modulate its activity in response to the cellular demand for mitochondrial rRNAs, on the one hand, and for mitochondrial tRNAs and mRNAs on the other.

Interestingly, a heteroplasmic A to G transition in the middle of the mtTERM binding site (A3243G) is frequently found in patients with the mitochondrial encephalomyopathy MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes) [87] and in patients with maternally inherited adult onset diabetes [88]. In vitro studies have shown that this mutation dramatically reduces the affinity of mtTERM for its binding site and causes a defect in transcript termination [82,83,89]. In contrast, steady-state transcript levels upstream and downstream of the termination site were not affected by the A3243G transition in cultured cells [89,90] or in tissues of patients [49,91,92]. Thus, it seems unlikely that the in vitro observed defective attenuation of mitochondrial transcription is of pathological significance.

3.3. Processing of primary transcripts

Once the RNA polymerase passes the 16S rRNA/$tRNA^{Leu(UUR)}$ boundary, H-strand transcription ap-

pears to be straightforward. As no intron sequences are present in vertebrate mtDNA and intergenetic sequences are minimal, processing of the long polycistronic H- and L-strand messengers is thought to be a relatively simple process, requiring only a few enzymes. Genes for tRNAs flank the two rRNA genes and nearly every protein gene (Fig. 1). This unique genetic organisation has led to the proposal that the secondary structure of the tRNA sequences provide the punctuation marks in the reading of the mtDNA information [27]. Precise endonucleolytic excision of the tRNAs from the nascent transcripts will concomitantly yield correctly processed rRNAs and, in most cases, correctly processed mRNAs [27,93]. In those cases in which the mRNA termini cannot be accounted for by tRNA excision (e.g. the messenger for subunit I of cytochrome *c* oxidase [27,93]; Fig. 1), the processing enzyme possibly recognises a secondary structure which shares critical features with the typical cloverleaf structures of tRNAs.

Maturation of mitochondrial tRNAs involves three enzymatic activities which were recently identified by Rossmanith and colleagues [94] in an in vitro HeLa cell mitochondrial tRNA processing system. Their experiments showed that cleavage at the 5′-end precedes that at the 3′-end. The endonuclease responsible for 3′-end cleavage has not been characterised. Cleavage at the 5′-end is performed by a mitochondrial RNase P (mtRNase P). The enzyme containing fractions prepared by Rossmanith and colleagues [94] cut mitochondrial tRNA precursors at the correct 5′-end, but, unlike the preparations by others (see, e.g. [95]), do not cleave $tRNA^{Tyr}$ precursors of *Escherichia coli* correctly. This suggests that previous preparations were contaminated with a cytosolic isoform of RNase P which is apparently able to accurately process bacterial tRNA precursors. Yeast mtRNase P has been characterised in detail. The enzyme of *Saccharomyces cerevisiae* is composed of a nuclear-encoded protein and a mtDNA-encoded RNA species [96,97]. The RNA moiety of the ribonucleoprotein complex is AU-rich and forms the catalytic core of the enzyme. Comparison of mtRNase P RNA from different yeast species has revealed a remarkable variation in size from 490 to 140 nucleotides [98].

Maturation of the excised tRNAs is completed by addition of the sequence CCA to their 3′-end catalysed by ATP(CTP):tRNA nucleotidyltransferase [94]. Mitochondrial mRNAs are polyadenylated by a mitochondrial poly(A) polymerase during or immediately after cleavage [99,100], whereas the 3′-ends of the two rRNAs are post-transcriptionally modified by the addition of only short adenyl stretches [78]. Mitochondrial messengers do not carry upstream polyadenylation signals as found in nuclear messengers.

4. Mitochondrial protein synthesis

4.1. Mitochondrial ribosomes

Early indications of the prokaryotic origin of mitochondria came from observations, now more than three decades ago, that the spectrum of antibiotics inhibiting mitochondrial protein synthesis resembles that of prokaryotic systems (reviewed in [101]). Subsequent research confirmed that nearly all constituents of the mitochondrial translation machinery are distinct from their cytosolic counterparts. Unique features of mitochondrial protein synthesis were first studied at the molecular level in mitochondrial ribosomes. The mitochondrial ribosomes, or mitoribosomes, are located in the matrix of the organelle. Steady-state rRNA levels in rat hepatocytes [102] suggest that there are <100 mitoribosomes per mitochondrion. The physical and chemical properties of mitoribosomes differ considerably from their cytosolic as well as their bacterial counterparts. Mammalian mitoribosomes have an unusually low RNA content and, consequently, a low sedimentation coefficient of $\sim$55S [103–106]. The $\sim$39S and $\sim$28S ribosomal subunits contain respectively the 16S and 12S rRNA species encoded by the mtDNA [103,104]. A 5S rRNA species, typically present in ribosomes, appears to be absent in mammalian mitoribosomes [103,104] but a 23-bp region at the 3′-end of the human 16S rRNA exhibits a 68% sequence identity to a portion of the *Bacillus subtilis* 5S rRNA. The structural similarity of the region and its mapping position have led to the suggestion that this piece represents a truncated 5S rRNA which remained part of the large rRNA component [107].

The low RNA content of mammalian mitoribo-

somes is compensated by a relatively high protein content and results in a total mass of mitoribosomes similar to that of bacterial ribosomes. Two-dimensional gel electrophoresis has allowed the resolution of 85 mitoribosomal protein spots from beef [108] and 86 from rat [106], however, the actual number of distinct mitoribosomal proteins may be lower as differences in staining intensity of some spots suggest possible proteolytic degradation and contamination with other proteins cannot fully be excluded. One can speculate that some mitoribosomal proteins have adopted structural and functional roles of the lost rRNA but experimental data as to the functions of mammalian mitoribosomal proteins are not available and most recent progress on the elucidation of the properties of the individual mitoribosomal proteins comes from studies in *S. cerevisiae* [109].

4.2. Initiation and elongation of translation

Although isolated intact mitochondria faithfully carry out protein synthesis, an in vitro mitochondrial translation system using only mitochondrial extracts is not available. Due to this persistent lack, many details of the mitochondrial protein biosynthesis are poorly understood and only a limited number of the mammalian auxiliary factors involved in initiation and elongation of translation have been characterised. Termination of the translation process has not yet been explored.

The mitochondrial translational apparatus is unique in many ways. As mentioned earlier, the rRNA and tRNA species are surprisingly small. The start of the translation process is intriguing because mammalian mitochondrial mRNAs have no upstream leader sequences to facilitate ribosome binding, unlike prokaryotic and eukaryotic cytosolic messengers, but start at or very near the 5′-end with the codon for the initiating *N*-formylmethionine [93]. In addition, the 5′-termini of mitochondrial mRNAs lack a 7-methylguanylate cap structure [110]. This excludes a cap recognition and scanning mechanism for directing the ribosome to the initiation codon as observed in the cytosol of eukaryotic cells. The low translational efficiency of mitochondrial messengers [102] may in fact be the result of the absence of a 5′-end ribosome recognition site and necessitate the observed abundance of mitochondrial messengers [111,112] to ensure that a sufficient level of translation occurs.

In vitro experiments with bovine mitoribosomes have indicated that the small (28S) ribosomal subunit has the ability to bind mRNA tightly in a sequence-independent manner and in the apparent absence of auxiliary initiation factors or initiator tRNA [113], unlike prokaryotic [114] and eukaryotic cytosolic [115] systems. Judging from the size of the RNA fragments protected from RNase T_1 digestion, the major interaction between the small subunit and the messenger occurs over a 30–80-nucleotide stretch, but $\sim$400 nucleotides are minimally required for efficient binding [113,116,117]. This may explain why the two shortest expressed open reading frames of mammalian mtDNA (*ATPase8* and *ND4L*; $<$300 bp) are both part of overlapping genes (*ATPase8*/*ATPase6* and *ND4L*/*ND4*; see Fig. 1). Both pairs of genes result in dicistronic messengers [100]. Monocistronic transcripts of the *ATPase8* and *ND4L* genes are possibly too short to interact effectively with the small subunit.

After binding of the small ribosomal subunit to the messenger, the subunit is assumed to move to the 5′-end of the mRNA mediated by yet unspecified auxiliary initiation factors [116,118]. The only initiation factor identified in mammalian mitochondria to date is mtIF-2 [118,119]. The cDNAs for bovine as well as human mtIF-2 have been cloned and sequenced [120,121]. The human factor shows 36% amino acid identity with *E. coli* IF-2. This monomeric protein factor belongs to the family of GTPases and promotes fMet-tRNA binding to the small ribosomal subunit in the presence of GTP and a template, reminiscent of the bacterial factor IF-2. Detailed in vitro characterisation of bovine mtIF-2 [119,122] has indicated that mtIF-2 may bind to the small ribosomal subunit prior to its interaction with GTP, however, GTP enhances the affinity between mtIF-2 and the small subunit and allows fMet-tRNA to join the complex. Hydrolysis of GTP is thought to facilitate the release of mtIF-2 and the concomitant association of the large (39S) ribosomal subunit to form the 55S initiation complex. Nonhydrolysable analogues of GTP can still promote formation of the initiation complex, indicating that GTP hydrolysis is not strictly required for subunit joining [119].

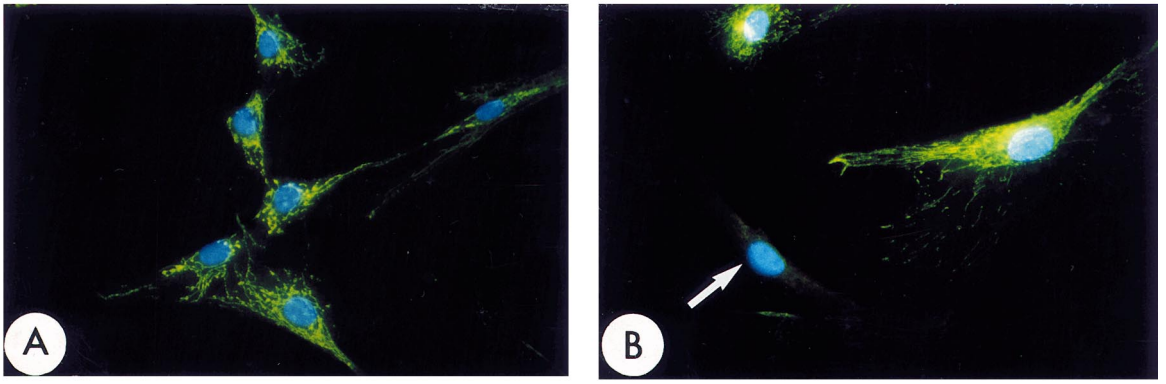

Fig. 3. Comparison of the expression of the mtDNA-encoded subunit I of cytochrome *c* oxidase in a control fibroblast culture (A) with that in a fibroblast culture from a patient with MELAS (B). More than 95% of the patient's mtDNA molecules harboured the $tRNA^{Leu(UUR)}$ A3243G mutation. The presence of subunit I was visualised with fluorescein isothiocyanate (FITC)-labelled antibodies (green fluorescence), while the cell nuclei were counter-stained with 4,6-diamidino-2-phenylindole (DAPI; blue fluorescence) [133]. A patient's cell not expressing subunit I is indicated with an arrow.

Three mitochondrial elongation factors, mtEF-Tu, mtEF-Ts and mtEF-G, have been purified from bovine liver [123,124]. The cDNAs for all three factors have been cloned and sequenced from mammalian sources [125–129] and the gene for human mtEF-Tu has been mapped to chromosome 16q11.2 [129]. The in vitro characterisation of the purified factors and the cDNA sequence information have revealed striking similarities with the corresponding prokaryotic factors. Consequently, elongation of the nascent mitochondrial polypeptide is assumed to proceed in a similar fashion as in *E. coli* [130]. Different from *E. coli* EF-Tu and EF-Ts, mammalian mtEF-Tu and mtEF-Ts form a tightly associated complex that, unlike the bacterial complex, cannot readily be dissociated by guanidine nucleotides alone [123]. However, it was recently demonstrated that the mtEF-Tu-Ts complex will dissociate in the presence of GTP and charged tRNAs [131].

4.3. *The effect of tRNA point mutations on mtDNA expression*

Pathogenic mutations in mitochondrial genes are generally heteroplasmic, i.e. wild-type and mutant mtDNA co-exist in the same cell. The wild-type mtDNA allows the mutant mtDNA with the otherwise lethal base change to persist. Maternally inherited, mitochondrial encephalomyopathies are often associated with point mutations in mitochondrial tRNA genes [132]. Muscle biopsies from patients show focal histochemical defects of cytochrome *c* oxidase associated with a non-random distribution of mutant and wild-type mtDNA [49,91]. This mosaic expression of the disease is also observed in primary cell cultures of these patients as illustrated in Fig. 3.

Recent progress on how mitochondrial tRNA mutations exert their effect has largely come from experiments in which patients' mtDNA has been transferred to ρ^0 control cells. These studies unequivocally demonstrated that tRNA point mutations, like the A3243G transition found in the $tRNA^{Leu(UUR)}$ gene of MELAS patients and the A8344G transition in the $tRNA^{Lys}$ gene commonly associated with MERRF (myoclonic epilepsy with ragged-red fibres) [134], are alone sufficient to impair intramitochondrial protein synthesis [89,90,135]. These and other cell culture studies [136] have revealed a steep threshold effect: mitochondrial translation remains unaffected by tRNA point mutations until $>85\%$ of the mtDNA molecules are mutated.

Different mechanisms can account for the observed impairment of protein synthesis. Apart from a potential effect on transcription termination, the $tRNA^{Leu(UUR)}$ A3243G transition appears to affect the processing of primary mitochondrial transcripts [90,92]. Cybrids resulting from re-population experiments of a human ρ^0 cell line with mtDNA carrying the A3243G transition showed a small (less than 2-fold) but consistent increase in the steady-state levels of a partially processed RNA species derived from

transcription of the 16S rRNA+tRNA$^{Leu(UUR)}$+ND1 genes [90,137], which are contiguous in the mtDNA (Fig. 1). Steady-state levels of mature 16S rRNA, tRNA$^{Leu(UUR)}$ and ND1 mRNA are not affected by the mutation [90,137]. Although the steady-state level of the immature transcript is extremely low in the cybrids compared to the level of mature 16S rRNA, the level showed a strong inverse correlation with the rates of oxygen consumption of the cybrids (an indicator of mitochondrial oxidative phosphorylation capacity [138,139]). These observations have led to the hypothesis [90,138] that the unprocessed transcript, which contains 16S rRNA, is incorporated into ribosomes rendering them functionally deficient. If this results in stalling of the translation of polyribosomal mRNAs, then a small increase of unprocessed transcript could interfere disproportionally with mitochondrial translation and explain the severe oxidative phosphorylation defects observed in the patients.

Alternatively, point mutations in tRNA genes may induce a conformational change of the tRNA resulting in a decreased stability of the molecule. A change in structure may also affect the identification by the cognate aminoacyl-tRNA synthase and lead to decreased levels of aminoacylation or even mischarging of mutated tRNAs. In prokaryotes, the TΨC region of the tRNA molecule is important for recognition and binding by EF-Tu and ribosomes, therefore, mutations in the TΨC region of mitochondrial tRNAs are likely to affect their affinity with mtEF-Tu and mitoribosomes. High resolution Northern (RNA) blot hybridisation experiments have indicated that these mechanisms are indeed likely to play a role in the aetiology of these diseases. In cell lines carrying either tRNA^{Lys} A8344G [140,141], tRNA$^{Leu(UUR)}$ C3256T [142], or tRNA^{Asn} G5703A mutations [143], markedly reduced steady-state levels of the affected tRNA have been found, suggesting an increased susceptibility to nucleolytic digestion. Moreover, a ~40% reduction in aminoacylation of tRNA^{Lys} has been demonstrated in cell lines with the tRNA^{Lys} A8344G mutation [141]. In contrast, cell lines with the tRNA^{Lys} C8356T mutation exhibited no significant decrease in tRNA^{Lys} content [140] but this mutation, which is located in the TΨC arm of the molecule, may interfere with the binding of mtEF-Tu or the mitoribosome [144].

5. Replication of mtDNA

5.1. Basic mechanism of mammalian mtDNA replication

The fortuitously slow rate of mtDNA replication has facilitated the isolation and characterisation of in vivo replicative intermediates and has provided the now generally accepted model of the replication cycle of mammalian mtDNA. Early studies, which predominantly relied on electron microscopic and centrifugal analysis of mtDNA molecules from cultured cells, indicated that mammalian mtDNA molecules replicated unidirectionally from two spatially and temporally distinct, strand-specific origins [23]. The origin of H-strand replication (O_H) is located downstream of the LSP in the D-loop region of the genome, whereas the origin of L-strand replication (O_L) is at two-thirds of the genomic distance away from O_H with respect to the polarity of H-strand synthesis (Fig. 1). A round of replication begins at O_H with the synthesis of a daughter H-strand and continues along the parental L-strand to produce a full H-strand circle. Only after the replication fork has passed the second replication origin, O_L, is synthesis of the L-strand initiated which proceeds in a direction opposite to that of H-strand replication (reviewed in [145]).

5.2. Initiation of H-strand synthesis

Fine mapping of RNA and DNA species in the D-loop region of human and mouse mtDNA have suggested that short mitochondrial transcripts, originating at IT_L, serve as primers for the initiation of synthesis of nascent H-strands (Fig. 2; [146,147]). Thus, replication of mammalian mtDNA appears to be intimately linked with mitochondrial transcription. There are no known differences between the initiation of L-strand transcription and the initiation of RNA primer formation for mtDNA replication [36] and it is not clear which mechanism decides between transcript elongation or H-strand synthesis. Transitions from RNA to DNA synthesis take place at several distinct sites that collectively constitute O_H in a region of three short, evolutionary conserved sequence blocks, named CSB I, II and III (Fig. 2; [34]).

As the precursor RNA primer extends beyond the transition sites of RNA to DNA synthesis, the primary transcript is believed to be enzymatically processed to yield the mature primer RNA 3′-termini. Because of their location, it has been speculated that CSB I, II and III direct the precise cleavage of primary transcripts to provide the appropriate primer species [36]. Recent in vitro transcription studies of the O_H region with mitochondrial RNA polymerase fractions [148] indicated that the precursor RNA primer exists as a stable and persistent RNA-DNA hybrid also known as an R-loop. Hybrid formation requires the CSB II element and is also affected by mutations in CSB III.

The search by Clayton's group for catalytic activity capable of processing L-strand transcripts containing O_H sequences led to identification of the enzyme called mitochondrial RNA processing endonuclease (RNase MRP; reviewed in [149]). In their initial studies, in which single-stranded O_H-containing RNA species were used as substrate, the in vitro RNase MRP cleavage sites did not match with all in vivo 5′-ends of the nascent human and mouse H-strands (cf. [146,147,150,151]). However, recently Lee and Clayton [152] demonstrated that mouse RNase MRP does cleave the precursor RNA in the context of a triple-stranded R-loop configuration in vitro at the majority of the 3′ priming sites found in vivo.

RNase MRP is a ribonucleoprotein [149]. The bulk of the enzyme is present in the nucleolus where it plays a direct role in processing of precursor 5.8S rRNA [153–155]. The predominant nucleolar location of the enzyme has led to controversy as to its mitochondrial function [156,157]. However, recent ultrastructural in situ hybridisation experiments indicated a preferential localisation of RNase MRP RNA to nucleoli as well as mitochondria in comparison to the nucleoplasm and cytosol [158], consistent with a dual role of RNase MRP in maturation of nuclear rRNAs and mitochondrial RNA primers. Null mutant analysis of the RNase MRP RNA gene in *S. cerevisiae* indicated that the gene is essential for cellular viability [159], agreeing with the nuclear function of the factor. Genetic evidence linking RNase MRP to mitochondrial biogenesis was recently provided by a strain of *Schizosaccharomyces pombe* with a functional dominant mutation in its RNase MRP RNA gene. The strain was shown to require the mitochondrially associated, nuclear mutation *ptp-1* for viability [160].

Another nuclease implicated in processing of precursor RNA primers for H-strand replication is endonuclease G [161]. The enzyme was first isolated from bovine heart mitochondria as a homodimer of a ~29 kDa polypeptide [162] and cDNA sequences specifying endonuclease G of several mammalian species, including human, were recently reported [163]. The mitochondrial location of endonuclease G is undisputed but the enzyme is also found in the nucleus [161,164]. Endonuclease G has a rather wide spectrum of nucleolytic activities: it cleaves GC-rich double-stranded and single-stranded DNA tracts, RNA and an RNA-DNA heteroduplex containing the mouse O_H [161]. The in vitro RNA cleavage sites of the heteroduplex, however, do not align with all predicted in vivo priming sites. Deletion of the homologous gene in *S. cerevisiae* does not seem to affect mtDNA metabolism [165]. Thus, any conclusion as to the function of endonuclease G in mitochondrial biogenesis appears premature.

In vertebrates, most H-strand synthesis events stall shortly after initiation. Arrested nascent H-strands remain annealed to their template L-strand and create the triplex D-loop structure [145]. The 3′-ends of prematurely terminated H-strands map ~50 nucleotides downstream of a short (15 bp) conserved sequence element, called the termination-associated sequence (TAS [149]). The number of TAS elements and H-strand termination sites vary per species. The human mitochondrial genome contains only a single TAS element and there is only one major H-strand termination site, with two minor termination sites mapping immediately adjacent to the major site [166]. The mechanism which determines whether a nascent H-strand ends downstream of the TAS element or elongates over the entire length of the genome is not known but is likely to be a key regulator of the mtDNA copy number in a cell. Recently, in vivo and in organello footprinting studies indicated multiple protein binding sites in the TAS region of human and rat mtDNA [167]. Furthermore, a 48-kDa protein has been isolated from bovine mitochondria with a TAS-specific DNA binding activity [168]. These data suggest that nuclear-encoded *trans*-acting proteins interact with the *cis*-acting TAS ele-

ments and regulate the equilibrium between D-loop formation and H-strand replication.

5.3. Initiation of L-strand synthesis

The replication origin O_L was first identified in mouse and later in human mtDNA by 5′-end mapping of in vivo nascent L-strands [169,170]. The origin is located in a non-coding region of ~30 nucleotides and is flanked by five tRNA genes (Fig. 1). O_L is only activated when the parental H-strand is displaced as a single strand by the growing daughter H-strand. After strand displacement, O_L is thought to adopt a distinctive stem-loop structure [36]. In vitro run-off replication studies of O_L have suggested that this configuration serves as the recognition structure for a mitochondrial DNA primase which provides a short RNA primer for L-strand synthesis [171,172]. However, the location of O_L within a cluster of tRNA genes and the fact that the potential stem-loop structure might be absent in mtDNA of some vertebrate species [149] suggest that additional secondary structures contribute to DNA primase recognition in vivo.

RNA priming starts at the T-rich portion of the predicted O_L-loop and the transition from RNA to DNA synthesis takes place at a specific site near a critical GC-rich element at the base of the hairpin [171,172]. The mitochondrial DNA primase involved in priming of the L-strand synthesis has only been partly purified [173]. The physical properties of the mitochondrial enzyme are distinct from the major nuclear DNA primase. Mitochondrial DNA primase is associated with RNA. Treatment with ribonuclease A leads to rapid inactivation of primase activity and suggests a crucial role for the associated RNA. The predominant RNA moiety co-fractioning with primase activity is the nuclear gene product 5.8S rRNA [173]. However, as only crude fractions of mitochondrial DNA primase have been analysed, the co-fractionation of 5.8S rRNA might have been the result of an adventitious contamination.

5.4. Trans-acting factors involved in elongation and maturation of progeny strands

DNA polymerase γ is the only DNA polymerase present in mitochondria [174]. Disruption of the DNA polymerase γ gene in *S. cerevisiae* has demonstrated that the enzyme is necessary for mtDNA synthesis but has no basic function outside the organelle [175]. DNA polymerase γ accounts for only a small fraction of the total cellular DNA polymerase activity, yet its unique enzymatic properties, its resistance to aphidicolin and its sensitivity to dideoxynucleoside triphosphates make it relatively easy to identify the mitochondrial DNA polymerase [176]. The enzyme has been isolated from humans [177] and several other organisms (reviewed in [149]). DNA polymerase γ appears to be prone to proteolytic degradation and its subunit structure is still a matter of debate. Most recent data suggest that the enzyme is a heterodimer in vertebrates comprised of a 125–140-kDa polypeptide and a 35–54-kDa polypeptide (see, e.g. [178]). In addition to its $5' \rightarrow 3'$ polymerase activity, DNA polymerase γ has an intrinsic $3' \rightarrow 5'$ exonuclease activity which is highly mispair-specific and ensures faithful replication of mtDNA [176]. In recent years, cDNA sequences specifying the major subunit have been identified from a number of vertebrate species, including human [179–181]. Sequence comparisons with the polypeptide from *S. cerevisiae* have shown that the major subunit is evolutionarily conserved and have allowed identification of both the polymerase and the exonuclease proof-reading domain. Recent kinetic studies with the recombinantly expressed, 140-kDa human subunit showed that the major subunit is a relatively poor polymerase compared to other DNA polymerases, suggesting that this core subunit may require accessory factors to increase the enzymatic rate [182]. Nevertheless, the kinetic parameters indicated that the major subunit could replicate the mitochondrial genome in a physiologically relevant time frame [182].

Replicative intermediates of mtDNA feature extensive single-stranded DNA regions [145]. A mitochondrial single-stranded binding protein (mtSSB) has been isolated and cloned from human [183] and several other species (reviewed in [149]). Mutation analysis in *S. cerevisiae* has demonstrated that mtSSB is required for maintenance of mtDNA [184]. Binding of mtSSB to the exposed single-stranded mtDNA is thought to maintain the integrity of these regions and to stimulate the overall rate of DNA synthesis by DNA polymerase γ in vivo (see, e.g. [185,186]). The mitochondrial protein is distinct

from the nuclear single-stranded binding protein but resembles the *E. coli* single-stranded binding protein in structure as well as in DNA-binding properties [187,188]. The protein interacts with single-stranded DNA as a homotetramer [187–189]. The crystal structure of human mtSSB suggests that single-stranded DNA wraps around the tetrameric complex through electropositive channels guided by flexible loops [189].

Several additional enzymatic activities are considered to be essential for mtDNA replication but only a few supplementary enzymes of the mitochondrial replication machinery have been identified. Helicases catalyse the unwinding of duplex DNA by disrupting the hydrogen bonds that hold the two strands together to provide single-stranded templates for DNA polymerases [190]. An ATP-dependent helicase has been identified and partly purified from mitochondria of bovine brain [191] and sea urchin eggs [192]. The enzyme shows biochemical similarities with Rep helicase of *E. coli* and shares its $3' \rightarrow 5'$ polarity of unwinding with respect to the single-stranded portion of the partial duplex DNA. This direction would place the mitochondrial helicase on the template strand ahead of DNA polymerase γ during mtDNA replication.

Type I topoisomerases catalyse the relaxation of supercoiled DNA, while type II topoisomerases are responsible for the introduction of supercoils. Topoisomerases change the topology of DNA by transiently breaking the backbone bonds, using a mechanism involving either single strand (type I) or double strand (type II) cleavage [193]. Mitochondrially associated, type I topoisomerases have been identified from various sources, including human leukemia cells [194] and human platelets [195]. The mitochondrial enzyme is immunologically related to its nuclear counterpart [196,197] and yeast mitochondrially associated type I topoisomerase activity is abolished when the gene that encodes the nuclear type I topoisomerase is disrupted [198]. Despite the apparent immunological and genetic relationship between the nuclear and mitochondrial type I topoisomerases, the enzymes can be distinguished from each another by a differential response to chemical reagents and inhibitors, both in yeast and humans [195,197].

Mitochondrially associated type II topoisomerases have been identified in *Dictyostelium discoideum* [199], rat liver [200] and human leukemia cells [194]. Further support for the existence of a distinct mitochondrial type II topoisomerase comes from findings with cell cultures grown in the presence of ciprofloxacin. This antibiotic inhibits prokaryotic type II topoisomerases much more than the eukaryotic nuclear enzyme. Treatment of cultured cells with ciprofloxacin causes double-stranded breaks in mtDNA and results in loss of the mitochondrial genome [201]. These observations suggest the presence of a bacterial-like type II topoisomerase in mitochondria.

5.5. A role for mtTFA in mtDNA maintenance

In addition to its role in transcription and, consequently, primer formation for H-strand synthesis, mtTFA appears to have a function in maintenance of mtDNA. Mitochondria do not contain histones [202,203]. Yeast mtTFA [204] contains a tandem of HMG DNA-binding motifs, similar to mtTFA of vertebrates, but yeast mtTFA lacks the basic C-terminal region shown to be important for promoter selection in vertebrates [60]. The yeast homologue is not considered to play a key role in initiation of transcription [37] but is thought to have a histone-like function in coating the entire mitochondrial genome [66,202]. Likewise, binding of vertebrate mtTFA is not restricted to the regions upstream of the HSP and LSP. The factor is inherently flexible in its recognition of DNA sequences [52,65,71] and a comparative in organello and in vitro footprinting study indicated that human mtTFA binds at regularly spaced intervals throughout a 500-bp region encompassing O_H and the two major promoters [61]. Binding is largely excluded from CSB II and III but mtTFA shows a strong interaction with CSB I [61,65]. As some of the intervals of mtTFA binding align with prominent 5′-ends of nascent H-strands, it has been suggested that the phased binding of the protein functionally organises the D-loop and facilitates access of other *trans*-acting factors involved in the transition from RNA to DNA synthesis [37,61,65].

There exists a direct correlation between mtTFA and mtDNA steady-state levels. Heterozygous mtTFA knockout mice exhibit reduced mtDNA copy numbers and homozygous knockout embryos

lack mtDNA and die [205]. This demonstrates the importance of mtTFA in maintaining mtDNA levels in vivo. Conversely, in ρ^0 cell cultures mtTFA levels are low [206–208], while in patients with mitochondrial myopathies increased levels of mtTFA are found in ragged-red muscle fibres with accumulated levels of mtDNA [206]. Although mtTFA protein levels are low in ρ^0 cells, mtTFA mRNA levels are normal [206,207], indicating that mtTFA protein levels are post-transcriptionally regulated by mtDNA levels. Possibly, binding to mtDNA protects mtTFA from proteolytic degradation. Similarly, mtSSB protein levels appear to correlate with mtDNA content but the expression of mtSSB is at least in part regulated at the level of transcription [209]. On the other hand, DNA polymerase γ is constitutively expressed [209]; even in ρ^0 cells, the steady-state level of the polymerase protein is unaffected [208].

Interestingly, the expression of mtTFA is reduced during mammalian spermatogenesis, most likely due to the production of alternate, testis-specific mtTFA transcript isoforms [210,211]. The low mtDNA copy number observed in sperm cells may, therefore, be a direct result of a decreased expression of mtTFA which, together with other mechanisms, may prevent paternal transmission of mtDNA [211].

6. Prospects

Transcription, translation and replication of mtDNA are controlled by many factors. *Cis*-acting mtDNA sequences have been identified in mapping studies and by mutation analysis. Characterisation of *trans*-acting factors proved more difficult because of their low cellular concentration and the potential risk of contamination with analogous enzyme activities of nuclear or cytosolic origin in cell fractionation experiments. Still, some relatively abundant mitochondrial enzymes could be purified to homogeneity and their human coding sequences have been cloned utilising conventional molecular biological techniques. More recently, problems with purification have been circumvented by screening of human EST databases with sequences of yeast genes involved in transcription and replication [51]. As a result, several key enzymes involved in transcription and replication of human mtDNA have now been cloned and mapped to chromosomes (Table 1). Nevertheless, complementary strategies are needed to characterise the full repertoire of enzymes involved in mtDNA expression as evolutionary divergence between factors may prevent successful cyberscreening and certain factors could be unique to the mammalian mitochondrial system.

Patients with inborn errors of mitochondrial replication, transcription or protein synthesis present a challenge as well as a resource to the biochemist. The challenge is to understand the molecular mechanism of the disease, whereas the resource is as a naturally occurring mutant which provides an opportunity to characterise aspects of these processes in humans that are difficult to study by other means. Patients with mtDNA mutations are providing unparalleled insights into the roles of *cis*-acting elements. With respect to *trans*-acting factors involved in maintenance and replication of mtDNA, one group of patients has attracted special attention. In 1991, Moraes and colleagues [216] described several infants with marked depletion of mtDNA and defective oxidative phosphorylation. Since its discovery, more than 30 patients have been described with this syndrome (discussed in [217]). Most patients present soon after birth with muscle weakness and hepatic failure or renal tubulopathy associated with a severe depletion of mtDNA (88–99%) in affected tissues at

Table 1
Chromosomal location of putative human *trans*-acting factors involved in mitochondrial transcription and replication

Factor	Gene	Chromosome
Endonuclease G	*ENDOG*	9q34.1 [57]
DNA polymerase γ	*POLG*	15q24-q26 [180,212,213]
Mitochondrial processing endoribonuclease (RNase MRP) RNA	*RMRP*	9p21-p12 [214,215]
Mitochondrial RNA polymerase	*POLMRT*	19p13.3 [51]
Mitochondrial single-stranded binding protein (mtSSB)	*SSBP*	7q34 [57,183]
Mitochondrial transcription factor A (mtTFA)	*TFAM* (*TCF6*)	10q21 [54,57]

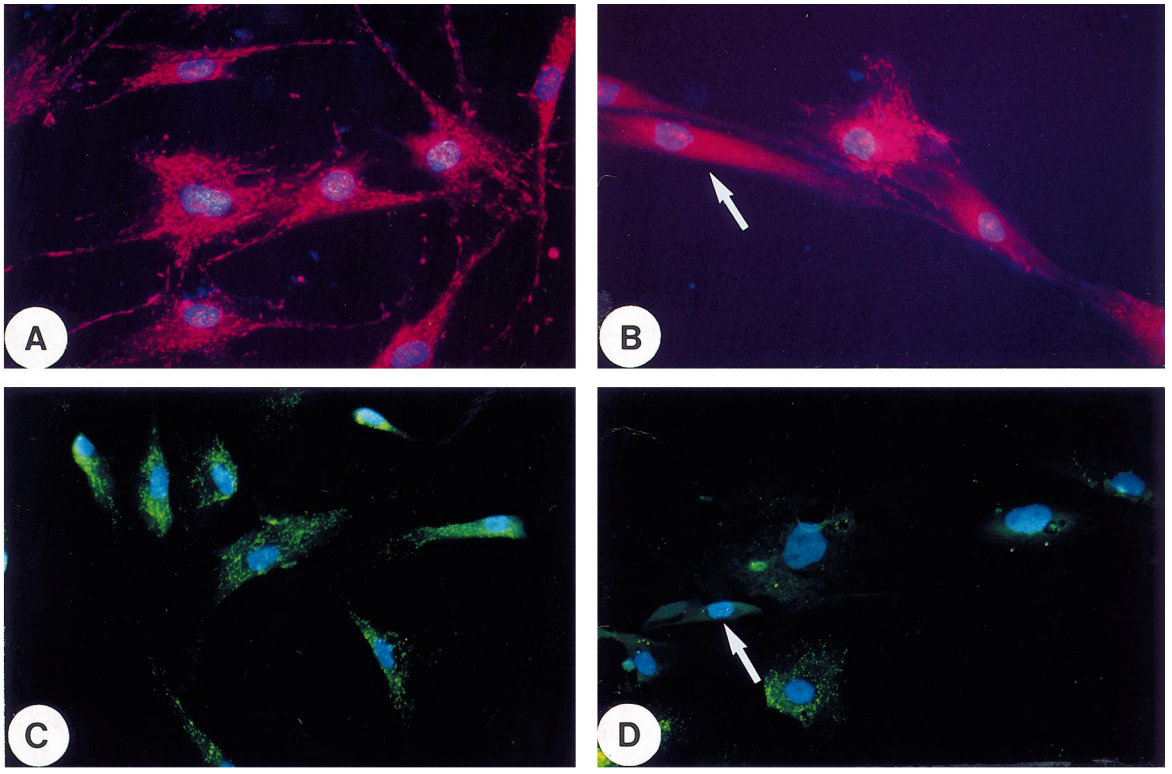

Fig. 4. Comparison of total mtDNA (A, B) and recently replicated mtDNA (C, D) levels in a control myoblasts culture (A, C) with that in a myoblast culture from a patient exhibiting mtDNA depletion (B, D). In (A) and (B), SYTO 17 (Molecular Probes) was used to visualise mtDNA; this cell-permeant, red fluorescent dye reveals the characteristic mitochondrial structures in the cell if mtDNA is present. In (C) and (D) replicating mtDNA was visualised by the incorporation of 5-bromo-2′-deoxyuridine (BrdU) and subsequent detection with an FITC-labelled antibody (green fluorescence). Nuclei were stained fluorescent blue with DAPI. Cells were either cultured for 1.5 h in medium containing 2 μg/ml of DAPI, followed by culturing for 30 min in 62.5 nmol of SYTO 17, 2 μg/ml of DAPI, 10 mM of HEPES·NaOH (pH 7.4), 150 mM of NaCl, 2 mM of $CaCl_2$ and 2 mM of $MgCl_2$ (A, B), or cells were cultured for 16 h in medium containing 15 μM of BrdU, 10 μg/ml of aphidicolin followed by immunological detection of BrdU (Detection kit I, Boehringer Mannheim) and counter staining with DAPI (C, D). Arrows indicate a patient's cell lacking mtDNA or a patient's cell devoid of mtDNA replication.

post-mortem. No mtDNA mutations have been documented in these infants and pedigree analysis is consistent with autosomal inheritance of the trait. A nuclear genomic involvement has been confirmed in two families by mtDNA transfer techniques [218,133]. The progressive loss of mtDNA in tissues is also observed in cell cultures of some of the patients [218,133]. In the depleting cultures, a fraction of the cells still contains apparently normal levels of mtDNA but mtDNA replication is more or less diminished in all cells (Fig. 4). Taken together, these observations strongly suggest a replication arrest due to a deficiency of a *trans*-acting, nuclear-encoded factor.

Patients with mtDNA depletion display decreased levels of mtTFA in tissue [206,207]. However, the fact that remnant mtDNA is still transcribed in cell cultures of the patients [133,219] suggests that the decrease in mtTFA levels is probably secondary to depletion of mtDNA. Other candidate genes, listed in Table 1, are currently being evaluated but, to date, no molecular defects underlying the mtDNA depletion have been reported. A deficiency of one of the key enzymes of mtDNA replication is likely to be lethal early in embryonic development [205]. The apparent tissue-specific and neonatal expression of symptoms indicates a less drastic defect and points to the deficiency of a factor involved in regulation of mtDNA copy numbers. Regulatory factors are generally present at very low cellular concentrations and only at certain stages of development, making it exceedingly difficult to characterise these factors by tra-

ditional means. It may, however, be feasible to identify the deficient factor in complementation studies of mtDNA depleting cell cultures with a human cDNA expression library. This approach is facilitated by the fact that the patient's cells, because of impairment of the oxidative phosphorylation complexes, become auxotrophic for uridine and pyruvate [218,133], which provides selectable markers to identify complementing cDNAs.

One can anticipate that future progress in the field of replication, transcription and translation of human mtDNA will increasingly come from detailed case studies as exemplified above for mtDNA depletion. This research will not only enrich science but will also permit the development of diagnostic tools as well as new pathogenic insights to minimise the devastating consequences of a defective expression of the mitochondrial genome.

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