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Does the mitochondrial transcription-termination complex play an essential role in controlling differential transcription of mitochondrial DNA?

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Abstract

The mechanism of mitochondrial transcription is well documented although the method of regulation remains obscure. The mitochondrial transcription-termination complex, mTERF, holds a key position in determining the fate of heavy-strand promotor-initiated transcripts and has been suggested as a candidate in the regulation of mitochondrial DNA (mtDNA) transcription. We report here the first example of a modulation of mTERF-complex binding activity concomitant with a differential mtDNA transcription rate. We suggest that these observations are indicative of a method of intra-organellar transcriptional fine tuning.

Introduction

Many protozoa, a few fungi and mature mammalian red blood cells do not harbour mitochondria. This organelle is ubiquitous in all other eukaryotic cells and functions to transform the energy released during substrate oxidation into ATP. Mitochondria contain a compact, extranuclear genome, mitochondrial DNA (mtDNA; Figure 1). In humans, this is an approximately 16.5-kb, circular, closed covalent genome that is transcribed from both stands. It encodes 13 essential polypeptides represented in four of the five respiratory chain complexes, and all of the mitochondrion-specific rRNAs and tRNAs necessary for intra-mitochondrial protein synthesis. The remaining 90 or so proteins constituting the respiratory chain and all proteins involved in mitochondrial function and homoeostasis (greater than 1000), including those required for mtDNA replication, transcription and translation are encoded by the nucleus, translated in the cytosol and imported into the mitochondrion [1].

As the respiratory chain is composed of subunits encoded by two distinct genomes and holds the central role in vertebrate energy production, its correct assembly, activity and response to local energy demands and other external stimuli are likely to require complex but tightly co-ordinated regulatory mechanisms. Supporting this view, cellular mitochondrial content, activity and mtDNA copy number are generally related to the energy demand of the tissue type [2]. Hence, myocytes and neuronal cells contain amongst the largest cellular mtDNA copy number and mitochondrial volume. mtDNA copy number has been shown to increase with increased cellular mitochondrial content and metabolic activity as induced by continual exercise [3], electrical stimulation of cardiomyocytes [4], adaptive thermogenesis of myotubes [5] or after thyroid hormone (TH) treatment of liver cells [6].

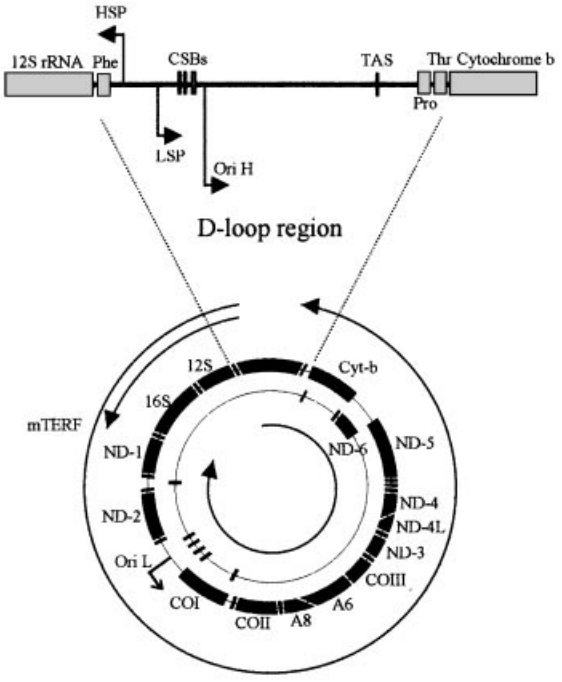
Key words: D-loop, mitochondrial stress, mtDNA, mtDNA transcription.
Abbreviations used: mtDNA, mitochondrial DNA; mtRNA, mitochondrial RNA; MRP-RNase, mtRNA processing RNase; mtTFA, mitochondrial transcription factor-A; TH, thyroid hormone; mTERF, mitochondrial transcription-termination complex; TAP, thiamphenicol; HSP, LSP, heavy- and light-strand promoters.

The molecular mechanisms underlying mitochondrial proliferation are beginning to be dissected and may share a common pathway for numerous stimuli. The general mechanism suggested is a temporal cascade: stimuli, induction of transcription factor(s), increased expression of mitochondrial and related genes, and mitochondrial proliferation. A candidate that appears central to co-ordinating the proliferative response is a nuclear transcription factor, NRF-1 [7,8].

Figure 1

Schematic diagram of mtDNA, the major polycistronic RNAs and the displacement (D-) loop region and its *cis*-acting elements

The human mitochondrial genome is 16569 bp. The bold outer circle is representative of the heavy (H-) strand and the bold inner circle the light (L-) strand. Ori H and Ori L denote origins of replication and arrows indicate direction. Transcript-initiation sites are denoted HSP and LSP and their direction of elongation indicated by arrows. The three main polycistronic RNA species are shown as the two outer and one inner thin black lines. The premature termination site of HSP-initiated transcripts is denoted by mTERF at the 3′-end of the 16 S rRNA gene. Peptide and rRNA genes are denoted by bold black lines and annotation and the tRNA genes are denoted by bold thick black lines but are not annotated. The D-loop region is shown in closer detail. The conserved sequence blocks (CSBs), are the sites of LSP-initiated transcript maturation for DNA replication. Small RNA species initiated from LSP are used to prime DNA synthesis. Nascent DNA species are often terminated downstream of the termination-associated sequence (TAS) site. This premature replication termination produces the 7 S DNA D-loop. Subunit abbreviations are: ND, NADH dehydrogenase; CO, cytochrome *c* oxidase; A, ATP synthetase; Cyt-b, apocytochrome b.



This is an immediate-early transcription factor that is required for maximal transcription of many genes. Potential binding sites have been reported in over 50 genes encoding respiratory-related proteins [8]. Two key genes that are bound by and require NRF-1 for their maximal transcription encode mitochondrial transcription factor-A (mtTFA) and the RNA component of mitochondrial RNA (mtRNA) processing RNase (MRP-RNase). mtTFA is the only known transcription factor required, in tandem with mtRNA polymerase, for mammalian mitochondrial transcription (and replication priming). The amount of mtTFA in mitochondria appears to directly reflect mtDNA levels [9] and transcription rate [10]. MRP-RNase is believed to be a key component involved in RNA primer formation for mtDNA replication [11]. Collectively these two gene products alone could regulate mitochondrial transcription and replication.

Control of mitochondrial transcription is likely to be complicated. Transcription is initiated from at least two strand-specific promoters, the heavy- and light-strand promoters (HSP and LSP, respectively). Three major polycistronic RNA species are produced, two from the HSP and one from the LSP (Figure 1). The majority of transcripts initiated from the HSP are terminated just 3′ to the end of the 16 S rRNA by a mitochondrial transcription-termination complex, mTERF, producing an excess of mitochondrial ribosomal RNAs over other RNA subtypes [12]. The majority of LSP-initiated transcripts are believed to be cleaved within the conserved sequence block (CSB) region by MRP-RNase to generate primers for mtDNA heavy-strand synthesis, resulting in RNA–DNA hybrid formation [13]. The majority of these hybrids are subsequently terminated 500–700 nucleotides downstream, generating a 7 S DNA species that remains bound to its template, forming a triplex, displacement (D-) loop structure. This is continually being turned over and may represent a replicative control mechanism [1]. The majority of mitochondrial transcripts initiated from both promoters are thus thought to be prematurely terminated.

Although the longer-term consequences of TH treatment on TH-sensitive tissue promotes an increase in mtDNA copy number and general mitochondrial proliferation, a distinct early effect of TH is to cause an increase in mtDNA transcription and mitochondrial respiratory activity without a concomitant increase in mtDNA load [14,15]. The exact mechanism of this early

TH-mediated increase in mitochondrial transcription is unknown, but it is postulated to follow other short-term signalling pathways. Reports of the relatively rapid TH-mediated effect on respiratory activity, of high-affinity TH-binding sites located in the mitochondrial outer and inner membranes and of a putative TH-receptor element on mtDNA support this hypothesis [6,15].

We recently showed that inhibiting mitochondrial translation with thiamphenicol (TAP) causes an increase in correctly processed mtRNA species from the two full-length polycistronic RNAs, without a concomitant increase in mt-rRNAs, a strikingly similar response to the initial TH-mediated increase in mitochondrial transcription [15,16]. Further, mtDNA copy number, NRF-1/mtTFA-binding activity and protein level were unaffected, consistent with TAP- treatment inducing an increase in mitochondrial transcription without causing mitochondrial proliferation (S. P. Selwood, Z. Chrzanowska-Lightowlers and R. N. Lightowlers, unpublished work). Here we report data, based on differential electrophoretic mobilities of DNA–protein complexes, that TAP treatment of HepG2 cells also causes a modulation in the HSP-initiated mTERF. From these results we suggest a novel fine-tuning mechanism for the regulation of mtDNA transcription.

Materials and methods

Culture and TAP treatment of HepG2 cells

Human HepG2 liver cells were grown in Eagle's minimal essential medium supplemented with 10 % fetal calf serum, 2 mM L-glutamine and non-essential amino acids. TAP medium was prepared to a final concentration of 50 μg/ml TAP by dissolving TAP in 100 % ethanol and the concentration was spectroscopically assessed at 273 nm ($\varepsilon_{mM} = 0.64$). An equal volume of 100 % ethanol was added to control cells. All medium was filter sterilized.

Cytosolic protein lysate extraction

HepG2 liver cells were harvested by stripping the cells in PBS/1 mM EDTA and centrifugation at 223 ***g*** for 4 min. Cell pellets were resuspended in 50 mM Tris, pH 7.5, 130 mM NaCl, 2 mM $MgCl_2$, 2 % (v/v) Nonidet P40, 1 mM PMSF, 50 μg/ml leupeptin, 5 μg/ml antipain and 1 mM *p*-a-benzamidine. This was vortexed for 30 s and the nuclear pellet removed by bench-top centrifugation at 600 ***g*** for 2 min. The supernatant was removed and diluted in an equal volume of 50 mM Tris, pH 7.5, supplemented with protease inhibitors. The samples were snap frozen in liquid nitrogen. Protein concentrations were determined using the Bradford method [18].

Mobility-shift assay

Binding reactions were performed as in [19] using cytosolic protein lysates and with the addition of 5 μg of BSA, 10 μg of sheared salmon sperm DNA and 1 μg of poly(dI-dC). Double-stranded oligonucleotides complimentary in sequence to the mTERF-binding site [19] were internally labelled with [α-^{32}P]dCTP following a standard PCR protocol. Cytosolic protein lysate (5–50 μg) was incubated with 30–50 fmol of wild-type or deletion-labelled probe, or with internal control probes of the HSP- and LSP-mtTFA-binding regions [1]. One strand of sequence of each double-stranded probe is shown below with the mTERF footprint region underlined [20]: wild-type mTERF, 5′-AGAACAGGGTTTGTTAAGATGGCAGAGCCCGGTAATCGCATAAAAAGAACA-3′; delete sequence mTERF, 5′-AGAACAGGGTTTGTTAAGATGGTAATCGCATAAAAAGAACA-3′; LSP-mtTFA, 5′-ACTTTTAACAGTCACCCCCCAACTAAC-3′; HSP-mtTFA, 5′-ACCGCTGCTAACCCCATACCCCGAACC-3′.

After incubation, 0.1 vol. of 80 % (v/v) glycerol/0.2 % (w/v) Bromophenol Blue/0.2 % (w/v) Xylene Cyanol was added and the samples immediately loaded on to a 5 % polyacrylamide gel (79:1, acrylamide/bisacrylamide). The running buffer was 0.5 × TBE (where 1 × TBE = 45 mM Tris-borate/1 mM EDTA) and the samples were separated at 5 V/cm.

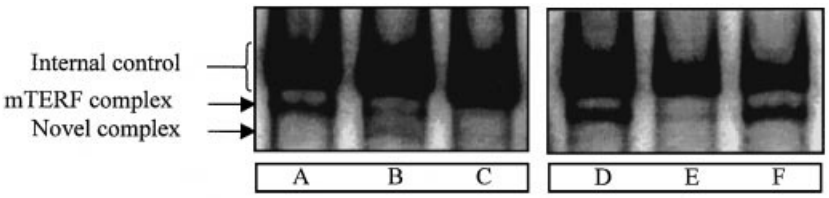
Results

We have previously reported that inhibition of mitochondrial translation in human HepG2 liver cells by treatment with TAP induces a series of responses, some of which are common to those seen in the early stages of TH treatment: a 3–5-fold increase in the steady-state transcript levels of both light- and heavy-strand mRNAs and tRNAs without a concomitant increase in the 12 S and 16 S rRNA species [15–17]. In TAP-treated cells (at least), there is no increase in the half-life of processed mtRNAs and so this represents a real transcriptional upregulation. One key protein that is immediately implicated in modulating differential rates of mtDNA transcription is mTERF. This transcription-termination factor binds across a tridecamer region of mtDNA,

Figure 2

A novel mTERF complex is formed in TAP-treated cytosolic protein lysate

Protein lysate (30 μg) was added to radiolabelled probe and resolved by gel electrophoresis as detailed in the Materials and methods section. Lanes A and C–F contain control lysate, with lane B containing lysate from TAP-treated cells. A second complex with faster mobility is noted in lysate extracted from TAP-treated cells (lanes A and B). Formation of the mTERF complex is specific to the nucleotide sequence as the delete probe is not bound (lane C). The labelled probe is specifically competed out by a 500 × excess of unlabelled mTERF probe (lane E) but not by a 3000 × excess of unlabelled HSP-mtTFA site. All reactions contained LSP-mtTFA radiolabelled probe as an internal control and all complexes were removed upon the addition of 1 μg of proteinase K to the binding reactions.



nucleotide positions 3237–3249, and is believed to be responsible for terminating the majority of HSP-initiated primary transcripts. As the steady-state levels of mt-rRNAs are unaffected in TAP-treated cells and all genes downstream of the mTERF-binding site show an increase in transcription, the most likely candidate for mediating this control in transcription is mTERF. Western analysis using antibodies raised to a major polypeptide of the complex however, revealed that TAP treatment had no effect on at least one subunit of mTERF (results not shown).

The minimum protein complex responsible for terminating transcription *in vitro* is now known to require at least two separate polypeptides [20] and whereas the steady-state level of an mTERF polypeptide subunit is unaffected by TAP treatment, this may not rule out altered termination activity for the complex. We therefore performed mobility-shift assays using a radiolabelled double-stranded DNA probe with a nucleotide sequence corresponding to the highly conserved mTERF-binding site. Figure 2 shows a PhosphorImage of a typical mobility-shift assay. A complex corresponding to mTERF is clearly resolved when using lysate extracted from HepG2 control cells. When lysate extracted from 11-day TAP-treated HepG2 cells is subjected to similar analysis, a decreased amount of mTERF complex is seen as well as a second complex of a higher mobility. The summation of both TAP complexes is similar to that calculated for the single control complex.

Discussion

From our previous results we know that TAP treatment drastically reduces mitochondrial respiratory activity, alters the ATP/ADP ratio and selectively increases the steady-state levels of mt-mRNA and mt-tRNAs transcribed from genes downstream of the termination site for the major heavy-strand primary transcript [17]. We have also clearly shown that the half-lives of these RNA species are unaffected in TAP-treated cells. What mechanism(s) could therefore be responsible for controlling the relative amounts of primary transcripts in cells treated with TAP?

There are two hypotheses: (i) an increase in selective transcription initiation; and (ii) a decrease in the premature termination of nascent transcripts allowing a greater number to continue the full length of the genome. On the basis of *in vivo* data, Attardi and colleagues have argued that a second HSP site exists, HSPII [21]. Transcripts from this promoter are believed to be refractive to premature termination by the mTERF complex and transcribe all genes downstream of the termination site. One possible explanation for the relative increase in heavy-strand mRNA transcribed from genes downstream of the termination site is that TAP induces an increase in transcription initiation from this second promoter. This hypothesis was recently strengthened by Enriquez et al. [16], who showed that in cells or isolated mitochondria treated with TH, mtDNA footprinting around the HSPII site was substantially altered. We have now addressed the second hypothesis by investigating the transcription-termination complex in TAP-treated cells. Our results demonstrate a modulation of normal mTERF complex in lysate extracted from TAP-treated HepG2 cells compared with control. A higher-mobility complex is also apparent in the

TAP lysate. The sum of the amounts of both complexes in the TAP lysate is comparable with the total amount of complex formed in the control.

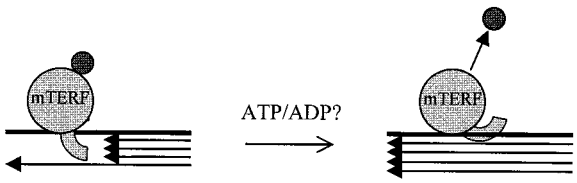
The position held by mTERF in mtDNA transcription is likely to be central to the fate of HSP-initiated transcription downstream of the rRNA genes and possibly as the final termination point for LSP-initiated transcription. As such it has often been highlighted as a potential transcription-control point. Characterization of this termination complex is still incomplete. It has, however, already been reported to consist of at least three polypeptide subunits *in vivo*, two of which confer the majority of the binding activity [20] and its maximal termination activity has additionally been shown to be dependent upon a dissociable factor [22]. It has further been shown to possess a high level of occupancy at its binding site [23]. The higher-mobility complex present in the TAP-treated lysate could be representative of the termination complex less its dissociable factor required for maximal transcript-termination activity. The slower-migrating complexes in each case represent the nucleic acid probe bound by the mTERF holoenzyme. As has already been discussed, steady-state levels of one mTERF polypeptide are unaffected by TAP treatment. However, termination activity may be regulated by a mitochondrial effector binding to the mTERF complex. Concentrations of this effector may fluctuate as a consequence of the energy poise of the mitochondria, explaining how transcription termination may be regulated by energy demand (Figure 3). Consistent with this general concept, previous reports have shown that, in isolated mitochondria, mRNA synthesis can be inhibited in the presence of high concentrations of ATP [24,25]. Additionally, altered mt-mRNA and mt-rRNA transcription patterns in response to ATP changes have been previously documented [25,26]. Inhibition of mitochondrial translation causes a selective increase in the steady-state mtDNA transcript levels, and shows a marked alteration in ATP/ADP ratios [17].

Therefore, our data suggest that mitochondrial gene expression can be controlled at the level of transcription termination and may be determined, at least in part, by modulation of the mTERF complex.

Figure 3

Schematic representation of a potential mtDNA transcription-termination control mechanism

In the normal state the majority of HSP-initiated transcripts are terminated by the termination complex mTERF. A physiological effector, e.g. ADP/ATP ratio, may cause a component of the termination complex to dissociate, causing a decrease in the termination activity, thereby increasing the level of nascent transcripts to proceed the full length of the genome.



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Muscle fibres: applications for the study of the metabolic consequences of enzyme deficiencies in skeletal muscle

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Abstract

Mitochondrial function in saponin-permeabilized muscle fibres can be studied by high-resolution respirometry, laser-excited fluorescence spectroscopy and fluorescence microscopy. We applied these techniques to study metabolic effects of changes in the pattern of mitochondrial enzymes in skeletal muscle of patients with chronic progressive external ophthalmoplegia or Kearns–Sayre syndrome harbouring large-scale deletions of mitchondrial DNA (mtDNA). In all patients combined deficiencies of respiratory chain enzymes containing mitochondrially encoded subunits were observed. The citrate synthase-normalized activity ratios of these enzymes decreased linearly with increasing mtDNA heteroplasmy. This indicates the absence of any well-defined mutation thresholds for mitochondrial enzyme activities in the entire skeletal muscle. We applied metabolic control analysis to perform a quantitative estimation of the metabolic influence of the observed enzyme deficiencies. For patients with degrees of mtDNA heteroplasmy below about 60 % we observed at almost normal maximal rates of respiration an increase in flux control coefficients of complexes I and IV. Permeabilized skeletal-muscle fibres of patients with higher degrees of mtDNA heteroplasmy and severe enzyme deficiencies exhibited additionally decreased maximal rates of respiration. This finding indicates the presence of a 'metabolic threshold' which can be assessed by functional studies of muscle fibres providing the link to the phenotypic expression of the mtDNA mutation in skeletal muscle.

Key words: genotype–phenotype relations, mitochondrial (mt) myopathy, mtDNA deletion.

Abbreviations used: mt DNA, mitochondrial DNA; CPEO, chronic progressive external ophthalmoplegia; KSS, Kearns–Sayre syndrome; COX, cytochrome *c* oxidase; CS, citrate synthase.

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Introduction

The metabolic consequences of large rearrangements of mitochondrial DNA in skeletal muscle of patients with mitochondrial myopathies remain unclear [1,2]. So, previous attempts to correlate biochemical findings in skeletal muscle of patients with chronic progressive external ophthalmoplegia (CPEO) or Kearns–Sayre syndrome (KSS) to the degree of the mitochondrial DNA (mtDNA) mutation failed to establish a clear-cut relationship between genotype and the biochemical phenotype [3–8]. These problems may be attributed to various reasons. First, the heteroplasmic occurrence of the mtDNA mutation; second, the unpredictable mosaic distribution of mtDNA mutations in the affected tissue; third, unknown individual effects of various mutations; and fourth, difficulties in the quantitative determination of respiratory chain-enzyme activities and the degree of heteroplasmy of the mtDNA in skeletal muscle. In contrast, in cell cultures containing well-defined amounts of heteroplasmic mtDNA for different mutations, so-called threshold values have been defined beyond which each mutation had an effect on the activity of mtDNA-encoded enzymes. For various tRNA point mutations this threshold has been determined to be above 85 % of mutated mtDNA [9]. In contrast, the 4977-bp 'common deletion' had a 50–55 % threshold [10,11].

In this report we studied the metabolic consequences of deficiencies of enzymes of the mitochondrial respiratory chain in skeletal muscle by applying different techniques: enzyme-activity measurements with improved methods and investigation of saponin-permeabilized muscle fibres