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Inhibition of mitochondrial protein synthesis promotes autonomous regulation of mtDNA expression and generation of a new mitochondrial RNA species

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Abstract Mammalian mitochondria are known to proliferate in response to several stimuli. Proliferation requires an increase in expression of genes encoding proteins involved in mitochondrial biogenesis, as well as in the replication and expression of mitochondrial DNA (mtDNA). In contrast, we report that inhibiting mitochondrial protein synthesis causes a modulation in mtDNA gene expression without the concomitant increase in proliferative markers. Further, inhibition results in the production of a previously unidentified light-strand mitochondrial RNA that spans the entire displacement loop, the function of which is currently unknown.

Key words: Mitochondrial proliferation; Autonomous mitochondrial DNA expression; Regulation of mitochondrial DNA expression; Mitochondrial translation inhibition

1. Introduction

Mitochondria are known to proliferate in response to several external stimuli and in certain mitochondrial disorders [1–4]. It is, therefore, highly likely that a mechanism exists for co-ordinating the expression of numerous nuclear genes with expression of the mitochondrial genome. As all the proteins involved in mitochondrial gene expression are encoded by the nucleus, one way to co-ordinate such a mechanism is for genes encoding mitochondrial proteins crucial for metabolic function and for regulating mitochondrial DNA (mtDNA) gene expression to share a common binding site for a transcription factor. Several candidates have been proposed, one of which is nuclear respiratory factor-1 (NRF-1, [5]). In response to electrical stimulation of isolated cardiomyocytes, conditions that are known to increase the mitochondrial content of the cell, NRF-1 has been shown to modulate expression of several nuclear genes encoding mitochondrial proteins [6]. Stimulation of NRF-1 has also been shown to increase expression of mtTFA, a mitochondrially destined protein required for mtDNA replication, consistent with NRF-1 playing a pivotal role in co-ordinating expression [7,8].

Although an increase in NRF-1 and mtTFA has clearly been linked with mitochondrial proliferation, there is some evidence that regulation of mtDNA expression can occur without proliferation and may be a mechanism for fine-tuning the amounts of respiratory chain components in response to chronic energy demands [9]. Most notably, a fluctuation in the levels of RNA products processed from the three major polycistronic RNA units has been reported in isolated mitochondria on addition of thyroid hormone or in response to varying concentrations of ATP [10,11]. Whilst the evidence for autonomous regulation of mtDNA transcription is compelling, the exact molecular mechanisms that are responsible remain to be elucidated.

The steady state level of mitochondrial messenger RNAs (mt-mRNAs) is known to be substantially increased when mitochondrial translation is terminated by protein synthesis inhibitors such as thiamphenicol (TAP) [12,13]. In our current study, we have addressed the question of whether this increase is orchestrated at least in part by the proliferation mechanism or whether the process is autonomous to the mitochondrion. We were unable to detect any change in the levels of candidate nuclear-encoded transcripts or proteins. However, a new species of polyadenylated transcript was shown to accumulate in mitochondria of liver cell lines after treatment with TAP. This accumulation was temporally distinct from the global increase in mtDNA transcription and may indicate a novel mechanism for regulating mtDNA copy number.

2. Materials and methods

2.1. Materials and cell lines

All radiochemicals were from Amersham UK. Chemicals including TAP, tissue culture medium, foetal calf serum (FCS) and supplements were from Sigma-Aldrich, UK, whilst chick embryo extract was from ICN-Flow. Tissue culture plastic was from Corning-Costar UK Ltd. Cells were cultured in Eagle's minimal essential medium, 10% FCS, 2 mM L-glutamine and non-essential amino acids, and where required, supplemented with 1 mM pyruvate and 50 μg ml^{-1} uridine. TAP was dissolved in ethanol, the concentration calculated spectrophotometrically ($\varepsilon_{mM} = 0.64$ at 273 nm) and administered at 50 μg ml^{-1} final concentration; control cells were supplemented with an equal volume of ethanol. Oligonucleotides (30 nmol) were synthesised in the molecular biology facility (University of Newcastle upon Tyne, UK).

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Abbreviations: TAP, thiamphenicol; SDS, sodium dodecyl sulphate; mtDNA, mitochondrial DNA; D-loop, displacement loop; I_{H1} and I_{H2}, mtDNA heavy-strand promoters; LSP, mtDNA light-strand promoter

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2.2. RNA and DNA

2.2.1. RNA extraction and Northern analysis. Total cytosolic RNA was isolated using Ultraspec II RNA (Biotex lab Inc., USA) following manufacturer's instructions. DNase treatment of RNA was performed with RNase-free DNase I (22 U, Boehringer Mannheim), followed by phenol extraction and precipitation. mRNA was extracted from total cytosolic RNA preparations following manufacturer's instructions (Qiagen Oligotex mRNA mini kit). RNA samples (10 μg) were subjected to Northern analysis as detailed in [13], blots were hybridised with DNA probes at 42°C or RNA probes at 55°C followed by stringent washes and PhosphorImage analysis (Molecular Dynamics). Blots were stripped as detailed in [13].

2.2.2. Fine mapping of RNA DL-L (RNA D-loop L-strand). To determine the 3′-terminus of this polyadenylated species, reverse transcription (RT) was performed on 5 μg of DNase-treated RNA from TAP-treated cells as recommended using Superscript II pre-amplification kit (Gibco BRL), with a T_{30}A/C/G-tagged (5′-CTACCAACTCGAGAGGATCT$_{30}$A/C/G-3′) oligodeoxynucleotide primer mix. Subsequent polymerase chain reaction (PCR) was performed with a primer identical to residues 467–486 of the light (L)-strand (5′-CCCATACTACTAATCTCATC-3′) in tandem with a primer specific to the T_{30}A/C/G tag (5′-CTACCAACTCGAGAG-3′). The resultant approximately 170 bp product was cloned into pCR-Script (Stratagene) and a number of clones subjected to automated DNA sequence analysis. To map the 5′-terminus, a primer extension method was employed, described in [14]. A primer mapping to heavy (H)-strand residues 16 215–16 196 (5′-TGCTGTACTTGCTTGTAAGC-3′) was end-labelled with [γ-^{32}P]ATP (3000 Ci mmol^{-1}) using T4 polynucleotide kinase and 100 ng was hybridised with 10 μg RNA overnight at 37°C in 80% (v:v) formamide, 40 mM PIPES pH 6.4, 1 mM EDTA and 0.4 M NaCl. This was precipitated twice and resuspended according to Superscript II pre-amplification kit instructions for RT. Superscript II RT enzyme (50 U) was added and RT allowed to proceed for 1 h at 42°C. The reaction was ethanol-precipitated and electrophoresed through a 6% polyacrylamide 7 M urea gel, together with a sequencing ladder derived from M13–40 oligomer-primed M13 ssDNA (Sequenase version 2.0). The gel was dried and analysed by phosphorImage.

2.2.3. Southern analysis. DNA was isolated from control and TAP-treated cells by overnight incubation with proteinase K (2 mg ml^{-1}) and sodium dodecyl sulphate (SDS; 1% (w:v)) at 37°C, followed by phenol extraction and precipitation. Aliquots were digested with *Pvu*II and electrophoresed through a 0.7% agarose gel in 1×TAE buffer (40 mM Tris–acetate, 1 mM Na_2EDTA, pH 8.0). Transfer to Genescreen Plus membrane (NEN DuPont) and probing were performed following manufacturer's instructions.

2.2.4. DNA and RNA probe production. DNA probes for Northern and Southern analyses were prepared by random primer extension (20–30 ng of template DNA) in the presence of [α-^{32}P]dCTP (3000 Ci mmol^{-1}) and unincorporated nucleotides removed (Pharmacia nick columns). Template DNA was generated by PCR for mitochondrial sequences or RT-PCR for the remainder. Primers used to generate mtDNA probes corresponded to: **probe 2d**, L-strand residues 467–486 in tandem with H-strand residues 680–665; **probe 3d**, L-15 950–15 964 with H-16 060–16 033; **probe 4d**, L-371–389 with H-530–548; **ND1 probe**, L-3384–3403 with H-4233–4250; **ND-6 probe**, L-14 201–14 217 with H-14 650–14 631; **12S mtDNA**, L-915–933 with H-1289–1272. Primers were designed for RT-PCR of transcripts encoding mtTFA, NRF-1 and mt-SSB from sequences in the GenBank database. PCR-generated fragments were cloned into pCR-Script and confirmed by DNA sequence analysis. The remaining probes were gifts.

Riboprobes were generated by in vitro transcription using an SP6/T7 Stratagene kit supplemented with 50 μCi [α-^{32}P]UTP (800 Ci mmol^{-1}) and template (1 μg linearised plasmid or 300 ng PCR product). Probes were purified following separation on 5% denaturing polyacrylamide gel, the radiolabelled RNA excised, eluted (0.5 M ammonium acetate, 1 mM EDTA, 0.2% SDS (w:v)) and then precipitated. To determine the strand specificity of RNA DL-L, two probes were in vitro transcribed from a PCR-derived template (PCR primers: 5′-CCCTATAGTGAGTCGTATTACTCCACCATTAGCACCCAAAG-3′ with 5′-GATTTAGGTGACACTATAGATTTCACGGAGGATGGTG-3′). This 482 bp PCR fragment spans the mtDNA displacement (D)-loop from residues 15 974–16 419 and incorporates phage T7 and SP6 recognition sites at opposite termini, allowing the synthesis of riboprobes that bind transcripts with H-strand or L-strand sequences, respectively.

2.3. Mobility shift assays

NRF-1 and AP-1 mobility shift assays were performed by the method of [5] on nuclear protein extracts prepared at 4°C as described in [15]. Reactions were separated on 5% non-denaturing polyacrylamide gel and PhosphorImage analysed.

2.4. Western analysis

Cell lysates were prepared by shearing cells at 4°C in 2% (v:v) Nonidet P-40, 50 mM Tris pH 7.5, 130 mM NaCl before adding an equal volume of 50 mM Tris–HCl pH 7.5. Both buffers contained the following protease inhibitors at final concentrations of 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 1 μg/ml antipain, 1 mM *p*-aminobenzamidine. Aliquots of cell lysate from control and TAP-treated cells were electrophoresed for 1200 V h through 15% (w:v) SDS–polyacrylamide gel and proteins transferred to Immobilon PVDF membrane (Millipore) in CAPS buffer (10 mM CAPS, 10% methanol, pH 11) for 3 h at 295 mA. Membranes were probed with antisera (mtTFA, p43, crotonase, COX lV, cpn10, cpn60, mTERF) and secondary antibodies conjugated to horseradish peroxidase. Signals were detected by enhanced chemiluminescence (Amersham, UK).

2.5. Estimation of cell doubling times

Hep G2 cells were seeded at 10% confluency in 16 mm wells. Cultures were fed every alternate day and split at 80% confluency. Each time point was duplicated and the experiment repeated such that each point was assayed in quadruplicate. Cell number measurements were made on a Z1 Coulter counter (100 μm aperture size, 1 ml metered volume).

3. Results

3.1. TAP treatment does not affect the cellular level of mtDNA

In Hep G2 cells treated with TAP for 11 days, the steady state levels of mt-mRNA transcripts increase without a concomitant increase in mitochondrial ribosomal RNAs (mt-rRNAs) [13]. There was no measurable effect on mt-mRNA stability, but without determining the cellular content of mtDNA, it could not be concluded unequivocally that TAP treatment resulted in an upregulation of transcription. Southern analysis (Fig. 1) shows that the relative levels of nuclear and mtDNA are similar in control and TAP-treated cells, demonstrating that the previously reported increase in steady state mt-mRNA levels was due to a selective increase in the rate of production of the two H-strand polycistronic RNA units.

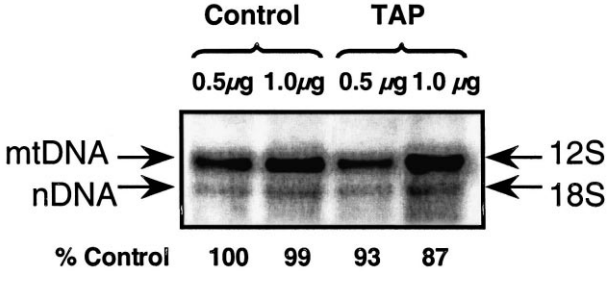

Fig. 1. Treatment with TAP does not affect total cellular mtDNA content. DNA was isolated from both untreated (Cont) and 11 day TAP-treated cells and the indicated amount was subjected to Southern analysis as described in Section 2. A representative result is shown. Relative levels of nuclear and mtDNA were determined following hybridisation with probes to the 18S rDNA and 12S rDNA, respectively. Signal intensities were calculated for both probes in the control (0.5 μg) sample and the ratio normalised to 100%. Relative intensities were calculated for each sample and compared to the control (shown under each lane).

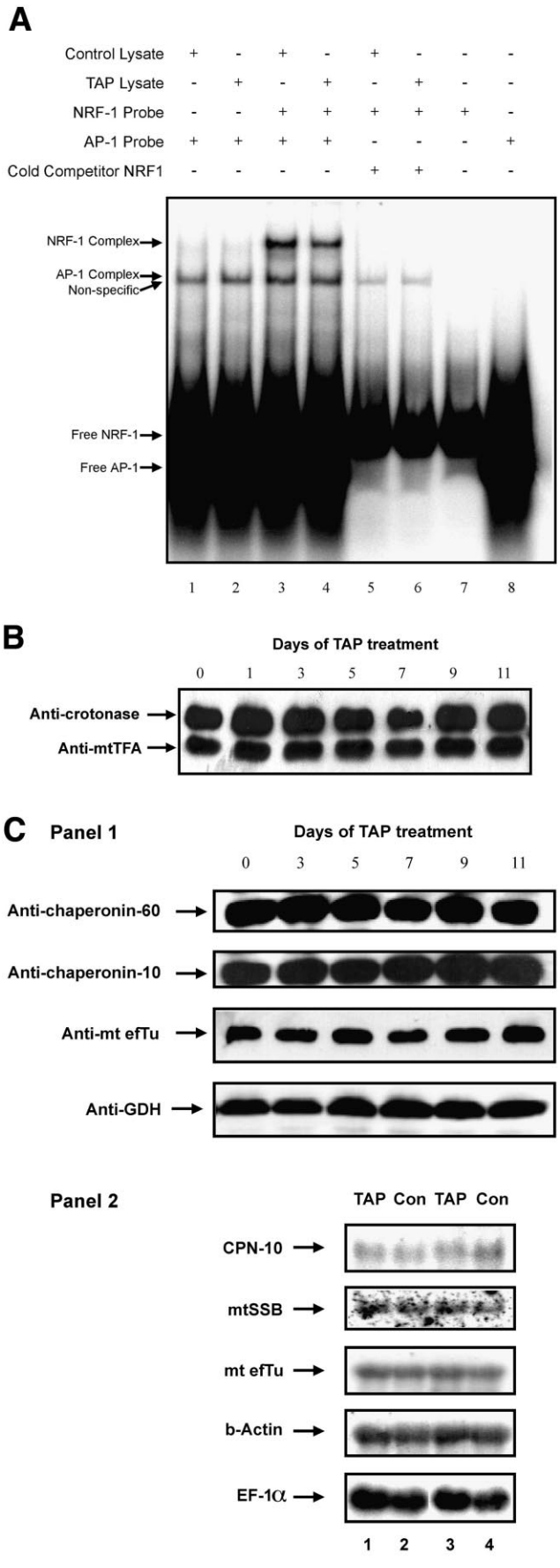

Fig. 2. TAP treatment does not affect the levels of products from various candidate nuclear genes. In all cases, examples shown are representative of at least three repeat time courses. A: NRF-1 DNA-binding activity is not affected by TAP. Nuclear lysate (40 μg) from control and 11 day TAP-treated Hep G2 cells was incubated with radiolabelled probes as detailed in Section 2. No appreciable difference in NRF-1-binding activity could be shown (cf. lanes 3 and 4). Probe specific for AP-1 binding was used as an internal control. A small amount of non-specific protein binding to the NRF-1 probe was found, with the resultant complex having a similar mobility to the AP-1 complex (lanes 5 and 6). B: TAP does not induce a modulation in the levels of mtTFA. Cytoplasmic lysate was prepared from both control and TAP-treated Hep G2 cells after the indicated number of days in TAP. Samples (75 μg) were subjected to Western analysis as detailed in Section 2. Comparison of the protein levels revealed by antibodies specific to mtTFA and to the internal control (the mitochondrial β-oxidation enzyme crotonase) showed no effect upon TAP treatment. C: Steady state levels of candidate nuclear gene products were unaltered by growth of cells in TAP. Panel 1: As with (B), cytoplasmic lysates were subjected to Western analysis with antibodies to chaperonin 60, chaperonin 10, mitochondrial efTu and glutamate dehydrogenase (as a control) with no evident effect with TAP treatment. Panel 2: RNA (10 μg) from both 11 day TAP-treated and control Hep G2 cells were subjected to Northern analysis. Neither TAP treatment (lanes 1 and 3) nor pyruvate and uridine addition (lanes 3 and 4) appeared to affect the steady state level of the transcripts studied. ←

3.2. *Levels of NRF-1 and mtTFA are unaffected in cells treated with TAP*

The relative levels of NRF-1 DNA-binding activity were assessed by gel-mobility shift analysis using nuclear extracts isolated from TAP-treated and control Hep G2 cells. No appreciable difference in the amounts of binding active-NRF-1 protein (Fig. 2A) or transcript (RPA assay, data not shown) was detected between control and TAP-treated cells. In agreement both with this and with the absence of any TAP-induced increase in the steady state levels of mtDNA shown in Fig. 1, Western analysis of whole cell lysates revealed that TAP treatment did not alter the levels of mtTFA protein (Fig. 2B). Further, there was no appreciable difference in transcript levels or the encoded polypeptides of other mitochondrial proteins that have been postulated to play a role in modulating mitochondrial gene expression (Fig. 2C).

3.3. *TAP treatment results in the production of a novel mitochondrial RNA species*

In light of previous suggestions that antisense molecules encoded within the D-loop region may regulate mammalian mtDNA replication and transcription [16,17], a D-loop-specific probe was made and used to assess transcription through this region of the genome. As illustrated in Fig. 3, no D-loop transcript was detectable in the untreated Hep G2 RNA. 11 day treatment with TAP, however, resulted in marked expression of an approximately 1200 nucleotide (nt) species and, to a lesser degree, an approximately 900 nt species. Further, both species were detectable in mt-mRNA fractions purified through oligo dT affinity columns, confirming that they were polyadenylated (data not shown). Strand-specific RNA probes showed that both species were transcribed from the H-strand (Fig. 3A). Whilst an approximately 800 nt D-loop species has been reported in human HeLa cells [16], to our knowledge the larger D-loop species reported here has never been documented. Crude mapping experiments were at-

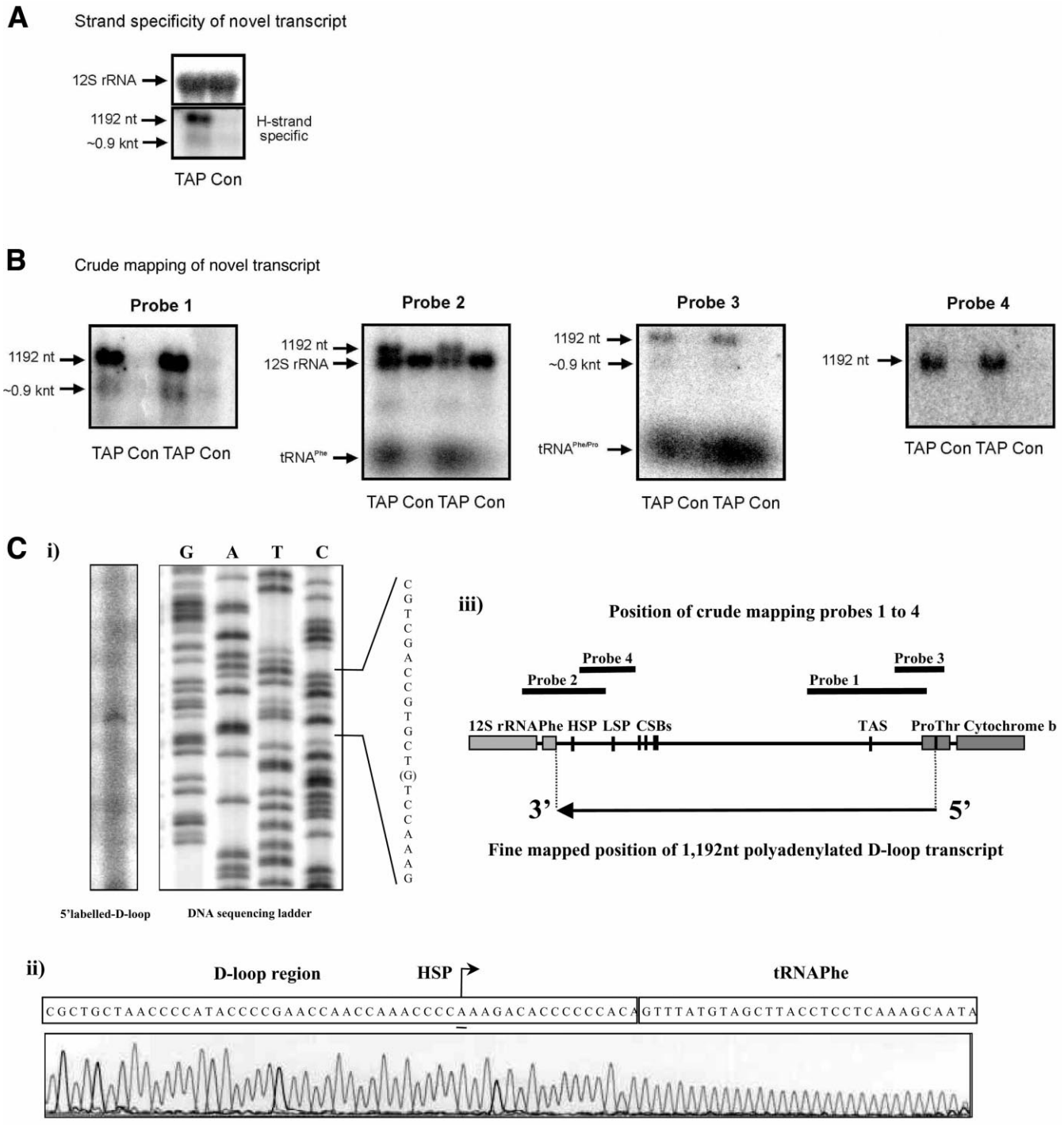

Fig. 3. A novel D-loop-encoded RNA species is induced by TAP treatment. Total cytoplasmic RNA (10 μg) isolated from TAP-treated and control cells was subjected to Northern analysis with various probes. A: Determination of strand specificity of D-loop transcripts. Riboprobes specific to region 1 of either D-loop strand were prepared and hybridised to the membrane as detailed in Section 2. Results show that two D-loop transcripts were present and both were H-strand in origin. A 12S rRNA probe and β-actin (not shown) were used as controls. B: Crude mapping of the D-loop transcripts. Crude mapping of transcript positioning was performed by using probes designed to specific regions of the D-loop (as indicated in C(iii)) and specified in Section 2. C: Fine mapping of the 1200 nt D-loop transcript. The 5′-terminus was determined by primer extension by standard methods. To determine the 3′-terminus of the polyadenylated D-loop species, RT-PCR was performed as detailed in Section 2 and the subsequent products were subjected to DNA sequence analysis. A schematic of the relative position of this 1192 nt RNA is shown. The initiating 5′-residue corresponds to the nucleotide between mt-tRNAs ^{Pro} and ^{Thr} and terminates beyond the HSP initiation site (the precise initiating base is underlined) at the final residue preceding mt-tRNA^{Phe}.

tempted with three other D-loop probes as shown in Fig. 3B, showing the 1200 nt species to span approximately the entire D-loop. Probes 2 and 4 did not detect the 900 nt species, consistent with it terminating within the conserved sequence box CSB1, reported to be the natural termination site for the large H-strand polycistronic transcript [16]. To accurately map the 5′- and 3′-termini of the major 1200 nt transcript, primer extension and RT-PCR were used. The 5′-proximal

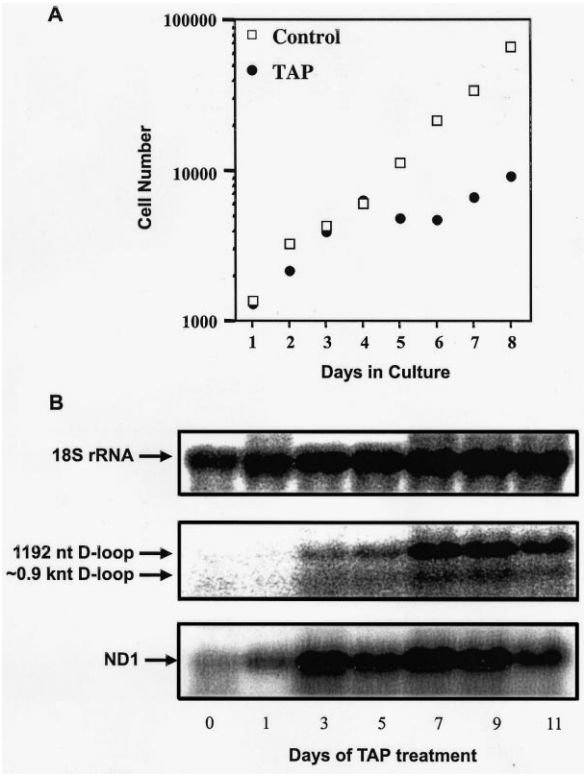

Fig. 4. The increase in steady state levels of RNA DL-L is temporally distinct from the TAP-induced global increase in mt-mRNA. A: Effect of TAP treatment on growth of Hep G2 cells. Identical numbers of cells were seeded into culture wells and incubated with control or TAP medium. Wells were seeded in duplicate and harvested at daily intervals, the contents were counted using a Coulter counter. Each point on the semi-log plot represents the mean of four measurements. Control and TAP-treated cells behave in an identical fashion for the first 4 days after which TAP-treated cells dramatically decrease the growth rate. B: Temporal analysis of RNA DL-L following TAP treatment. A Northern blot was prepared with 10 μg samples from control and TAP-treated (1–11 days) Hep G2 cells. The 18S rRNA was used as a loading control. Probes to RNA DL-L reveal an induction of expression after 3 days in TAP, reaching maximal expression after 7 days. Probes to mt-mRNA species (ND1 is shown) show the expected increase in steady state levels even after a single day, but maximum expression in each case occurs after 3 day TAP exposure.

residue was determined to be nt 15954, the single residue between the genes encoding mt-tRNA^{Pro} and mt-tRNA^{Thr}. The 3′-terminal residue, nt 576, was the first base 5′-proximal to mt-tRNA^{Phe} (Fig. 3C). Thus, the complete 1192 nt H-strand transcript runs through the entire D-loop and past the major H-strand promoter I_{H1}, consistent with this transcript being processed from a larger polycistronic unit that may have been generated by run on transcription through the D-loop. We have termed this unique species RNA DL-L.

3.4. Temporal production of RNA DL-L is distinct from the increased production of mt-mRNAs

The identification of this novel RNA species in TAP-treated Hep G2 and other liver cell lines (data not shown) raises the possibility that RNA DL-L could remain bound to the H-strand, occluding I_{H1}, thus promoting initiation from a second H-strand promoter I_{H2}. Transcriptional initiation from this second promoter has been implicated in the differential H-strand transcription that occurs in response to thyroid hormone treatment [18]. To address this possibility, total cytoplasmic RNA was isolated from untreated Hep G2 cells and after various incubation times in TAP (Fig. 4). Although RNA DL-L becomes detectable at day 3, the major induction of this species did not occur until 7 day treatment, well after the increased transcription of the mt-mRNAs which reaches a maximum after 3 days. The induction of RNA DL-L is, therefore, likely to involve a mechanism distinct from that responsible for the increase in mt-mRNAs.

No substantial reading frame is present in the D-loop region of the corrected Cambridge Reference mtDNA sequence [19]. As the function of RNA DL-L is unknown, one potential explanation is that RNA DL-L is edited to produce a mt-mRNA that could encode a protein induced under conditions of mitochondrial stress. RNA editing is a common mechanism in the mitochondria of numerous species [20], although there has never been a report of such a process in human mitochondria. DNA sequence analysis was performed for the D-loop region of Hep G2 mtDNA and was compared to the sequence of RNA DL-L after RT-PCR. As shown in Table 1, although there were several changes from the reference sequence, all the alterations found in RNA DL-L were also found in the corresponding mtDNA. An alternative role for this molecule is in the regulation of mtDNA replication. Fig. 4 shows the standard growth curve of Hep G2 cells in TAP-treated and control cells. Cell division time in untreated cells is 30 h, a rate that is similar to cells grown in TAP for up to 4 days. However, the major effect on cell turnover occurs around the 4–5 day mark, just as the expression of RNA DL-L becomes apparent.

4. Discussion

TAP treatment causes an increase in mtDNA transcription without the modulation of nuclear gene expression that can affect mtDNA expression. What, therefore, is the mechanism that underlies this autonomous regulation? It has been speculated that autonomous regulation could be controlled by the transcriptional termination activity of the termination complex, mTERF [21]. A decrease in binding affinity for this site might be predicted to attenuate transcription termination, leading to increased synthesis of the mt-mRNA species downstream. The components of this complex and indeed the molecular mechanisms underlying truncation of H-strand transcription have not been fully described. Originally, a partially purified mtDNA DNA-binding and transcription termination activity termed mTERF [22] (or mtTERM [23]) was identified in a group of proteins ranging from 31 to 36 kDa [24]. The

Table 1
Changes from the mtDNA reference sequence found in both RNA DL-L and D-loop

Nucleotide position	Base change	Nucleotide position	Base change
16182	A→C	16519	T→C
16183	A→C	73	G→A
16189	T→C	263	A→G
16217	T→C	317	C→G
16223	C→T	332	C→G
16295	C→T		

most abundant of these species, a 34 kDa polypeptide, is also known as mTERF. This 34 kDa component alone is capable of DNA-binding activity but is not sufficient to terminate transcription in vitro [25]. It was also speculated that modulating intramolecular interactions could alter DNA-binding affinity of this component. In cytoplasmic lysates, we were able to detect a protein complex that bound specifically to a probe carrying the tridecamer mTERF DNA-binding sequence [26]. The control probe carrying a short deletion was not bound, as predicted. A decrease in binding complex was consistently noted in cell lysates following TAP treatment (data available on request). However, following exhaustive immunological experimentation we were unable to confirm the presence of the 34 kDa subunit in this complex. As we had attempted to protect the intact binding complex, it is possible that polyclonal antibodies raised against purified mTERF could not access the component. An alternative explanation is that the binding complex we see either does not contain mTERF or is an artifact.

A second possibility to explain the TAP-induced differential in H-strand transcription is that TAP promotes initiation from the second H-strand promoter I_{H2}, in a comparable manner to that proposed for thyroid hormone treatment, resulting in a similar differential increase in mt-mRNA over mt-rRNA gene transcription. This selective increase is believed to be mediated by a truncated form of c-erbA-α1 acting as a mitochondrial T_3 receptor [27]. Binding to the receptor may promote transcription from I_{H2}, resulting in a product, which is not terminated by mTERF. Further experimentation will be necessary to elucidate the exact mechanisms responsible for this differential transcription.

In response to TAP treatment, a novel mtRNA species RNA DL-L is generated. In contradistinction to the increase in the levels of mt-mRNAs, which reached a maximum after 3 day treatment, maximal levels of RNA DL-L are present at between 5 and 7 days, just after the TAP-induced decrease in cell turnover. As the cell turnover rate is diminished, the rate of mtDNA replication or turnover must compensate to maintain the cell copy number of mtDNA. One potential role for this novel species is, therefore, to inhibit the rate of mtDNA replication. It is well documented that the L-strand promoter (LSP)-initiated RNA transcribed through the CSBs within the D-loop has an unusually high affinity for template DNA and remains bound, forming an R-loop hybrid [28]. The bound RNA acts to prime DNA synthesis from the LSP [29]. RNA DL-L may invade the RNA/DNA hybrid to form a very stable RNA duplex. Blocking the formation of the RNA/DNA hybrid around the CSB regions would prevent maturation of the LSP-initiated RNA primer by MRP RNase, consequently preventing mtDNA replication. Although we have limited data to support this speculation, such an antisense mechanism is known to regulate the copy number of the ColE1 replicon in bacteria [30]. How is RNA DL-L formed? Thirty years ago, Attardi et al. reported that, at least in HeLa cells, the entire mtDNA molecule was transcribed [31]. It is therefore possible that this species is normally generated but has a very short half-life. Stabilisation of RNA DL-L by a currently unknown mechanism may therefore be all that is necessary for it to function as an antisense regulator.

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