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Transcription of rat mitochondrial NADH-dehydrogenase subunits Presence of antisense and precursor RNA species

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bstract We have characterized the transcriptional pattern of the rat mitochondrial ND6-containing region in vivo. We have identified a stable olyadenylated RNA species complementary for the full length of the ND6 mRNA. The analysis of the ND5 region has revealed the presence of antisense RNA only at its 3′ end. The presence of these stable antisense species complementary to structural genes is intriguing and suggests a ossible regulatory function. The quantitative analyses have demonstrated that the H transcripts, both codogenic and non-codogenic, are more stable an the L transcripts.We have defined the 5′ end of the ND6 mRNA at the level of the ATG downstream of the $tRNA^{Glu}$. The mapping of the ND1 end has demonstrated that GTG is the first codon of the mRNA. Our findings suggest that the post-transcriptional mechanisms involved in the pression of the mt genome are much more numerous and complex than those already described in the literature.

ey words: Mitochondria; Mitochondrial genome; Transcription; Antisense RNA; Regulation

Introduction

The main peculiar feature of metazoan mitochondrial (mt) NA is its extremely compact genome organization; the genes ck introns and flanking non-coding regions. This feature is flected in its mode of expression. The two strands, heavy (H) d light (L), are symmetrically transcribed in a polycistronic ay, starting from the two promoters, which are located in the ain regulatory region (D-loop) upstream the replication ori- n of the H strand (for review see [1–3]). According to the odel accepted so far, the polycistronic transcripts are proc- ssed at the level of the tRNAs which, scattered along the olecule, make up a sort of punctuation. The L strand, which for the major part non-codogenic, is transcribed two or three mes more frequently than the H strand. However, the major- y of the L strand transcripts has a half-life which is much orter than that of the H strand and do not accumulate in itochondria to any significant level [4]. This model of expres- on appears reasonable but the details of processing and regu- tion remain to be established. Apparently, the extremely com- act organization of the mt genome does not leave much space the non-coding regions containing regulatory signals. The vestigation of these signals, which are very likely superim- osed to the coding regions, has focused our interest. In the ourse of our recent studies on the mammalian mitochondrial enome and its expression, we have discovered new aspects and eatures of mt transcription. We have identified RNAs, which re precursors of the mature species, in the Ori L region in rat nd in the ATPase region in human, demonstrating that the rocessing of the polycistronic transcripts occurs by steps [5,6]. hese data have led us to consider the processing mechanism s a further possible regulatory element in the expression of the t genome. In addition to the precursor RNAs, we have identified stable antisense RNA species in the two regulatory regions, D-loop and Ori L, and we have suggested their possible regulatory role [7,5].

In the light of these new findings, we have believed it resonable to investigate in more details the transcription mechanisms proposed by the punctuation model and we have analysed, through more strict experimental approaches, several key genome regions some of which already described by other authors [8].

In this paper, we have focused our attention on the in vivo studies of mRNAs of the mt NADH-dehydrogenase (ND) complex (complex I) in rat. All vertebrate mt DNAs sequenced so far (rat, mouse, cow, whale, seal, human, *Xenopus*, chicken) contain seven genes coding for the ND subunits: ND1, 2, 3, 4L, 4, 5, 6. These genes are non-contiguous and interspersed along the genome. The order of the genes is the same in all organisms with the exception of chicken where a gene rearrangement has occured [9]. In all cases ND4L and ND4 subunits are translated starting from a common mRNA in two out-of-phase frames overlapping for 7 bases. The ND6 subunit is coded for by the L strand in all vertebrates. The 3′ ends of ND6 and ND5 on the two strands are adjacent (man) or overlapped (with the exception of chicken).

In particular, we have addressed our interest to the detailed fine mapping of the ND6 containing region where, in HeLa cells, about 2,000 bp downstream the L promoter, the 5′ ends of the three long L transcripts (called 1, 2, 3) have been mapped [10].

2. Materials and methods

2.1. Preparation of mitochondrial RNA

RNA has been purified from the liver of male albino *Rattus Norvegicus* of the Wistar strain [11]. Poly(A)$^+$ mtRNA has been purified by using Dynabeads (Dynal A.S. Oslo, Norway) according to the methods indicated by the suppliers. The RNA has been treated with RNase-free DNase before hybridization. Any residual contaminating DNA can be excluded as riboprobes 3(L), 4L(L), 4(L), 5(L) do not reveal hybrids (Fig. 5).

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2.2. Cloning, transcription and sequencing of ND subunits

Subfragments of all ND subunits have been cloned in Bluescribe vector (Stratagene San Diego, CA); the nucleotide (nt) positions are those relative to the rat sequence [12]:
fragment 3 (ND3) *NdeI–HinfI* = 128 bp, nt 9,544–9,669;
fragment 4L (ND4L) *RsaI–RsaI* = 214 bp, nt 9,909–10,123;
fragment 4 (ND4) *HinfI*–EcoRI = 378 bp, nt 10,776–11,151;
fragment 5 (ND5) *AluI–AluI* = 153 bp, nt 12,274–12,426;
fragment 5(3′) (ND5) *DraI–AvaI* = 360 bp, nt 13,178–13,533;
fragment 6 (ND6) *AvaI–AccI* = 308 bp, nt 13,534–13,839;
fragment 7(ND6 207 bp, $tRNA^{Glu}$ 69 bp, Cyt *b* 317 bp) *AccI–BamHI* = 598 bp, nt 13,840–14,433;
fragment 8 (ND5 379 bp, ND6 314 bp) *DraI–AccI* = 664 bp, nt 13,178–13,839.

Differences of few bases in the length of the fragments 3, 4, 6, 7, 8 are due to fill-in before cloning.

To obtain the transcripts of both strands of the fragments, we have linearized the plasmid upstream or downstream of 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 transcripts has been checked by sequencing the DNA templates with the dideoxynucleotide chain-terminator Sanger method adapted to double strand templates [13].

The labelled riboprobes have been hybridized with total rat liver mtRNA, RNase-free DNase treated, in qualitative and quantitative experiments.

2.3. Northern blot analysis of mtRNA

mtRNA (10 μg), RNase-free DNase treated, has been denatured and fractionated on a 1.2% agarose-formaldehyde slab gel. mtRNA has been transferred onto nitrocellulose and hybridized in 50% formamide, 1 × Denhardt's, 5 × SSPE, 0,1% SDS, 50 μg/ml tRNA for 20 h at 42°C with riboprobes complementary to H or L transcripts (2×10^8 dpm/μg). The washes have been carried out at 65°C with 2 × SSPE, 0.1% SDS (30 min twice) followed by two washes with 0.2 × SSPE, 0.1% SDS (30 min).

2.4. RNase mapping of H and L transcripts

Total mtRNA (30 μg), RNase-free DNase treated, has been hybridized with different riboprobes (2×10^8 dpm/μg) as described [5,7].

2.5. Reverse transcriptase experiments

Experiments have been performed using synthetic 20-mer primers, whose nucleotide positions reported in the results, are relative to the rat sequence [12]. The experimental conditions have been carried out as described [7].

2.6. Quantitative analyses of the transcripts

Hybridizations have been 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 results were controlled by repeating the hybridization 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 experimental conditions have been carried out as described [7]. The concentrations of all the transcripts at saturation are shown in Table 1.

3. Results

3.1. Characterization of rat ND mRNA subunits

To characterize the transcripts of the region under investigation we have used probes containing ND5, ND6, $tRNA^{Glu}$ and Cyt *b* genes (riboprobes 6, 7 and 8). The riboprobes used to identify the transcripts of the heavy and light strands are defined H and L, respectively.

The Northern blot experiment performed with riboprobes 7 and 8 is shown in Fig. 1. The riboprobe 7(H) identifies the mature Cyt *b* mRNA (1,143 bp) and two precursor RNA species (2,400 bp and 3,500 bp, respectively) which correspond to processing intermediates of the polycistronic transcript. Since

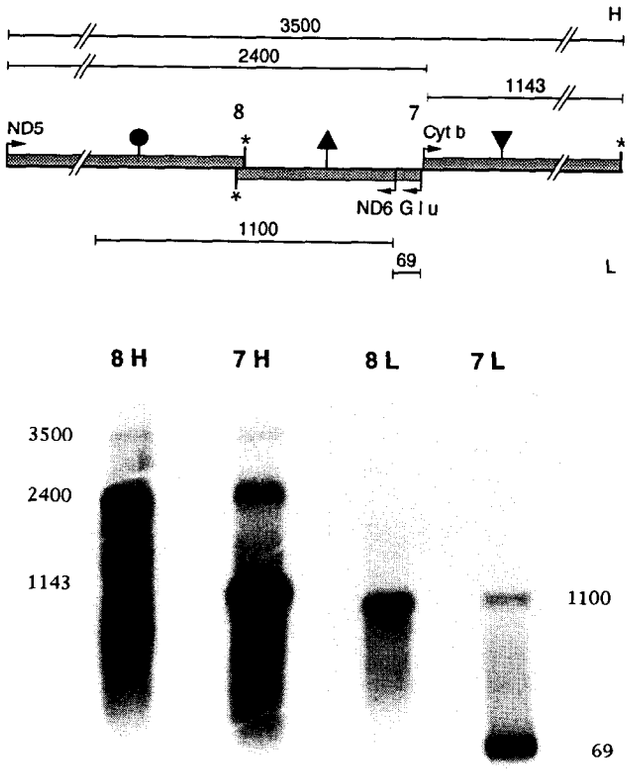


Fig. 1. Northern blot analysis of the H and L transcripts of the region containing ND5, ND6, $tRNA^{Glu}$, Cyt *b*. Gene organization and restriction map are reported above the autoradiogram. The arrows indicate the direction of the transcription of each gene; *stop codon. Clone 8 (*DraI–AccI* fragment) = ND5, ND6; clone 7 (*AccI–BamHI* fragment) = ND6, $tRNA^{Glu}$, Cyt *b*. The RNA species identified by the respective riboprobes are indicated on the map. H = heavy transcripts; L = light transcripts. ● = *DraI*, ▲ = *AccI*, ▼ = *BamHI*

these precursors are present also in the hybridization performed with riboprobe 8(H), we presume that the 2,400 bp RNA species corresponds to the ND5 coding region (1,836 bp) and to its trailer of about 600 bp (RNA 5 in HeLa cells [14,15]); the 3500 bp RNA species represents a longer precursor, containing also the Cyt *b*. Both riboprobes 7(L) and 8(L) identify a transcript 1,100 bp long, corresponding to the ND6 coding region (519 bp) and to its trailer (about 600 bp) (see below RNase mapping and reverse transcriptase experiments) demonstrating the presence of the ND6 mRNA in vivo. The riboprobe 7(L) identifies also the $tRNA^{Glu}$ (69 bp). Even with longer exposures no other RNA species is dectable, as shown in the previous report [8].

In the attempt to characterize these transcripts, we have used different experimental approaches. RNAse protection experiments, performed with riboprobes 6(L) and 7(L) show the presence of the ND6 mRNA. In particular, the riboprobe 7(L) identifies two transcripts (Fig. 2 lane 7L) which have been interpreted as ND6 5′ end (207 bp) and $tRNA^{Glu}$ (69 bp). No transcripts complementary to the Cyt *b* mRNA are visible. Several bands have been detected in the 69 bp region. The largest band corresponds to the processed $tRNA^{Glu}$, whereas the smaller bands are most likely due to the incomplete unfolding or refolding of the tRNA during RNase protection [16]. The

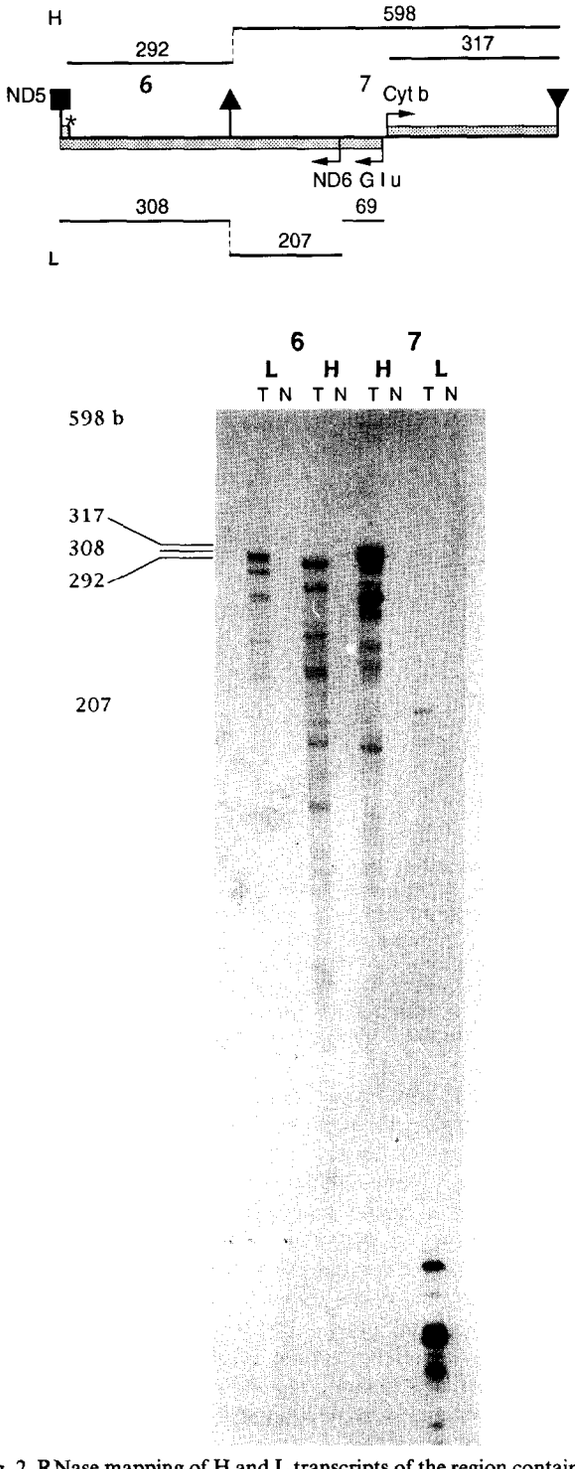


Fig. 2. RNase mapping of H and L transcripts of the region containing the ND6, $tRNA^{Glu}$ and Cyt *b*. Gene organization and restriction map are reported above the autoradiogram. Clone 6 (*Ava*I–*Acc*I fragment) = ND6 (308 bp); clone 7 (*Acc*I–*Bam*HI fragment) = ND6 (207 bp), $tRNA^{Glu}$ (69 bp), Cyt *b* (317 bp). The arrows indicate the direction of the transcription of each gene; *stop codon. The RNA species identified by the respective riboprobes are indicated on the map. ■ = *Ava*I, ▲ = *Acc*I, ▼ = *Bam*HI. T = hybridization performed with total mtRNA; N = RNase treated probe as negative control; H = heavy transcript; L = light transcript.

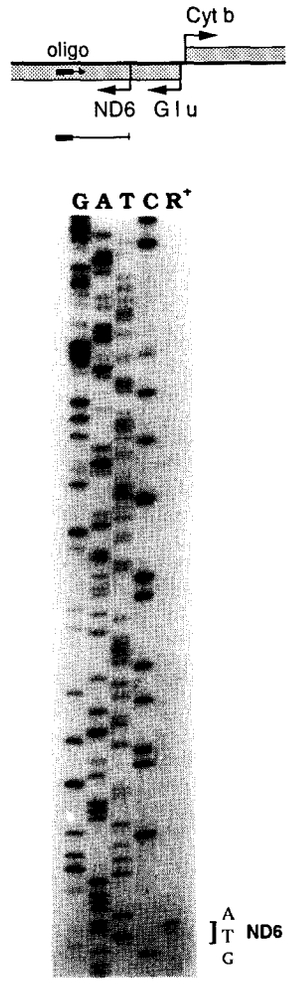


Fig. 3. ND6 mRNA 5′ end mapping. The oligo used for the reverse transcriptase and the elongation product are indicated on the analytical scheme. GATC = sequence reaction of the corresponding mtDNA region. R^+ = reverse transcriptase reaction performed with poly(A)$^+$ mtRNA.

riboprobe 6(L), containing the 3′ end of the gene, is completely protected (308 bp), demonstrating the presence of the full-length ND6 mRNA (Fig. 2, lane 6L).

The ND6 5′ end has been mapped precisely by primer extension experiment using poly(A)$^+$ mtRNA and the oligo complementary to nucleotides 13,964–13,983 of the rat sequence [12]. The elongation product corresponds to the ND6 ATG initiation codon downstream the $tRNA^{Glu}$(Fig. 3). No RNA species extending upstream the ND6 5′ end is detectable. Therefore the transcript 1,100 bp long, identified by the Northern blot analysis, longer than the ND6 coding region (Fig. 1, lanes 7L and 8L), contains a trailer corresponding to the antisense of ND5 3′ end region. This result is confirmed by RNase mapping experiments performed with the riboprobe 5(3′)(L) containing the 3′ end of the mRNA (data not shown). Instead, the RNase mapping performed with riboprobe 5(L), corresponding to the ND5 central domain, demonstrates the absence of an antisense RNA (see below Fig. 5, lane 5L).

The RNAse mapping experiments with riboprobes 6(H) and 7(H) give clear signals, demonstrating the presence of an ND6 mRNA antisense species (Fig. 2, lanes 6H and 7H). In particu-

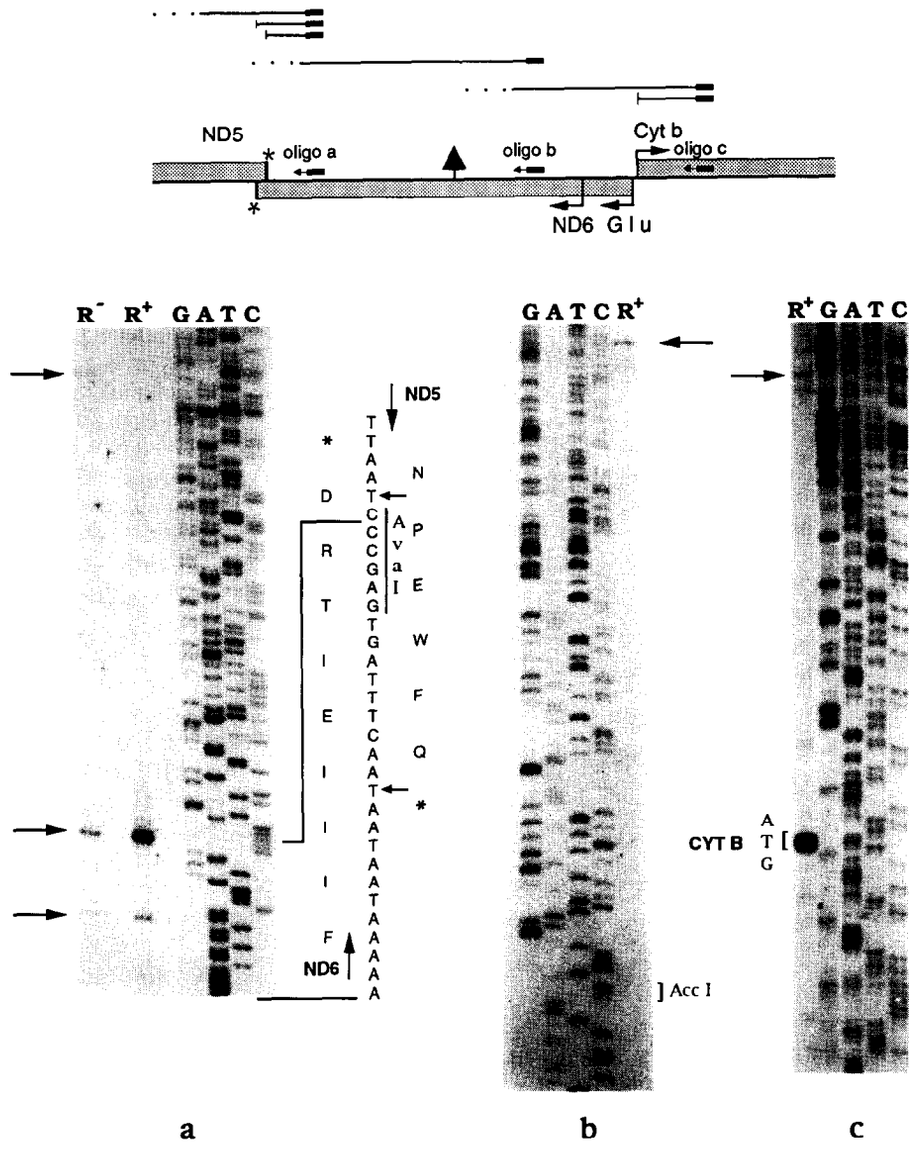
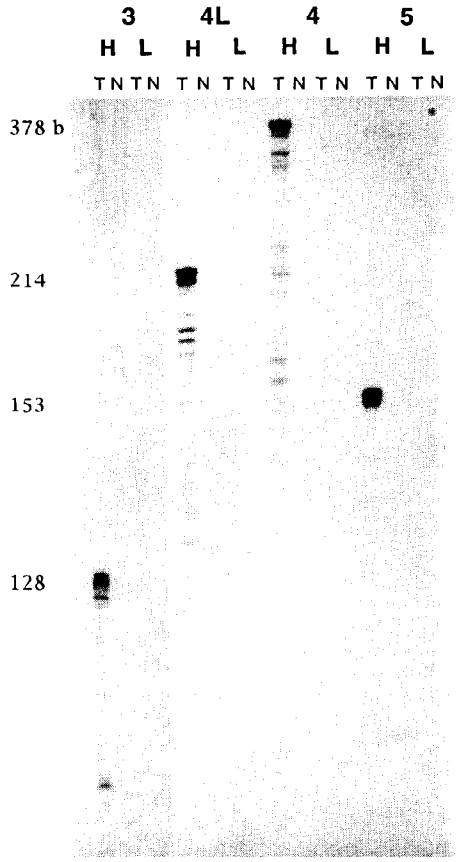


Fig. 4. Antisense ND6 mRNA mapping. Gene organization and restriction map of the region containing the ND5, ND6, $tRNA^{Glu}$ and Cyt *b* genes. The arrows indicate the direction of the transcription of each gene; *stop codon. The oligos used for the reverse trascriptase experiments and the elongation products are indicated above the scheme. ▲ = *Acc*I site. GATC = sequence reactions of the corresponding mtDNA regions. R^+ = reverse transcriptase reaction performed with $poly(A)^+$ mtRNA. R^- = reverse transcriptase reaction performed with $poly(A)^-$ mtRNA. (a) Reaction primed by oligo a. The arrows on the left indicate the primer extension products which goes into the ND5 coding region (top of the autoradiogram) and two major antisense ND6 5′ ends (bottom). The primary sequence and the reading frames relative to the rat ND5-ND6 overlapping region are reported. The *Aval* cloning site is underlined; the sequence reactions upstream the *Aval* site correspond to the Bluescribe vector. The smaller arrows on the right indicate the two major antisense ND6 5′ends on the sequence. (b) Reaction primed by oligo b. The arrow indicates the extension product which outstretches the *Acc*I site. The sequence reactions upstream the *Acc*I site correspond to the Bluescribe vector. (c) Reaction primed by oligo c. The Cyt *b* ATG initiation codon is indicated. The arrow indicates the precursor RNA species.

lar, the riboprobe 6(H) protects a transcript 292 bp long corresponding to the 5′ end of the ND6 antisense RNA (Fig. 2, lane 6H). The riboprobe 7(H) identifies the Cyt *b* mRNA (317 bp) and a precursor RNA species (598 bp corresponding to the Cyt *b* 5′ end and to the antisense of the $tRNA^{Glu}$ and of the ND6 3′ end) (Fig. 2, lane 7H). This precursor RNA species is barely visible but its presence is confirmed by the Northern blot (Fig. 1, lanes 7H and 8H) and the reverse transcriptases primed by oligos a, b and c. No transcripts complementary to the mature tRNA are detected.

Reverse transcriptase experiment primed by oligo c (14,225–14,206) allows us to map the Cyt *b* initiation codon (position 14,121) (Fig. 4c). The bands marked with an arrow can be attributed to the limit of elongation of the reverse transcriptase enzyme [17]. They cannot be termination points since they are not visible in all the experiments (data not shown). Yet, these bands demonstrate there is an RNA precursor which goes beyond the ATG of Cyt *b*. The existence of an RNA precursor which includes the full-length ND6 antisense transcript is anyway described above by Northern blot and RNase mapping experiments (Fig. 1, lanes 7H, 8H; Fig. 2, lane 7H), below by reverse transcriptase experiments (Fig. 4a). Reverse transcrip-



ig. 5. RNase mapping of H and L transcripts of the 3, 4L, 4, 5 ND ubunits. riboprobe 3 = ND3, riboprobe 4L = ND4L, riboprobe = ND4, riboprobe 5 = ND5. T = hybridization performed with total ıtRNA; N = RNase treated probe as negative control; H = heavy ranscript, L = light transcript.

ase primed by oligo b (13983–13964) proceeds through the *Acc*I site confirming that the antisense of the ND6 spans along ıll the ND6 region (Fig. 4b).

Primer extension analyses, performed by using poly(A)$^+$ and ɔoly(A)$^-$ mtRNA preparations and oligo a (13632–13612) (Fig. la), reveal a long transcript which goes into the ND5 coding egion (see Fig. 1 lanes 7H, 8H). Moreover, the reverse tran-criptase reveals two additional extension products not identi-ied with Northern blot (the experiment was carried out at least n triplicate using several batches of RNA and the results were :onfirmed in all cases). In particular, the shorter product maps mmediately downstream the ND5 stop codon, whereas the ɔther product, 16 bp longer, maps inside the ND5 gene coincid-ng with the ND6 3′ end. This result demonstrates that the ND6 ıntisense RNA (ND5 trailer) is processed at the junction be-:ween ND5 and ND6.

To verify if other antisense RNA species are present in the :egions containing the ND genes, we have used subfragment probes, corresponding to the central domain of the ND3, ND4L, ND4, and ND5 in RNAse protection experiments as shown in Fig. 5. The H riboprobes are completely protected by total mtRNA and identify the ND mRNAs (Fig. 5, lanes 3(H), 4L(H), 4(H), 5(H); the results relative to the ND1 and ND2 are in Sbisà et al. [5]). Whereas, the L riboprobes indicate that there are no detectable full-length transcripts complementary to the central domains of ND3, ND4, ND4L and ND5 mRNAs, (Fig. 5, lanes 3(L), 4L(L), 4(L), 5(L)).

3.2. *In vivo dosage of the ND subunit mRNAs*

The presence of stable antisense RNA species have prompted us to determine their steady state concentration and to compare these with the concentration of the structural ND mRNAs. We used all the riboprobes of both strands corresponding to the central domain of the ND coding regions, as in RNase mapping experiments (Fig. 5). In particular, for ND5 we used two different riboprobes: namely riboprobe 5 which corresponds to the central domain, and riboprobe 5(3′) corresponding to the 3′ end of the gene. The relative concentrations of the ND subunits and antisense RNAs are shown in Table 1. The immediate major observation is that the concentrations of the ND mRNAs are different. The ND6 mRNA is less abundant with respect to the other ND subunits, but the concentration of its antisense is three times higher and indeed its concentration is comparable to those of other H strand coded mRNAs. The ND5 mRNA has an antisense only in its 3′ end region (about 600 bp) which is less abundant than its mRNA and has a concentration similar to the ND6 mRNA. The level of ND4L and ND4 are comparable as expected since they belong to the same messenger. ND1, ND2 and ND3 are present at the highest concentrations, whereas ND6 is the least abundant species [18].

3.3. *Mapping of the ND1 mRNA*

We have proposed GTG rather than ATT as the initiation codon in rat and mouse based on the sequence comparison of the ND1 5′ ends [19]. In order to test experimentally our proposal, we have performed reverse transcriptase experiments. As shown in Fig. 6 a single strong product terminating at the GTG codon was obtained with the oligo complementary to nucleotides 2,780–2,799. Thus, our mapping experiment has demonstrated that the mRNA 5′ end is immediately contiguous to the $tRNA^{Leu(UUR)}$ and that GTG could indeed be the initiation codon.

4. Discussion

In this paper, we have performed a detailed analysis of the in vivo transcripts of rat ND subunits. The ND6 containing region has revealed interesting results which, though far from conclusive, offer new hints for further experimental approaches. We have found the remarkable presence of a polyadenylated antisense RNA for the full length of the ND6 mRNA. This species is distinct from the RNA 5 reported previously [14,

Table 1
Concentration (pmol/μg RNA × 10^{-6}) of the H and L ND mRNAs

	ND1	ND2	ND3	ND4L	ND4	ND5	ND5-3′	ND6
H	463.6 ± 37.8	626.4 ± 29.5	574.3 ± 15.2	332.7 ± 22.9	370.3 ± 13.6	310.9 ± 53.8	294.6 ± 23.9	339.8 ± 31.3
L	–	–	–	–	–	–	145.08 ± 4.0	132.9 ± 16.7

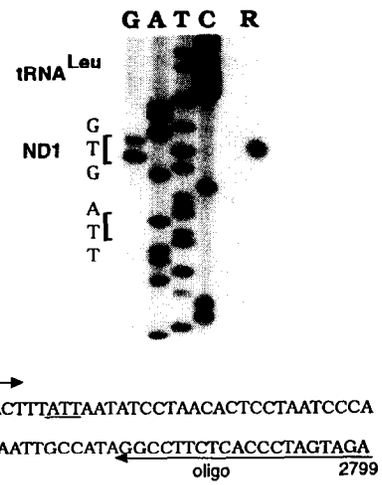


Fig. 6. ND1 mRNA 5′ end mapping. GATC = sequence reactions of the corresponding mtDNA region. R = reverse transcriptase reaction performed with total mtRNA. The nucleotide sequence of the region containing the 5′ end of ND1 gene and the oligo used for both the reverse transcriptase and sequencing experiments are reported (the nucleotide position is that relative to the rat sequence). The GTG and ATT codons are underlined.

15], because it is processed at the level of the junction between ND5 and ND6 genes. Interestingly, its 5′ end is heterogeneous, showing two major initiation sites located in a tract containing TAAT repeats, the longest inside the ND5 mRNA (Fig. 4a).

We have also confirmed the presence of the ND6 mRNA trailer (about 600 bp), that is the antisense of ND5 mRNA at the 3′ end (Fig. 1, lanes 7L, 8L) [8]. This mRNA lacks a stable antisense in its central domain (Fig. 5, lane 5L). The quantitative analyses allowed us to compare the concentrations of the antisense RNAs with the ND mRNAs (Table 1). The results demonstrate that H transcripts, both codogenic and non-codogenic, are more stable than L transcripts, and that the mRNAs of subunits belonging to the same complex have different concentrations in the cell, thereby suggesting different turnover rates.

In our previous work we have analyzed in rat the two regulatory regions (D-loop, Ori L), and several structural genes (CoI, 12S rRNA, ND1 and ND2 subunits) [5,7]. We have identified antisense RNAs only in the two regulatory regions and in the ND6 containing region. Although the transcription analysis of the remaining genes of mtDNA is still to be performed, the presence of such antisense species only in some tracts is intriguing as non-coding RNAs (deriving from the polycistronic transcripts) complementary to structural genes are rapidly degraded [4]. The presence of such antisense species indicates also the existence of a selective post-transcriptional mechanisms regulating the stability and degradation of non-codogenic RNAs in the two strands. The possible physiological role of such antisense species could be inferred by analogy with other systems [20]; the interaction of sense and antisense RNAs might play a regulatory role in mtDNA replication and gene expression.

The fine mapping of the messanger RNAs for ND6 and ND1 reported here is another new finding since both transcripts have never been characterized in detail previously.

By reverse transcriptase experiment (Fig. 3) we demonstrate that the 5′ end of the ND6 mRNA coincides with the ATG codon downstream $tRNA^{Glu}$ (position 14,046).

The mapping in vivo of ND1 mRNA is important for the particular involvement of this gene in the minor histocompatibility antigen (Mta) in mice. Fisher Lindahl has reported that an in vitro translation product of the ND1 gene which starts from GTG is active in the maternally transmitted component (MTF) of Mta [21]. In mouse, it has been proposed, only on the basis of the sequence analysis, that the ND1 gene starts with ATT, 9 bp downstream the GTG [22]. However, the alignment of the 5′ region of vertebrate ND1 genes reveals that if ATT is the initiation codon in rodents, the ND1 mRNA would be three amino acids shorter than in other mammalian species. The length is, however, normalized (318 aa) if the reading frame starts immediately downstream the $tRNA^{Leu(UUR)}$ gene at the level of GTG. On the basis of such theoretical observations, we suggested that this non-canonical codon might act as initiator in the rat mt genome similarly to other mt genes like *D. yakuba* ND5 [23], sunflower Cyt *b* (Gallerani R., personal communication), sea urchin ATPase 8 [24], blue whale ND4L [25] and to some prokaryotic genes [26]. The results reported here demonstrate that there is only one mRNA for ND1 starting from GTG (Fig. 6).

Finally, we report here the presence of another RNA precursor species containing the Cyt *b* and the antisense of the $tRNA^{Glu}$ and of the ND6 (Fig. 1, lanes 7H, 8H; Fig. 2, lane 7H; Fig. 4c). As in the case of precursor RNAs reported by us in human [6], termination codons present upstream the initiation codon prevent the translation of a product longer than the mature mRNA. The finding of this precursor confirms our previous hypothesis that the processing proceeds stepwise [5,6]. Precursor products are likely to have functional consequences on the expression of the mammalian mitochondrial genome and to play a role in development and disease [27].

In conclusion, all these findings suggest that the mammalian mt genome expression is subject to much more complex mechanisms than those previously reported and stimulate further investigation on the post-transcriptional events.

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