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Detection of novel transcripts in the human mitochondrial DNA region coding for ATPase8–ATPase6 subunits

Maria Nardelli[a], Stefania Tommasi[b], Anna Maria D'Erchia[a], Filomena Tanzariello[a], Apollonia Tullo[c], Anna Teresa Primavera[b], Mario De Lena[b], Elisabetta Sbisà[c], Cecilia Saccone[a,*]

[a]*Dipartimento di Biochimica e Biologia Molecolare, Università di Bari, Via Orabona 4, 70125 Bari, Italy*
[b]*Istituto Oncologico, Via Amendola 209, 70126 Bari, Italy*
[c]*Centro di Studio sui Mitocondri e Metabolismo Energetico C.N.R., Bari, Via Orabona 4, 70125 Bari, Italy*

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Abstract

We have analyzed the $tRNA^{Lys}$, ATPase8, ATPase6, COIII region of mitochondrial DNA in several human tissues. Beside the mature $tRNA^{Lys}$, ATPase8 and ATPase6 common mRNA, and COIII mRNA, we have characterized two new transcripts, called RNA 20 and RNA 21. The RNA 20 is a precursor species which contains the $tRNA^{Lys}$ plus the ATPase8 and ATPase6 common mRNA; the RNA 21 is an RNA species shorter than the ATPase8 and ATPase6 common mRNA. The relative concentration of the mature with respect to that of the new species proved different in the various tissues. These findings provide new insights into the mitochondrial transcription mechanism opening the question of a possibly regulatory role of the processing on the expression of the mitochondrial genome.

Key words: Mitochondria; Genome (mt); Transcription; Processing; Expression

1. Introduction

The mitochondrial (mt) transcription mechanism in animal cells shows peculiar properties. The two strands of mt DNA are transcribed in opposite directions [1], starting from the promoters located at the 5′ end of the main regulatory region, called D-Loop. Then, the two polycistronic transcripts are processed to give the mature RNA species.

The concentration of different RNA species in the cell depends on several regulatory mechanisms modulating, at different stages, the expression of the mt genome. In the first instance, the rate of RNA synthesis is regulated at the level of the initiation (promoters) and termination sites, then several post-transcriptional factors such as the stability of mature RNA species come into play.

The model considered until now postulates that the polycistronic transcripts are processed at the level of the tRNAs which, regularly interspersed along the genome, make up a sort of punctuation [2,3]. An enzyme similar to an RNase P should process the transcripts and mature the products by adding a poly(A) chain for mRNAs allowing, in some cases, the formation of the termination codons.

This model, which has remained unchanged since its descovery, implies that the processing mechanism plays an important regulatory role despite no experimental evidences have been provided so far.

Recent studies of our group, aimed at the precise mapping of mt transcripts in vivo, have demonstrated the presence in rat of new mt RNA species. In particular, in the D-loop region we have detected transcripts which encompass the whole region and stable antisense RNAs [4]; in the Ori-L region we have identified again antisense RNAs and precursor species of tRNAs ($tRNA^{Ala}$–$tRNA^{Asn}$ and $tRNA^{Cys}$–$tRNA^{Tyr}$) as well as a precursor containing an mRNA plus a tRNA (ND2–$tRNA^{Trp}$) [5]. In human cells a new precursor species called RNA 19 which contains the 16S rRNA, $tRNA^{Leu(UUR)}$ and ND1, has been detected and related to a mitochondrial desease [6].

In this paper, we have studied in detail, in HeLa cells and in different human tissues, the transcriptional pattern of an interesting mtDNA region coding for the $tRNA^{Lys}$, the ATPase8, the ATPase6 and the Cytochrome oxidase III (COIII) subunits. The peculiar features of this region are a partial overlapping (46 bases) between the ATPase8 and the ATPase6 genes which are translated from a common mRNA, and the lack of the canonical tRNA punctuation signal between the ATPase6 and COIII genes. Our data relate to a possible regulatory role of the processing in the expression of the mitochondrial genome.

* Corresponding author. Fax: (39) (80) 243317.
E-mail: SACCONE@MVX36.CSATA.IT.

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2. Materials and methods

2.1. Preparation of total RNA

Hela cells were grown in MEM medium added with non essential amino acids 1×, 10% of foetal calf serum-heat inactivated, 100 U penicillin and 100 μg streptomycin.

The normal (liver, colon, gastric) and tumour tissues were obtained from surgical removal from patients who underwent surgery at the Oncology Institute of Bari. Pathology of tissues was confirmed by routine histological analysis. Abortive placenta derives from voluntary interruption of pregnancy at about 7–12 weeks. Immediately after removal, the tissues were frozen (−80°C). Before extraction they were first pulverised.

Total RNA was purified using the phenol–chloroform/guanidinium isothiocyanate method [7].

2.2. Cloning and transcription of the mithocondrial fragments

The fragments used in RNAse mapping were cloned in Bluescribe vector (Stratagene San Diego, CA) and transcribed using [^{32}P]UTP and T7 and T3 RNA Polymerase (Promega) according to the methods indicated by the suppliers. The strand specificity of the transcripts has been checked by sequencing the DNA templates using the Sanger dideoxynucleotide chain-terminator method adapted to double strand templates [8]. The genetic content and the restricton map of the region under investigation are shown in Fig. 1.

2.3. RNAse mapping

Total RNA (10 μg), RNase-free DNase-treated, has been denatured and the hybridizations were carried out in 20 μl of 80% formamide, 400 mM NaCl, 40 mM PIPES (1,4-piperazine-diethanesulfonic acid) (pH 6.7), 1 mM EDTA, 10 μg tRNA at 50°C for 16 h with different riboprobes (2×10^8 dpm/μg). The hybridization mixture has been subsequently digested with RNase T1 (2 μg/ml) and RNase A (40 μg/ml) in 0.3 M NaCl, 10 mM Tris-HCl (pH 7.8), 5 mM EDTA for 60 min at 30°C. Further incubation has been performed by adding 50 μg of proteinase K and 10 μl of 20% SDS for 15 min at 37°C. After phenol extraction and ethanol precipitation, protected fragments were analyzed by electrophoresis in 6% acrylamide/8 M urea gels. The negative controls (Figs. 2–6, lanes N) for each probe were carried out on samples which had been hybridized alongside the other reactions under exactly the same conditions but without total RNA. In these samples, after the RNase digestion, the riboprobes had been completely degraded, following expectations. All experiments were carried out at least in triplicate using several batches of RNA together with several batches of riboprobes and the results reported were confirmed in all cases.

3. Results

3.1. Transcriptional mapping of the tRNA^{Lys}, ATPase8, ATPase6 region

The transcriptional analysis has been performed with human total mt RNA and riboprobes 1 and 2 of mt genome whose content is shown in Fig. 1 (clones 1 and 2).

In Fig. 2 the RNAse mapping experiments, using HeLa cells, the main reference system for mt experimentation up till now [9], are reported. Riboprobe 1 (lane 1) reveals, as expected, the mRNA (231 b) common to ATPase8 and ATPase6 (RNA 14 according to Amalric et al.) [10] and the tRNA^{Lys} (70 b). It should be noted that the hybridization of the tRNA may show multiple bands, as already reported [11], due to an incomplete unfolding of the cloverleaf structure. Besides, riboprobe 1 also detects an additional new band (304 b) corresponding to a product never identified before. This band represents a precursor transcript, which includes the tRNA^{Lys} and the mRNA for ATPase8 and ATPase6. The new transcript has been called RNA 20, as Attardi's group identified 18 RNA species [10] and King et al. discovered RNA 19 [12].

For a more precise mapping of the RNA species present in this region, RNase mapping with the shorter riboprobe 2 (lane 2), containing only 204 b of the 3′ end region of probe 1, has been performed. We have obtained only one band corresponding to the complete protected fragment 204 b long. This result demonstrates that the RNA 14 and RNA 20, identified by riboprobe 1, have different 5′ ends and equal 3′ ends.

The transcriptional pattern of this region has been studied also in several human tissues, namely different donor's lymphocytes, abortive placenta, liver, colon and gastric tissues.

The RNase mapping experiments reported in Fig. 3 show that the riboprobe 1 (lanes 1) identifies the precursor (304 b), the ATPase8, ATPase6 common mRNA (231 b) and the tRNA^{Lys} (70 b) already detected in HeLa cells. In addition, we have identified in lymphocytes a new transcript of 209 b, we called RNA 21.

By using riboprobe 2 (Fig. 3, lanes 2), we have obtained also in these cases only one band 204 b long which allows us to conclude that the product of 209 b has the 3′ end coinciding with that of the RNA 14 and RNA 20. The RNA 21 is shorter than the mature RNA 14 and its 5′ end is located between the two initiation codons of the ATPase8 and ATPase6.

The transcriptional pattern is similar in all tissues but the relative abundance of the various transcripts appears

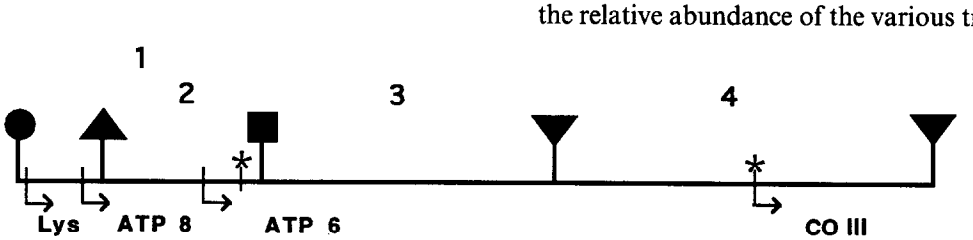

Fig. 1. Genetic organization and restriction map of the region containing the tRNA^{Lys} (Lys), ATPase8 (ATP8), ATPase6 (ATP6), cytochrome oxidase III (COIII) subunits. The arrows indicate the initiation codons and the direction of the transcription of each gene. The numbers above the line indicate the subfragments cloned in the Bluescribe vector. Clone 1, *XbaI–BclI* fragment (8,286–8,595): 310 bp (9 bp spacers, 70 bp tRNA^{Lys}, 162 bp ATPase8, 46 pb ATPase8–ATPase6 overlapping, 23 bp ATPase6). Clone 2, *Sau96I–BclI* fragment (8,393–8,595): 204 bp (135 bp ATPase8, 46 pb ATPase8–ATPase6 overlapping, 23 bp ATPase6). Clone 3, *BclI–TaqI* fragment (8,592–8,960): 369 bp (ATPase6). Clone 4, *TaqI–TaqI* fragment (8,957–9,446): 490 bp (250 bp ATPase6, 240 bp COIII). The nucleotide positions in brackets refer to reference [3]. (●) *XbaI*; (▲) *Sau96I*; (■) *BclI*; (▼) *TaqI*; (*) stop codon.

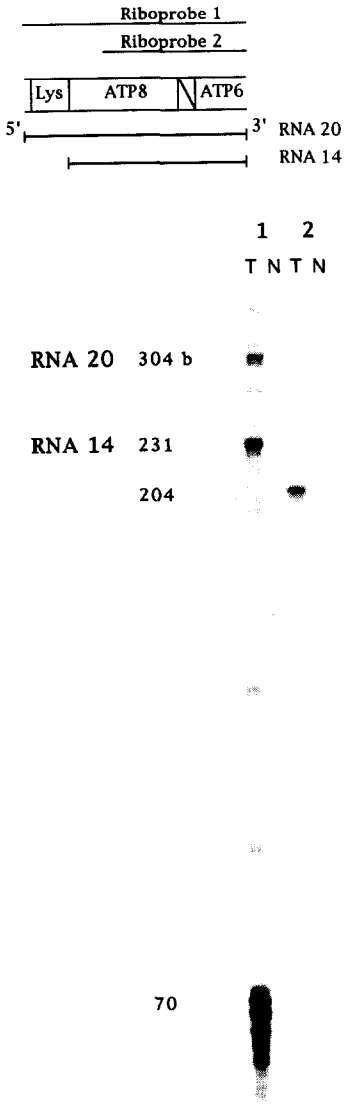

Fig. 2. RNase mapping in HeLa cells. The hybridizations are performed with riboprobe 1 (lane 1) and 2 (lane 2). T = hybridization performed with total RNA; N = RNase-treated probe as negative control. The analytical scheme is reported above the autoradiogram. The RNA species identified by the riboprobe 1 are indicated at the bottom of the map. The riboprobe 2 identifies one band (not shown in the scheme) corresponding to the 3′ end of both RNA 20 and RNA 14.

to be different. We have observed that the RNA 20 and 21 are particularly abundant in lymphocytes and RNA 20 in liver.

In further experiments we have analyzed the transcriptional pattern in several tumour tissues obtained from surgical removal. Figs. 4 and 5 show the RNAse mapping performed on three breast cancer RNAs and one synchronous metastatic lymphnode. We observed that in all tumours, as in placenta, the mature common mRNA (RNA 14) is the most abundant species whereas the $tRNA^{Lys}$ is less represented.

It should be noted that in studies on the stability of the different mtRNA species in HeLa cells [13] the presence of RNA precursors has never been reported and the tRNAs are usually in higher concentration than mRNAs (see also Fig. 2).

3.2. *Transcriptional mapping of the ATPase6, COIII region*

We have extended the transcriptional analysis to the 3′ end of the ATPase genes in order to verify whether the

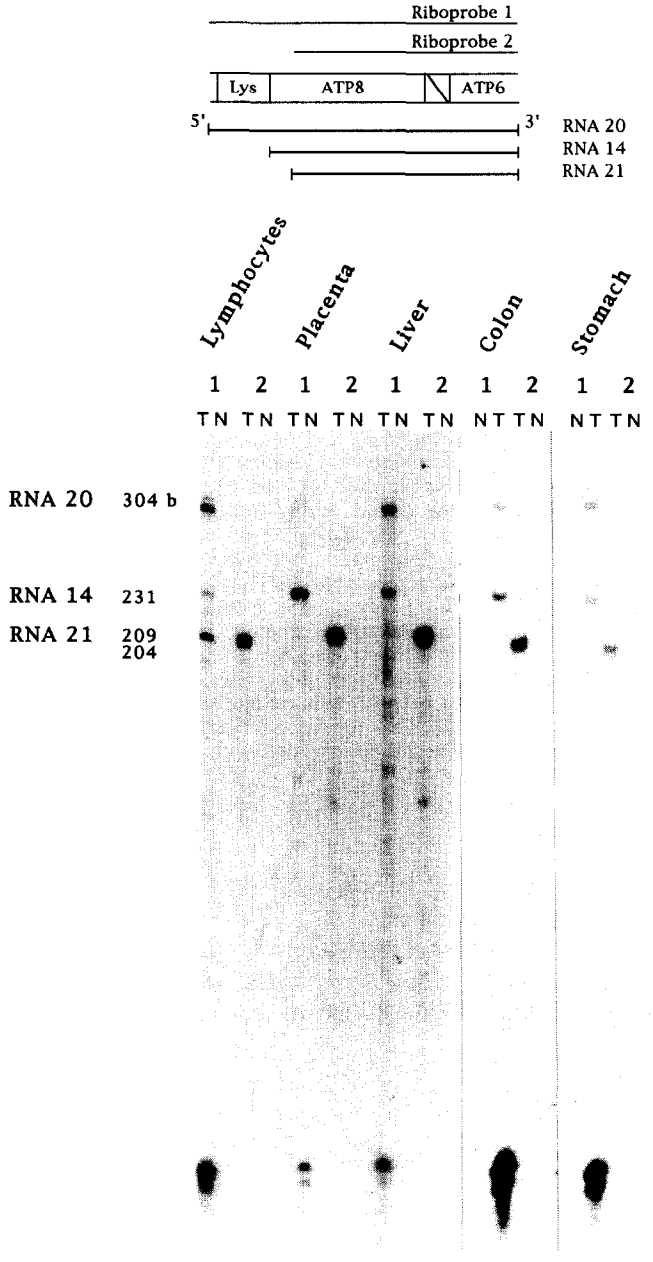

Fig. 3. RNase mapping in normal tissues. The hybridizations are perfomed using RNA of lymphocytes, placenta, liver, colon, gastric tissues with riboprobes 1 (lanes 1) and 2 (lanes 2). T = hybridization performed with total RNA; N = RNase-treated probe as negative control. The analytical scheme is reported above the autoradiogram. The RNA species identified by the riboprobe 1 are indicated at the bottom of the map. The riboprobe 2 identifies one band (not shown in the scheme) corresponding to the 3′ end of RNA 20, RNA 21 and RNA 14.

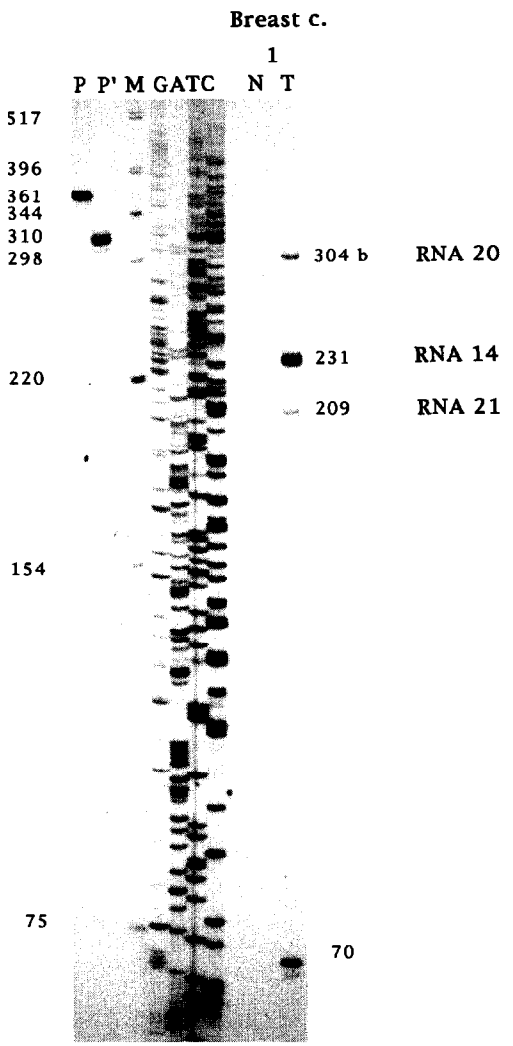

Fig. 4. RNase mapping in a tumor tissue. The hybridization was performed using RNA of a breast cancer tissue (breast c.) with riboprobe 1 (lane 1). P = Riboprobe 1 (361 b); P′ = riboprobe marker (310 b); M = pBR322 *Hin*fI digested; GATC = marker sequence; N = RNase treated probe as negative control; T = hybridization performed with total RNA. The larger dimensions of the riboprobe 1 with respect to the complete protected fragment are due to the transcription of the plasmid between the promoter site and the cloning site.

processing pattern at this level is as complex as at the 5′ end. We used riboprobes 3 and 4 (Fig. 1, clones 3 and 4) and total lymphocytes RNA. The riboprobe 3, corresponding to an internal region of the ATPase6 mRNA, shows a complete protected fragment 369 b long as expected (Fig. 6, lane 3). The riboprobe 4, corresponding to the junction of the ATPase6/COIII genes identifies two products of 250 and 240 b, respectively (Fig. 6 lane 4). The size of these RNA species coincides exactly with the length of the ATPase6 3′ end and with the COIII 5′ end present in riboprobe 4. We can therefore infer they represent the two mRNAs and confirm, by a different experimental approach, the mapping data previously reported [2,14]. These two mRNA species are equally represented. Differently from what was identified in the tRNA^{Lys}, ATPase8 region, no band corresponding to precursor RNA species is detectable at the ATPase6/COIII junction. The same results have been obtained by using total RNA purified from other tissues: placenta, fibroblast cell line (data not shown).

4. Discussion

In mitochondria, the processing of polycistronic transcripts and the polyadenylation are necessary to generate the ends of the mRNA to be translated. The mt mRNAs lack 5′ end leader regions and in some cases also the termination codons which are completed by the poly(A) addition. The data reported here indicate the processing is not always sufficiently efficient to generate mature species, as taken for granted so far.

It is difficult to speculate on the possible function of the precursor RNA species. The novel transcripts identified here and in previous studies [5,6] might represent processing intermediates, suggesting the polycistronic transcripts are not immediately processed and the RNA processing mechanism, at least in some cases, proceeds in steps. However, these precursors could be 'dead end'

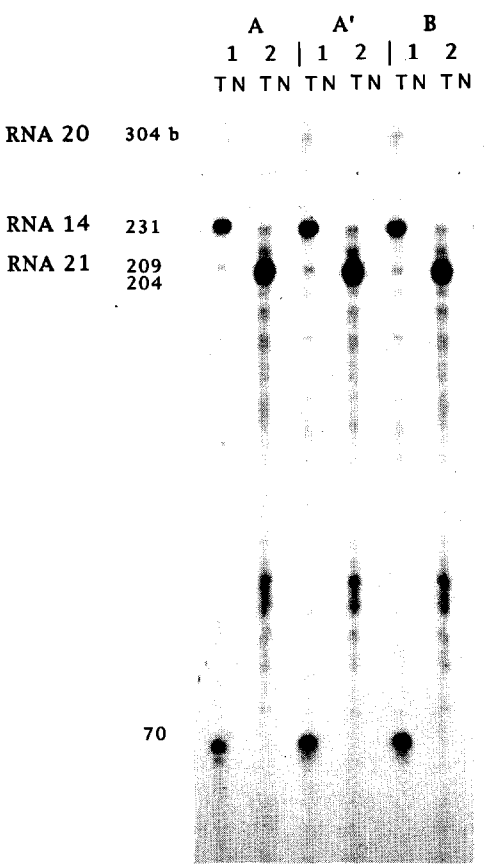

Fig. 5. RNase mapping in tumour tissues. The hybridizations are performed using RNA of two breast tumors (A and B) and of a metastatic lymph node (A′) of patient A with riboprobes 1 (lanes 1) and 2 (lanes 2). T = hybridization performed with total RNA; N = RNase-treated probe as negative control.

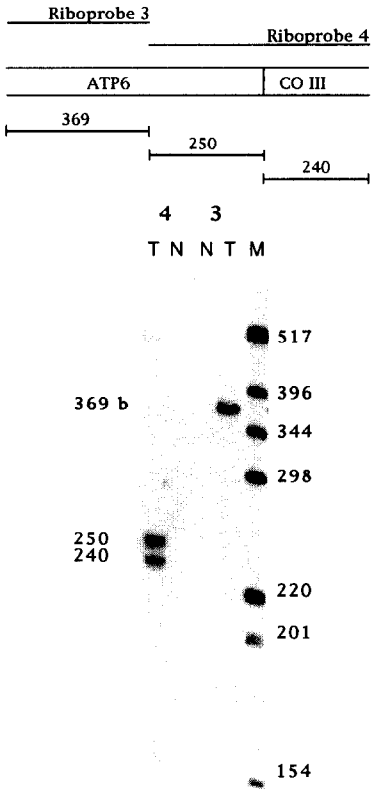

Fig. 6. RNase mapping of the ATPase6 and COIII region. T = hybridization performed with total RNA; N = RNase-treated probe as negative control; M = pBR322 *Hin*fI digested. The hybridizations are performed using RNA of lymphocytes with riboprobes 3 (lane 3) and 4 (lane 4). The analytical scheme is reported above the autoradiogram. Sizes of the full protected fragments are indicated below the map in the corrispondence of their riboprobes.

products generated also by alternative processing pathways.

The presence of precursors containing tRNA genes, confirms that tRNAs are not crucial in the processing. The polycistronic transcript is efficiently processed at the level of the ATPase6/COIII junction in the absence of a tRNA.

The ATPase8 and ATPase6 are translated from a common mRNA (RNA 14), though it remains unknown how the expression of the two subunits is coordinated. The RNA 21 contains only the complete frame of the ATPase6 subunit. Its translation, starting from the AUG of the ATPase6, might represent a mechanism for the translation of the ATPase6 subunit independently from that of the ATPase8. As far as the possible translation of the RNA 20 and RNA 21 they cannot generate products longer than the two mature ATPase subunits because of the presence of termination codons upstream of the AUG initiation codons of the ATPase8 and ATPase6.

The relative abundance of the RNA species differs in various normal and tumour tissues, in particular different precursor/mature and mRNA/tRNA ratios have been observed. The difference in the steady-state RNA levels can be ascribed to differential RNA stability as well as to differential processing efficiency. Though no conclusion can be drawn, it might be suggested that the concentration of the mt transcripts is correlated to specific factors such as the different metabolic pathways of the tissues (normal and pathological). This is supported by data showing a tissue-specific expression of both nuclear and mitochondrial subunits of the ATP synthetase [15].

It is noteworthy to mention that a human pathology (MELAS 3243 mutation) has been correlated to altered levels of mt transcripts [6]. An increased level of a precursor transcript (RNA19), has been observed in human mutant hybrids. The mutation does not affect the accuracy of the processing, rather it causes its lower efficiency and, consequentely, a higher level of this precursor.

These data clearly indicate that the processing mechanism is much more complex than previously reported and that it should be considered a further crucial element in the modulation of the mt genome expression.

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