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Transcription mapping of the Ori L region reveals novel precursors of mature RNA species and antisense RNAs in rat mitochondrial genome

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We have identified new transcripts in the region surrounding the L-strand replication origin (Ori L) of rat liver mitochondrial DNA. In particular, we have detected previously unidentified intermediates of RNA processing on both the heavy and the light strands, such as precursors of the ND2 mRNA plus the Trp-tRNA and precursors of the tRNAs clustered in the Ori L region. This indicates that the mechanism of RNA processing in mitochondria proceeds step-wise producing a variety of precursors of the mature forms. The other striking finding is the detection of antisense RNA species in the region of L-strand replication. Since a variety of antisense transcripts were also found in the D-loop region of rat mitochondrial DNA, we suggest that they might play a regulatory role in the replication and expression of the mitochondrial genome.

Mitochondrial DNA; Replication; Regulation; Antisense RNA; Transcript processing

1. INTRODUCTION

Mammalian mitochondrial (mt) DNA contains two distinct origins of replication at a distance of about 10 kb. The replication origin (Ori H) of the heavy (H) strand is located in the main non-coding segment of mtDNA, called the D-loop-containing region since in the majority of resting molecules, a newly nascent H strand creates a three-stranded structure with the displacement of the old H strand. This region, ranging from 0.9 to 1.2 kb in mammals, also contains the promoters for both light (LSP) and heavy (HSP) strand transcription. The non-coding region containing the origin of light (L) strand replication (Ori L) is only 30 b long and is flanked by five tRNA genes. This region can fold in a stable stem and loop structure which is very conserved in mammals.

The two origins of replication show a natural DNA curvature correlated with the periodic distribution of dinucleotides in the structure [1–2].

The replication of mtDNA in mammals has been studied extensively. It starts with the elongation of the nascent H strand and the concomitant displacement of the parental H strand. When two-thirds of the daughter H strand has been synthesized, the synthesis of the L strand begins on the displaced H strand template [3–4].

Both H- and L-strand replication require the presence of RNA primers which are produced by mtRNA polymerase at the level of Ori H and by a mt primase at the level of Ori L (and therefore transcription and replication mechanisms are strictly related). The switch from RNA synthesis to DNA synthesis is accomplished by a mtRNase MRP activity at the level of CSBs in the D-loop and at the level of a G-rich sequence at the base of a stem–loop structure in the L-strand replication origin [5].

As regards the mechanism of transcription, early data obtained by electron microscopy suggested that in HeLa cells both strands of mtDNA are symmetrically transcribed. Subsequent studies performed on various mammalian species in vivo and in vitro were mainly concerned with the characterization of the promoters and enzymes involved in transcription and with the mapping of structural transcripts [6].

We have recently demonstrated that the D-loop-containing region is symmetrically transcribed in rat mitochondria producing rather complex patterns of H and L transcripts [7].

In order to get a better insight into the transcription of the non-coding regions of mtDNA we have since extended our analyses to the replication origin of the L strand. In this paper we demonstrate the presence of novel unidentified precursors of mature RNA species and of stable complementary transcripts in the region containing the replication origin of the L strand. On the other hand no stable antisense RNAs were detected in

Abbreviations: mt, mitochondrial; Ori region, region containing the origin of replication; H, heavy; L, light; ND, NADH dehydrogenase; CO, cytochrome oxidase; D-loop, displacement loop; CSB, conserved sequence block.

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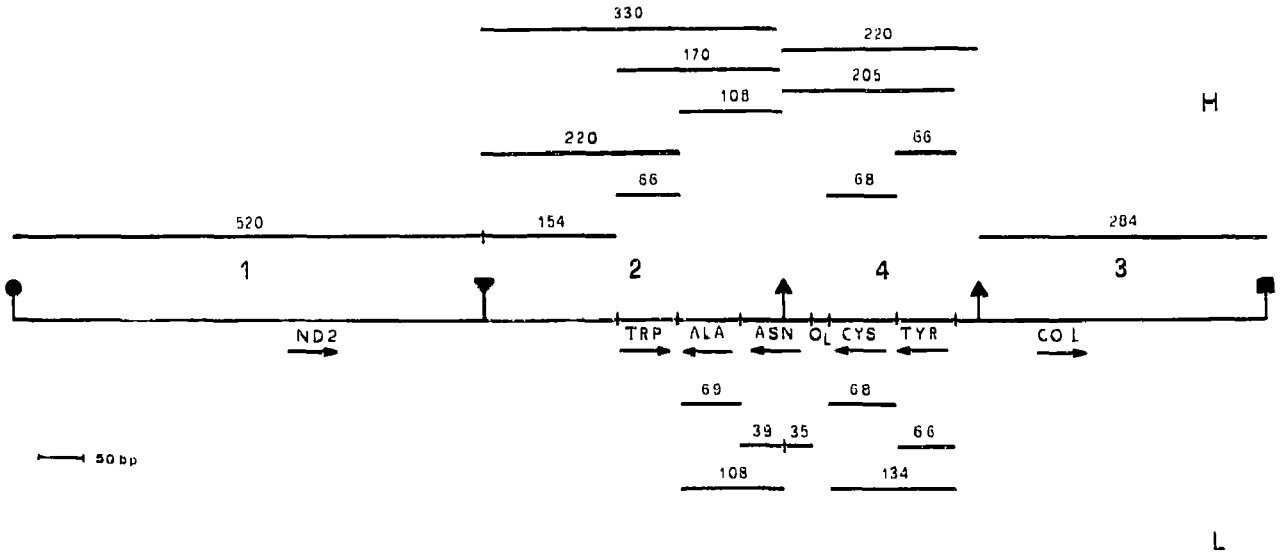


Fig. 1. Restriction map and genetic organization of the Ori L region. The numbers above the line indicate the subfragment cloned in the Bluescript vector. The arrows indicate the direction of the transcription of each gene. H, Heavy transcripts; L, Light transcripts; ND2, NADH-dehydrogenase subunit 2; Trp, Ala, Asn, Cys, Tyr: tRNA cluster; O_L, origin of replication of L strand; COI, cytochrome oxidase subunit I. Clone 1= 520 bp ND2; clone 2= 154 bp ND2, 66 bp Trp-tRNA, 69 bp Ala-tRNA, 39 bp Asn-tRNA; clone 4= 35 bp Asn-tRNA, 30 bp Ori L, 68 bp Cys-tRNA, 66 bp Tyr-tRNA, 15 bp COI; clone 3= 284 bp COI. ● = *Eco*RI; ▼= *Taq*I; ▲= *Hin*cII; ■= *Hin*dIII.

the surrounding segments coding for structural mRNA and rRNA genes.

2. MATERIALS AND METHODS

2.1. *Preparation of mitochondrial RNA*

RNA was purified from the liver of male albino *Rattus norvegicus* of the Wistar strain [8].

The RNA was treated with RNase-free DNase before the hybridization. We can exclude any residual contaminating DNA because several riboprobes (5(L), 1(L), 3(L), 6(L)) did not reveal any hybrid at all, and other riboprobes (2(L) and 4(L) revealed hybrids that are shorter than their complete sequences (Fig. 2).

2.2. *Cloning, transcription and sequencing of mitochondrial DNA*

The fragments: *Eco*RI-*Taq*I (fragment 1), *Taq*I-*Hin*cII (fragment 2), *Hin*cII-*Hin*dIII (fragment 3), *Hin*cII-*Hin*cII (fragment 4), *Eco*RI-*Sac*I (fragment 5), *Bam*HI-*Cla*I (fragment 6) were cloned in Bluescript vector (Stratagene San Diego, CA). The bluescript vector contains the T7 promoter-multiple cloning site-T3 promoter. To obtain the transcripts of both the strands of the fragments, we linearized the plasmid upstream or downstream from the cloned fragment and alternately used the T7 and T3 RNA polymerases (Promega) according to the methods indicated by the suppliers. The strand specificity of the transcripts was checked by sequencing the DNA templates using the dideoxynucleotide chain-terminator Sanger method adapted to double strand templates [9].

The exact size of the fragments are: fragment 1=520 bp, fragment 2=330 bp, fragment 3=284 bp, fragment 4=220 bp, fragment 5=331 bp, fragment 6=501 bp.

2.3. *RNase mapping of H- and L-transcripts in the Ori L region*

Total mtRNA (40 μg), RNase-free DNase-treated, was denatured and the hybridizations were carried out in 30 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 (2×10^6 dpm/μg). The hybridization mixture was subsequently digested with RNase T1 (173.5 U) and RNase A (0.3 μg) in 0.3 M NaCl, 10 mM Tris-HCl (pH 7.8), 5 mM EDTA for 30 min at 30°C. Further incubation was 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 for each probe were carried out on samples which had been hybridized alongside the other reactions under exactly the same conditions but without mtRNA. In these samples, after the RNase digestion, the riboprobes were completely degraded as expected. Optimum conditions for the reactions were set up using a riboprobe corresponding to cytochrome oxidase subunit I. All the riboprobes were eluted from the denaturing gel before the hybridization.

2.4. *Reverse transcriptase experiments*

Experiments were performed using three synthetic 20-mer primers: oligo a = 5117–5136, oligo b = 5360–5341, oligo c = 5180–5199; the nucleotide positions are those relative to the rat sequence reported by Gadaleta et al. [10]. The oligos have been purified by electrophoresis on a 20% polyacrylamide/8 M urea gel. 20 pmol of eluted primers were labelled with 60 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol, Amersham, UK) and 10 U of T4 polynucleotide kinase (Boehringer, Mannheim) [11]. For annealing, 7×10^6 cpm of these primers were combined with 10 μg of total rat liver mtRNA in 20 μl of 80% formamide, 0.7 M NaCl, 0.04 M HEPES (*N*-(2-hydroxyethyl)piperazine-*N'*-(2 ethanesulfonic acid)) pH 7.00 and incubated for 16 h at 37°C. After ethanol precipitation, the samples were suspended in 15 μl of 0.15 mM dATP, dCTP, dTTP, dGTP, 34 mM Tris-HCl pH 8.3, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT. The reverse transcription was started by adding 8 U of reverse transcriptase (Promega) and 30 U of RNase inhibitor (Promega), continued for 2 h at 42°C and terminated by adding 0.3 M NaOH and incubating at 56°C for 10 min. After neutralization and phenol/chloroform extraction, the nucleic acids were recovered by ethanol precipitation, suspended in 4 μl of 80% formamide, 10 mM EDTA, 0.3% xylene-cyanol and sized on denaturing 6% polyacrylamide/8 M urea sequencing gels.

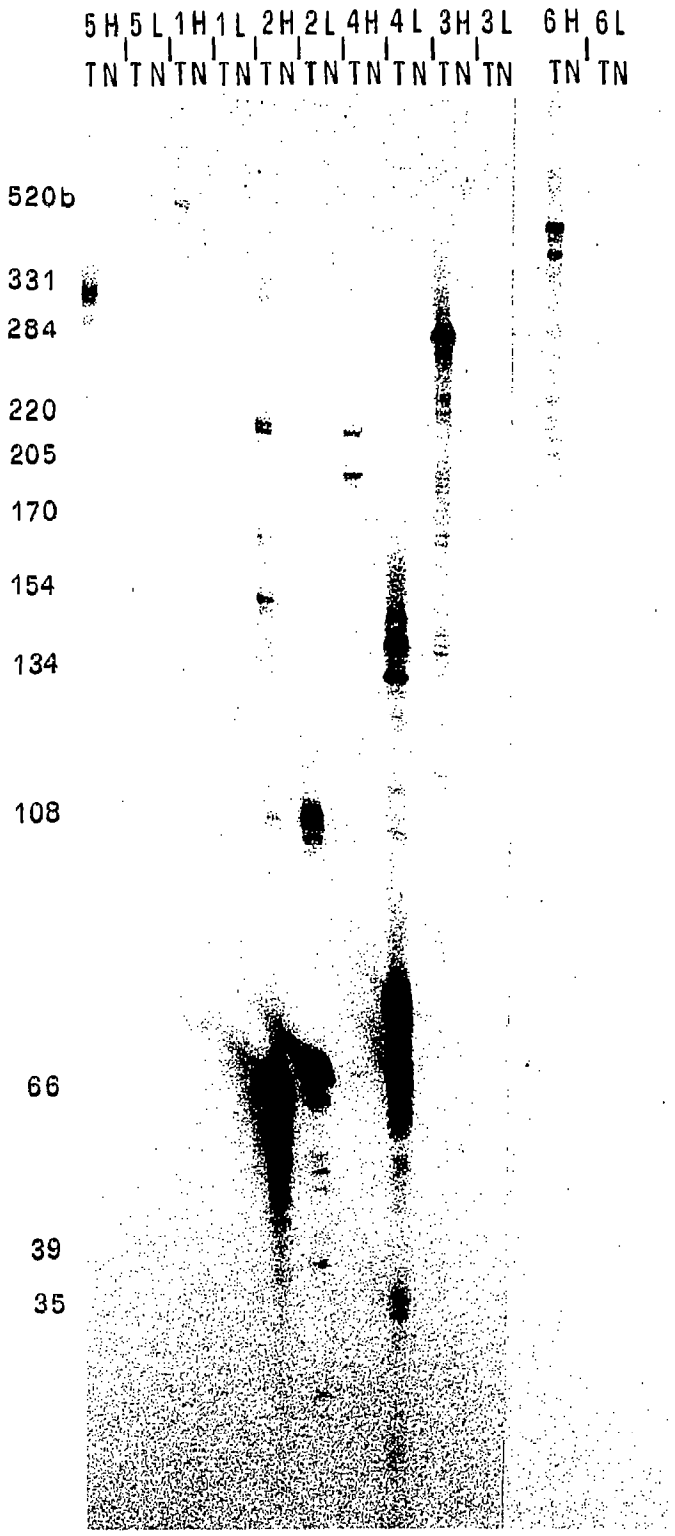


Fig. 2. RNase mapping of H- and L-transcripts in the Ori L region. Hybridization performed with total mtRNA, T; RNase-treated probe as negative control, N; H, heavy transcript; L, Light transcript. Clones 1–4, see legend Fig. 1; clone 5, 331 bp ND1; clone 6, 501 bp 12S rRNA. The protected fragments were sized against known sequence ladders.

2.5. Quantitative analyses of the transcripts

Hybridizations were performed by combining a fixed amount of RNase-free DNase-treated total mtRNA with increasing quantities of specific highly labelled riboprobes (0.1–1 ng; 2×10^8 dpm/μg). The saturation conditions were reached with 0.03 μg mtRNA for riboprobes 5(H) and 1(H); 0.4 μg for 4(H); 0.01 μg for 4(L) and 0.02 μg for 3(H). The amount of the hybrid was measured, after RNase digestion and TCA precipitation, from the difference between the TCA-insoluble radioactivity of samples containing mtRNA and that of the controls. Each experimental point was performed in quadruplicate. The relative concentrations of all the transcripts at saturation are shown in Table I.

3. RESULTS

Fragments of mtDNA containing Ori L and its surrounding genes were cloned in the Bluescript vector and transcribed in vitro in both directions by using T3 and T7 RNA polymerases. These riboprobes were hybridized with total rat liver mtRNA, RNase-free DNase treated and used in qualitative and quantitative analyses.

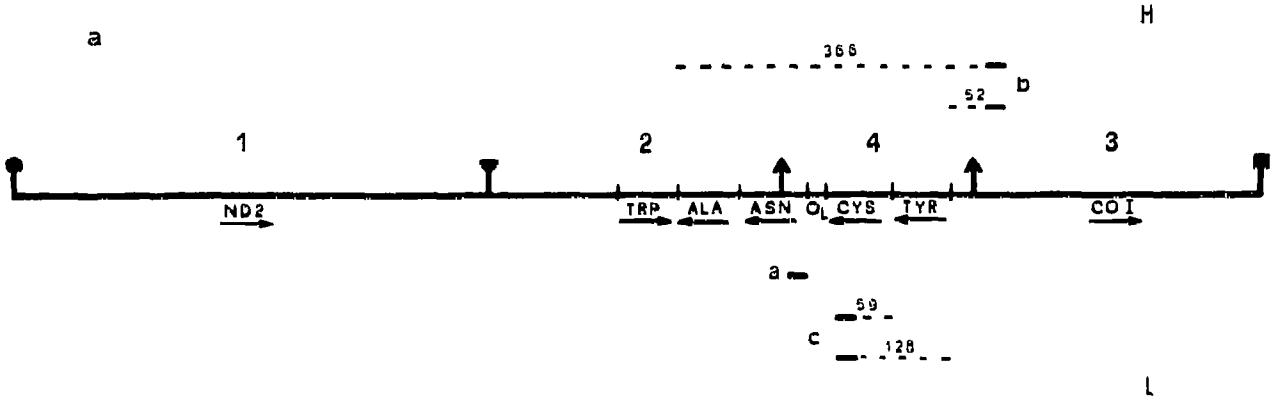
Fig. 1 shows the structural organization and the restriction map of the region under investigation. The H and L transcripts were characterized by RNase protection as shown in Fig. 2. The riboprobes used to identify the transcripts of heavy and light strands are defined H and L, respectively.

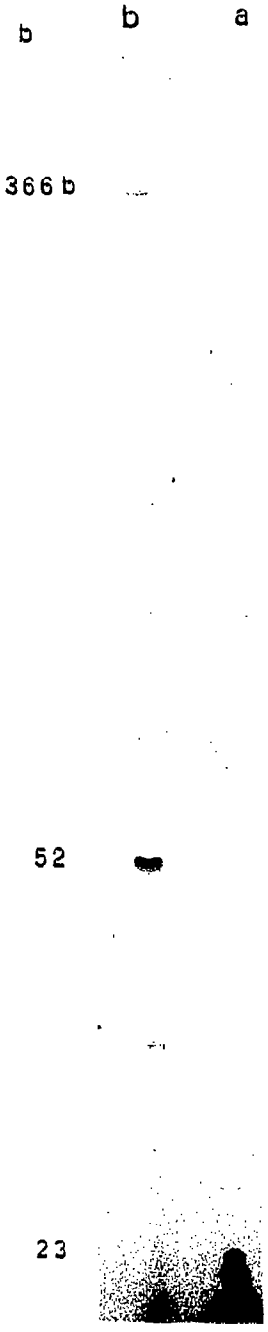
The riboprobes 5(H), not shown in Fig. 1, and 1(H) identify transcripts corresponding to ND1 and ND2 mRNAs respectively (331 b and 520 b long) (Fig. 2 lanes 5H and 1H). No transcripts complementary to these mRNAs are detectable (Fig. 2 lanes 5L and 1L).

Fragment 2 contains four structural genes: the ND2 3′ end and the Trp-tRNA coded by the H strand, and two tRNAs, Ala and Asn 3′ end, coded by the L strand. Riboprobe 2(H) identifies a peculiar transcription pattern (Fig. 2 lane 2H): the bands (154 b and 66b) correspond to the protection of the structural genes ND2 and Trp-tRNA, respectively; the faint bands represent several processing intermediates. On the basis of their dimensions the bands were interpreted as follows: the 330 b hybrid as the complete protected fragment, the 220 b hybrid as the H transcripts containing ND2 mRNA plus Trp-tRNA, the 170 b hybrid as a processing intermediate containing the structural Trp-tRNA plus antisense Ala-tRNA plus antisense Asn-tRNA, and the 108 b hybrid as the antisense Ala-tRNA plus Asn-tRNA. It should be noted that the hybridizations of tRNAs show multiple bands owing to an incomplete unfolding of the cloverleaf structure [12].

It should be stressed that the complete protected fragment (330 b) reveals the presence of a novel unprocessed RNA precursor containing an mRNA (ND2), Trp-tRNA and antisense tRNAs.

Riboprobe 2 (L) (Fig. 2, lane 2L) identifies Ala and Asn tRNAs (69 b and 39 b long) and their precursor (108 b) which migrates at the same level as its antisense RNA (Fig. 2, lane 2H). The concentrations of antisense





species appear to be much lower than those of the corresponding sense precursors. No transcripts complementary to ND2 mRNA are detectable.

Moreover, the reverse transcriptase experiment allowed us to map the 5′ end of the Asn-tRNA (23 b) (Fig. 3B lane a) by using the oligo a which anneals 3 bases downstream its 5′ termini (Fig. 3A); no longer transcripts have been detected, suggesting a processing of the polycistronic RNA at the level of the Ori L.

Riboprobe 4 (H) (Fig. 2, lane 4H) identifies a complete protected transcript, 220 b long, corresponding to the precursor of the COI (antisense RNA of Asn-tRNA, Ori L, Cys, Tyr-tRNAs 15 b of COI) and one transcript of 205 b very likely corresponding to a processing of the former at the 5′ end of the COI (15 b). The faint band 68 b long corresponds to the antisense tRNAs. These transcripts, like the transcript of 108 b identified with riboprobe 2 (H), do not contain genetic information.

These results have been confirmed by a reverse transcriptase experiment performed using the oligo b which anneals 31 bases downstream the ATG of the COI (Fig. 3A). The elongation products identify the 5′ end of the COI (52 b) and the precursor (366 b) (Fig. 3B, lane b).

It should be remembered that in HeLa cells a single transcript (RNA 6) present in this region has been identified as the precursor of COI [13]. The 5′ end of this transcript was mapped immediately downstream of the 3′ end of the Trp-tRNA whereas the 3′ end was not precisely determined. Our data determine the position of both the ends.

Riboprobe 4 (L) (Fig. 2, lane 4L) reveals a complex pattern with equally intense multiple bands. These bands have been interpreted as corresponding to the processed tRNAs of the cluster (68 b Cys-tRNA, 66 b Tyr-tRNA, 35 b Asn-tRNA) and to their precursors (134 b long). These results have been confirmed by

Fig. 3. Fine mapping of the transcripts in the Ori L region. A: schematic diagram of the probes used in the reverse transcriptase experiments (for details see Fig. 1). B: reverse transcriptase experiments. Lane a, mapping performed using oligo a; lane b, mapping performed using oligo b.

reverse transcriptase experiments, using oligo c, which anneal in the Cys-tRNA (Fig. 3A).

The elongation products identify the 5′ end of Cys and the precursor Cys-Tyr tRNAs (data not shown).

In the downstream region, hybridization with clone 3 (H) (Fig. 2, lane 3H) identifies a transcript corresponding to COI mRNA (284 b long) which is present in a much higher concentration compared to its precursor (Fig. 2, lane 4H). The 5′ end of the COI has been precisely mapped by using oligo b as previously reported (Fig. 3B, lane b). The mRNA starts from the triplet ATG as deduced from the nucleotide sequence.

Riboprobe 3 (L) does not reveal any hybrid at all, thus showing the absence of COI antisense RNA species.

Riboprobe 6 (H) (not shown in Fig. 1) identifies 12S rRNA (501 b) (Fig. 2, lane 6H). No antisense RNA species were detectable in the ribosomal region (Fig. 2, lane 6L).

By hybridizing in solution, RNase-free DNase-treated total mtRNA with an excess of radioactive riboprobes, we determined the relative concentration of the transcripts at saturation. The results were controlled by repeating the hybridizations with different amounts of total mtRNA (0.01–1 μg) so as to confirm that the ratio of the various products could be maintained.

The relative concentrations of all the transcripts at saturation are shown in Table I. It should be noted that the pg of the hybrid depends on the length of the riboprobe. For the structural genes ND1, ND2 and COI we confirmed the presence of the sense RNA only. The amounts of the mRNA of the two subunits ND1 and ND2 which both belong to complex I are comparable (when taking into account the different lengths of the riboprobes), whereas the concentration of the mRNA of COI is about three times higher. Only the riboprobes 4(H) and 4(L) identify symmetric transcripts. The tRNA antisense species are one and two orders of magnitude less abundant than the mature mRNAs and tRNAs species respectively.

4. DISCUSSION

The results shown in this paper indicate several novel features of the mitochondrial transcription mechanism in mammalian cells. We found that the tRNAs present in the region surrounding Ori L are synthesized from precursors which are subsequently cleaved to give the mature products. The identification of the ND2 mRNA plus Trp-tRNA precursor indicates that tRNAs are not immediately processed and that cleavage at the 5′ and 3′ ends may not be contemporary. This finding suggests that the mechanism of RNA processing in mitochondria proceeds step-wise producing several precursors of the mature forms. Very likely, these precursors have not been detected previously owing to both their lower abundance and their lack of polyadenylation. The 5′ and 3′ ends of the mRNA were mapped by Attardi's group [14,15] by using discrete poly(A) RNAs. In our case the use of total mtRNA and riboprobes with high specific activity overlapping two genes, allows the characterizations of the precursors.

The presence of the precursor ND2 mRNA plus Trp-tRNA raises the problem of its translation since it could produce a longer protein. In animal mitochondria, mRNAs usually end immediately before a tRNA and sometimes with an incomplete termination codon; the processing of the transcript upstream of the tRNA and its poly(A) addition create the termination codon TAA. However, in this case the ND2 mRNA terminates with one T and the Trp-tRNA starts with AG, thus creating a complete stop codon TAG.

It has been reported that RNA priming can occur within the T rich loop on the template strand and the role of poly(A) in serving a priming function has been suggested. The structural organization of the Ori L containing region renders the template DNA highly prone to elongation in vitro by DNA polymerase as a result of self priming in a reaction mixture that did not contain ribonucleotide triphosphates [5]. However the RNA primers for the initiation of the L strand replication have never been identified in vivo, although it has been shown that the nascent L-strand of mouse mtDNA contains alkali-labile sites due to the retention of ribonucleotides which may reflect inefficient removal of the RNA primer. The small primers have not been identified by our method because very likely they are immediately degraded.

In recent papers we analyzed the D-loop in detail in several mammals [16] and demonstrated that in rat this region is completely and symmetrically transcribed [7]. In particular we detected H and L transcripts which encompass the whole D-loop and abundant shorter H RNA species terminating downstream of the 3′ end of the last coded gene (Thr-tRNA), and also L RNA species actively processed at the level of both the CSBs (very likely representing RNA primers for the H strand replication) and of the D-loop 3′ end where the newly synthesized H strand terminates in resting molecules.

In this paper we show the presence of complementary transcripts in the other small regulatory region of mtDNA which contains the origin of replication of the L strand.

The simultaneous presence of H and L transcripts in

Table I

Relative concentration (pg hybrid/μg RNA) of the H and L transcripts detected in different regions of the rat mt genome

	Clone 4 cluster Ori L	Clone 5 ND1	Clone 1 ND2	Clone 3 COI
H	3.92± 0.36	50.5±4.12	107.5±5.07	182.5±0.14
L	398.75±54.47	–	–	–

the D-loop and in the region surrounding the Ori L is rather striking. It shows that a different turnover for the information-free transcripts of both H and L strands takes places in the coding and in the non-coding region of mtDNA and gives support to the hypothesis that antisense RNAs may play a regulatory role in the replication and expression of mtDNA.

It would be extremely interesting to investigate the molecular bases of these properties. To this end two approaches may be adopted. It should be determined whether the concentrations of antisense RNAs vary under different physiological conditions and in different tissues, and the synthesis and processing of mtRNA should be studied in vitro using isolated organelles or soluble systems. Both approaches are under investigation in our laboratory.

Recently, Clayton's group has suggested that the molecular defect associated with the MELAS subgroup of mt encephalomyopathies may be the inability to produce the correct type and quantity of rRNA relative to other mitochondrial gene products. This myopathy is in fact associated with a mutation in the gene for Leu-tRNA which is embedded in the middle of a tridecamer sequence necessary for the formation of the 3′ ends of 16S rRNA. This mutation causes severe impairment of rRNA transcription termination which correlates with a reduced affinity of the termination factor for the MELAS template [17]. Such experiments which demonstrate a link between a human disease and a molecular regulatory feature of mtDNA suggest that the level of the transcripts may play an important role for a correct functioning of the mitochondria within the eukaryotic cell.

It is clear that many peculiarities of the mt transcription mechanism still remain to be elucidated. The study of the organelle genome in both lower and higher eukaryotes has already revealed a number of unexpected features, but we should probably be prepared for further surprises.

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