Expression of the Cytochrome *b*–URF6–URF5 Region of the Mouse Mitochondrial Genome†

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ABSTRACT: The nature of RNA coded by the only light-strand (L-strand) open-reading frame unidentified reading frame 6 (URF6) was studied by using a variety of single- and double-strand DNA subclones derived from the 3.6-kilobase (kb) cytochrome *b* (cyt *b*)–URF5 coding region of the mouse mitochondrial genome. Northern blot experiments using single-strand-specific M13 clones indicate that both the heavy (H) and L strands of this genomic region are symmetrically transcribed and processed into poly(adenylic acid) [poly(A)] RNAs of comparable size. The 1.2- and 2.4-kb RNAs coded by the H strand, putative mRNAs for cyt *b* and URF5 reading frames, respectively, are derived from a common precursor of 3.6-kb RNA. The L-strand-coded 1.15-kb RNA, on the other hand, is derived from a short-lived precursor of 3.6-kb RNA by a multiple-step processing involving a 2.4-kb intermediate RNA. The S_1 nuclease protection experiments using both the 3′- or 5′-end-labeled DNA probes and also affinity-purified ^{32}P-labeled RNA probes indicate that the 1.15-kb RNA maps between the start of the URF6 reading frame (3′ end) and a region 590–600 nucleotides to the 5′ end of this reading frame. The 1.15-kb RNA thus contains the entire URF6 coding sequence and an about 590-nucleotide-long 3′ untranslated region. The molar abundance of the three mRNAs in the steady-state mitochondrial RNA varies markedly. The 1.15-kb URF6 mRNA is only one-tenth the level of 1.2-kb cyt *b* mRNA, although it is nearly as abundant as the 2.4-kb URF5 mRNA. The in vitro pulse–chase experiments using isolated mitoplasts show that the 1.15-kb URF6 mRNA is transcribed at 1.5–1.7 times faster rates than the 1.2-kb cyt *b* mRNA. The kinetics of hybridization to the H- and L-strand-specific M13 DNA probes show that the RNA coded by the URF6 region decays with a $t_{1/2}$ of 16–18 min while those coded by the cyt *b* gene turn over with a longer $t_{1/2}$ of 58–60 min. These rates are consistent with the rates of decay of ^{32}P label from the 1.15- and 1.2-kb poly(A) RNA during the pulse–chase of isolated mitochondria. Such differential turnover rates may account for the observed differences in the relative abundance of these mitochondrial mRNAs.

Recent DNA sequence analyses have established that the heavy strand (H strand)[1] of the mitochondrial genome of human, bovine, and mouse cells is virtually packed with information for 14 tRNAs, 2 rRNAs, and 12 potential mRNA species (Anderson et al., 1981, 1982; Bibb et al., 1981). The L strand of mitochondrial DNA in these cells, however, contains information for only one possible mRNA in addition to eight tRNAs (Anderson et al., 1981, 1982; Bibb et al., 1981). Although there are subtle structural and organizational variations in the mitochondrial genomes from different animal cells (Parker & Watson, 1977; Grosskopf & Feldman, 1981; Clary et al., 1982), there seems to be a remarkable conservation of mitochondrial genetic information within the animal kingdom (Anderson et al., 1981, 1982; Bibb et al., 1981; Parker & Watson, 1977; Grosskopf & Feldman, 1981; Clary et al., 1982; Dawid et al., 1976; Rastle & Dawid, 1979; Saccone et al., 1980). Physical mapping of mitochondrial RNA by S_1 nuclease protection (Battey & Clayton, 1978, 1980; Van Etten et al., 1982; Ojala et al., 1980) and nucleotide sequencing of RNA termini (Montoya et al., 1981; Ojala et al., 1981; Attardi et al., 1982; Dubin et al., 1982) have shown that all of the 12 reading frames on the H strand of both mouse and HeLa cell mitochondrial DNA are expressed in the form of discrete poly(A) RNAs having the properties of mitochondrial mRNAs. A recent study by Oliver et al. (1983) showed that HeLa cell mitochondria synthesize a 15-kDa polypeptide species having the properties of the URF6 gene product. Nevertheless, the putative mRNA coded by the only L-strand open-reading frame URF6 in both mouse and HeLa cells has escaped detection (Van Etten et al., 1982; Attardi et al., 1982; Bhat et al., 1984).

The URF5–cyt *b* coding region of the mouse mitochondrial genome is unique in that both the H and L strands of the 3.6-kb region contain potential mRNA reading frames. The URF5 and cyt *b* reading frames are localized on the H strand and are separated by a 573-nucleotide stretch which appears to be the only H-strand region besides the D-loop area not coding for defined structural information [see Bibb et al. (1981)]. The opposite strand of the 573-nucleotide region contains an open-reading frame designated as URF6 (Anderson et al., 1981, 1982; Bibb et al., 1981). In this study, we have determined the nature of steady-state and newly synthesized RNA coded by the URF5–cyt *b* region as well as the URF6 region of the mouse mitochondrial genome by using Northern blot analysis and S_1 nuclease protection. The URF6 reading frame of the mouse mitochondrial genome is

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[1] Abbreviations: URF, unidentified reading frame; cyt *b*, cytochrome *b*; cyt ox, cytochrome oxidase; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; H-strand DNA, heavy-strand DNA; L-strand DNA, light-strand DNA; kb, kilobase; kDa, kilodalton(s); EDTA, disodium ethylenediaminetetraacetate; poly(A), poly(adenylic acid); bp, base pair(s); Cl_3C-COOH, trichloroacetic acid.

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expressed as a presumptive mRNA of 1.15 kb which is derived from a large precursor of about 3.6-kb RNA.

Materials and Methods

Materials. Guanidinium thiocyanate, formamide, and formaldehyde were purchased from Fluka Chemical Corp. Restriction endonucleases, the nick translation kit, Klenow fragment of DNA polymerase I, and epoxy-activated cellulose were purchased from Bethesda Research Laboratories. AMV reverse transcriptase was obtained from Life Sciences, Inc. Poly(U)–Sepharose was obtained from Pharmacia. The M13mp8 and M13mp9 phage vectors, *Escherichia coli* K12 recipient strain, and T_4 DNA ligase were purchased from New England Biological Laboratories. [^{32}P]UTP (>600 Ci/mmol), [^{32}P]CTP (<600 Ci/mmol), [^{32}P]dCTP (>3000 Ci/mmol), and S_1 nuclease were purchased from New England Nuclear. Nitrocellulose membranes for RNA blot transfer were purchased from Schleicher & Schuell. Other chemicals and biochemicals were purchased either from Pierce Chemical Co. or from Sigma Chemical Co.

Cell System. Ehrlich ascites cells, grown in the peritoneal cavity of Swiss mice (Bhat et al., 1982), were used as the source of mitochondria.

Isolation of Mitochondria. Mitochondria were isolated from 7-day-old Ehrlich ascites cells by a differential centrifugation method using the sucrose–mannitol buffer (4 mM Hepes, pH 7.4, 7.0 mM sucrose, 220 mM mannitol, 2 mM EDTA, and 0.5 mg/mL BSA) as described before (Bhat et al., 1982; Kantharaj et al., 1983). Crude mitochondria were washed twice with mitochondrial isolation buffer containing 10 mM EDTA (Kantharaj et al., 1983) and treated with digitonin (70 μg/mg of protein) as described before (Bhat et al., 1982).

In Vitro Labeling and Isolation of Mitochondrial RNA. Mitochondrial RNA was pulse-labeled by using the in vitro mitoplast system described before (Kantharaj et al., 1983). Freshly isolated mitoplasts were incubated in the RNA synthesis buffer (Bhat et al., 1984; Kantharaj et al., 1983) at a final concentration of 10 mg of protein/mL and labeled with 200 μCi/μL each of [^{32}P]UTP and [^{32}P]CTP at 35 °C. In the pulse–chase experiments, pulse-labeled mitoplasts were pelleted at 10000*g* for 10 min, resuspended in the RNA synthesis buffer containing unlabeled UTP and CTP (0.5 mM each), and incubated at 35 °C for the required length of time in the presence or absence of 1 nM cordycepin triphosphate. RNA was isolated from the mitoplasts by the guanidinium thiocyanate method (Chirgwin et al., 1979) as described before (Kantharaj et al., 1983). The RNA was freed of contaminating DNA by sedimentation through CsCl (Kantharaj et al., 1983). Poly(A) RNA was isolated by binding to poly(U)–Sepharose (Ricca et al., 1981).

Cloning of Mitochondrial DNA. Mitochondrial DNA clones used in this study were derived from the pAM1 DNA which consists of the entire mouse mitochondrial genome cloned in pACYC 177 plasmid (Martens & Clayton, 1979) provided by David A. Clayton, Stanford University School of Medicine, Stanford, CA. A 3360-bp *Hin*dIII–*Bgl*II fragment from the cyt *b*–URF5 region (nucleotides 11 970–15 330) of mouse mitochondrial DNA (Bibb et al., 1981) was cloned in the *Hin*dIII–*Bam*HI sites of the pKS 451 plasmid vector provided by Dr. David Sherrat, Glasgow University, Glasgow, Scotland, and was designated as pHB 336. A 1945-bp-long *Hin*dIII fragment (nucleotides 9137–11 082) was cloned in the same restriction site of pUR 250 and was designated as pH 195. Single-strand-specific DNA clones were constructed by forced cloning in M13mp8 and M13mp9 phage vectors which contain the cloning sites arranged in opposite orientation (Messing & Vierra, 1982). The 1778-bp *Bgl*II–*Xho*I fragment was inserted through *Bam*HI and *Sal*I restriction sites, respectively, on the vector. The 1582-bp *Xho*I–*Hin*dIII fragment was cloned in the *Sal*I and *Hin*dIII sites, respectively, of the M13 vectors. The selected colonies or plaques were screened for the DNA inserts by electrophoretic analysis on 0.8–1.2% agarose gels, and the positive clones were further tested by hybridization to mitochondrial DNA by the dot hybridization procedure. The strand specificities of the three M13 clones designated as XB811, XB901, and HX905 have been described under Results. The cloning was carried out in accordance with NIH recommended guidelines.

Hybridization of Pulse-Labeled RNA to Cellulose-Linked DNA. The M13 phage DNA containing mitochondrial DNA inserts was covalently linked to epoxy-activated cellulose as described by Moss et al. (1981). RNA samples from ^{32}P-pulse-labeled and pulse-chased mitochondria were hybridized to cellulose-linked DNA as described before (Kantharaj et al., 1983; Moss et al., 1981).

Electrophoresis of RNA. RNA was electrophoresed through 1.6% agarose gels containing 10 mM methylmercuric hydroxide (Baily & Davidson, 1976) or 2.2 M formaldehyde (Lehrach et al., 1977). The gels were stained with ethidium bromide to visualize the RNA bands.

Northern Blot Analysis of RNA. In Northern blot experiments, RNA from the agarose gel was transferred to nitrocellulose by the method of Thomas (1980) and probed with ^{32}P-labeled DNA (Alwine et al., 1977). Specific restriction fragments generated from the pAM1 DNA or the DNA subclones were nick translated with [^{32}P]dCTP using a kit supplied by Bethesda Research Laboratories as described before (Kantharaj et al., 1983). The M13 clones containing single-stranded DNA inserts were labeled with [^{32}P]dCTP (20 μCi/25-μL reaction) by using AMV reverse transcriptase according to the method of Hu & Messing (1982). Under these labeling conditions, only the vector molecule is copied, and the inserted DNA remains in the single-stranded form, providing a powerful strand-specific probe for the detection of mRNAs of even very low abundance.

S_1 Protection Analysis. Single-stranded 5′- or 3′-end-labeled DNA probes were obtained either from a pAM1 DNA digest or from pHB 336 DNA digests. About 20 ng of ^{32}P-end-labeled DNA was hybridized with 15 μg of poly(A) RNA from Ehrlich ascites mitochondria in a buffer system containing 40 mM Hepes (pH 7.4), 1 mM EDTA, 0.5 M NaCl, and 80% formamide essentially as described by Berk & Sharp (1977). Hybridization was carried out in 20-μL volumes for 3 h at 46 °C. In some experiments, ^{32}P-labeled RNAs bound to DNA–cellulose columns (0.5–1.0 μg) were hybridized to 1.5 μg of single-stranded DNA cloned in M13 vectors at 42 °C for 3 h, or to 2.0–2.5 μg of double-stranded DNA at 46.5 °C for 3 h. After hybridization, reaction mixtures were diluted with 200 μL of S_1 nuclease buffer containing 0.25 M NaCl, 30 mM sodium acetate (pH 4.5), 2 mM $ZnSO_4$, and 4 μg of heat-denatured salmon sperm DNA. The nucleic acids were digested with 50–150 Vogt units of S_1 nuclease at 30 °C for 30 min. The reaction was stopped by adding 10 μL of 0.5 M EDTA, and the nucleic acids were precipitated with 3 volumes of ethanol along with 10 μg of carrier tRNA. The S_1-protected fragments were denatured in formamide at 65 °C for 10 min and electrophoresed on polyacrylamide gels containing 7 M urea (Maxam & Gilbert, 1977).

Results

Nature of DNA Probes. The structural organization and the restriction maps of the 3.6-kb region of the mouse mito-

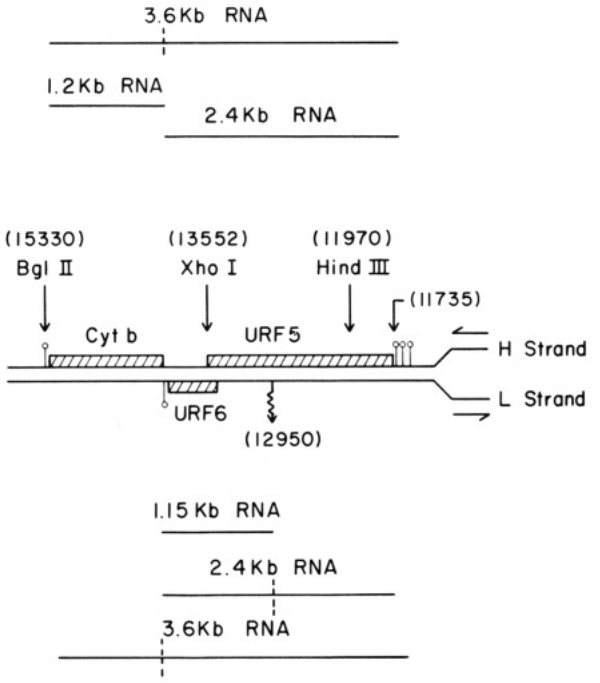

FIGURE 1: Restriction map of the mouse mitochondrial cyt *b*–URF5 coding region. The positions of URF5, URF6, and cyt *b* reading frame and tRNA genes (?) are according to Bibb et al. (1981). The restriction sites for *Hin*dIII, *Xho*I, and *Bgl*II are shown by the arrows. The half-arrows on each side of the H and L strands indicate the direction of transcription of the two strands. Various transcripts and their tentative map positions are shown by solid lines. The dashed lines across the 3.6- and 2.4-kb RNAs indicate the processing sites. The values in parentheses represent the nucleotide residues of the mouse mitochondrial DNA sequence reported by Bibb et al. (1981).

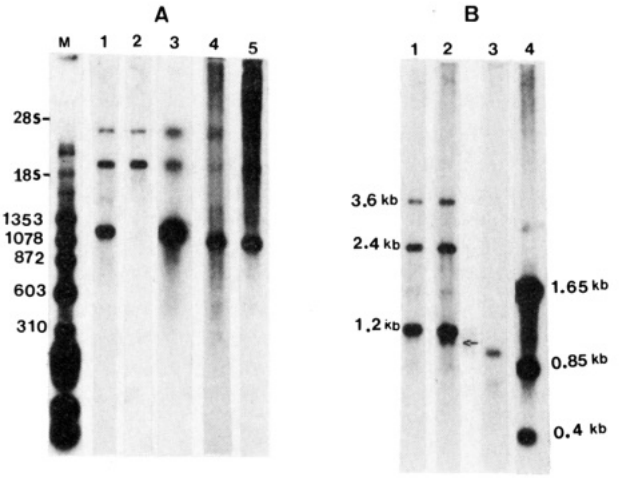

FIGURE 2: Northern blot analysis of mitochondrial RNA. Poly(A) RNA from mouse liver mitochondria (4 μg in each lane) was electrophoresed on formaldehyde-containing gels, blotted on cellulose nitrate membranes, and probed with ^{32}P-labeled DNA. Lanes in (A) were probed with DNA clones from the cyt *b*–URF5 region as follows: lane 1, nick-translated pHB 336 (3×10^7 cpm/μg of DNA); lane 2, nick-translated 1.58-kb *Hin*dIII–*Xho*I fragment (5×10^7 cpm/μg of DNA); lane 3, cyt *b* probe XB 811 (5×10^8 cpm/μg of DNA); lane 4, L-strand probe XB 901 (5.6×10^8 cpm/μg of DNA); lane 5, L-strand probe HX 905 (5.4×10^8 cpm/μg of DNA). The 5′-end-labeled *Hae*III fragments of ϕX174 DNA were run as molecular weight markers in the lane marked M. In addition, mitochondrial 12S and 16S rRNAs and cytoplasmic 18S and 28S rRNAs were also run as molecular weight markers. Lanes in (B) were probed with ^{32}P-labeled M13 clones from the cyt *b* coding region and nick-translated DNA from the areas flanking the cyt *b*–URF5 region: lane 1, probed with XB 811 DNA; lane 2, the blot in lane 1 was further hybridized with XB 901; lane 3, probed with p622 DNA; lane 4, probed with pH 195 DNA. *Hae*III-digested ϕX174 DNA was run as a marker. One microgram each of the M13 DNA probes and 1.5 μg of nick-translated DNA were used for hybridization. The blots for individual probes were exposed to X-ray films for different time periods to obtain optimal band intensity. Lanes 1–3 in (A) and 1 and 4 in (B) were exposed for 6 h. Lane 2 in (B) was exposed for 12 h, and lanes 4 and 5 in (A) and lane 3 in (B) were exposed for 24 h. The arrow in lane 2 (B) indicates the position of 1.15-kb RNA.

chondrial DNA containing the genes for cyt *b*, URF6, and URF5 are shown in Figure 1. The map positions of these three reading frames and the tRNA genes are based on the DNA sequence analysis of Bibb et al. (1981). The 1.77-kb *Xho*I–*Bgl*II fragment was cloned in M13mp8 and M13mp9 vectors which contain the *Bam*HI and *Sal*I restriction sites in opposite polarity (Messing & Viera, 1982). The cloning strategy is such that the H strand is cloned in M13mp8 and the L strand is cloned in M13mp9. The clones designated as XB 811 and XB 901 contain the H and L strands, respectively, of the 1.77-kb *Xho*I–*Bgl*II fragment. The clone HX 905 contains the L strand of the 1.58-kb *Hin*dIII–*Xho*I fragment (see Figure 1). In repeated attempts, however, the H strand of this DNA fragment could not be cloned in M13 vectors. The strand specificities of the M13 clones were confirmed by hybridization of ^{32}P-labeled clones to the H and L strands of the 3.36-kb *Bgl*II–*Hin*dIII DNA fragment, strand separated on an agarose gel (Hayward, 1972) by Southern blot hybridization (Southern, 1975). Clone XB 811 hybridizes to the L strand, and clones XB 901 and HX 905 hybridize to the H strand (results not presented).

Identification of URF6 mRNA by Northern Blot Analysis. The transcripts coded by the three reading frames under study were identified by Northern blot analysis of mitochondrial poly(A) RNA. As shown in Figure 2A (lane 1), the nick-translated probe pHB 336 which contains a 3.36-kb mitochondrial DNA insert identifies three transcripts of 3.6, 2.4, and 1.2 kb. The single-strand-specific probe XB 811, which contains the entire cyt *b* coding region in addition to the 573-nucleotide stretch separating the cyt *b* and URF5 reading frames, identifies the same three transcripts (i.e., 3.6-, 2.4-, and 1.2-kb RNA) as the probe pHB 336 (see lane 3). In addition to these, a very minor species of 4.7-kb RNA is also observed on longer exposure of the blots (results not provided). Since the H strand of the *Hin*dIII–*Xho*I fragment could not be cloned in M13 vectors, the 1.58-kb duplex DNA was used as the hybridization probe. As shown in lane 2, this probe identifies two RNAs of 3.6 and 2.4 kb but fails to hybridize with the 1.2-kb RNA. In agreement with published reports (Battey & Clayton, 1978, 1980; Van Etten et al., 1982; Montoya et al., 1981; Ojala et al., 1981), the 1.2- and 2.4-kb RNAs appear to be cyt *b* and URF5 mRNAs, respectively. The 3.6-kb RNA may be the common precursor coded by the URF5–cyt *b* region. Results presented in Figure 2A (lane 4) also show that the L-strand-specific probe XB 901 surprisingly detects three RNA species of comparable size (3.6, 2.4, and 1.15 kb) as the H-strand-specific probe. The L-strand origin for the transcripts identified by XB 901 is further supported by the fact that another L-strand probe, HX905, which exclusively hybridizes to the H strand identifies these same RNA species (see lane 5). However, the 2.4- and 3.6-kb transcripts hybridizing to the L-strand DNA probes are seen only after long exposure of the autoradiograms, suggesting their low abundance in the steady-state RNA. Probing the same RNA blot successively with XB 811 (see Figure 2B, lane 1) and then with XB 901 (Figure 2B, lane 2) reveals two closely migrating species of 1.2- and 1.15-kb RNAs (lane 2) in addition to two large-size transcripts of 3.6 and 2.4 kb. It should be noted that the 1.2-kb RNA is identified by the H-strand probe XB 811 (Figure 2B, lane 1) and the 1.15-kb band is seen only with

Table I: Determination of ^{32}P Radioactivity in RNA Bands Identified by Various DNA Probes[a]

probe used	RNA detected (kb)	^{32}P cpm in band expt I	expt II
XB 811	4.7	558	497
	3.6	2219	2225
	2.4	2652	2467
	1.2	19126	15720
XB 901	3.6	321	269
	2.4	663	569
	1.15	1878	1587
HX 905	3.6	386	369
	2.4	690	696
	1.15	1820	1940
pHB 336	3.6	130	126
	2.4	246	219
	1.2	1968	1970
*Hin*dIII–*Xho*I fragment	3.6	152	
	2.4	259	

[a] Various RNA bands from the Northern blots shown in Figure 2A were cut out and assayed for radioactivity with 10 mL of scintillation mixture. The background values from blank filter areas ranged from 48 to 59 cpm in different experiments.

probe XB 901 as indicated by the arrow in lane 2. These results indicate that the 1.15-kb band is a unique L-strand transcript from the URF6 coding region.

To verify if the 3.6-kb RNA species are exclusively coded by the cyt *b*–URF5–URF6 regions under study or if they overlap with the 3′ or 5′ flanking DNA regions, Northern blot experiments were carried out with p622 and pH 195 DNA probes. Clone p622 contains a 622-bp DNA from the 3′ flanking region of the cyt *b* reading frame (nucleotide residues 15 330–15 973) cloned in the pUR 250 plasmid vector. Probe pH 195 contains a 1.95-kb DNA (nucleotide residues 9137–11 082) fragment containing the 3′ end of the cyt ox III reading frame, the complete URF3 and URF4L reading frames, and most (921 nucleotides) of the URF4 coding area [see Bibb et al. (1981)]. Results presented in Figure 2B (lane 3) show that probe p622 hybridizes with two transcripts of 940 and 830 nucleotides of relatively low abundance. These transcripts are distinct from the cyt *b*–URF6 region coded 1.2- and 1.15-kb RNAs and may represent portions of the L-strand primary transcript. Probe pH 195, on the other hand, identifies a 1650-nucleotide URF4–URF4L RNA, an 850-nucleotide-long cyt ox III mRNA, and an approximately 400-nucleotide-long URF3 mRNA (Figure 2B, lane 4). It should be noted that both of these probes fail to hybridize to the 3.6-, 2.4-, 1.2-, and 1.15-kb RNAs identified by the cyt *b*–URF6–URF5 region of mitochondrial DNA. The 3.6-kb species coded by both the H and L strands may therefore be the common precursors to the 1.2- and 1.15-kb RNAs, respectively.

A quantitative analysis of data from the Northern blot experiment shows that the 1.2-kb transcript is present in about 8–9 times greater abundance than the 3.6- and 2.4-kb RNAs coded by the H strand (see Table I). Similarly, the 1.15-kb RNA, identified by the L-strand probes XB 901 and HX 905, is about 3–10-fold more abundant than the corresponding 3.6- and 2.4-kb RNAs identified by these probes. However, since the specific activities of the three M13 probes are nearly identical [i.e., (5–6) × 10^8 cpm/μg of DNA], the results of these experiments (Figure 2A and Table I) indicate that the 1.2-kb cyt *b* mRNA in steady-state mitochondrial RNA is about 9 times more abundant than the 1.15-kb RNA hybridizing with the L-strand open-reading frame URF6.

Synthesis and Decay of mRNAs in an in Vitro System. The mode of transcription of 1.2-kb cyt *b* mRNA and 1.15-kb

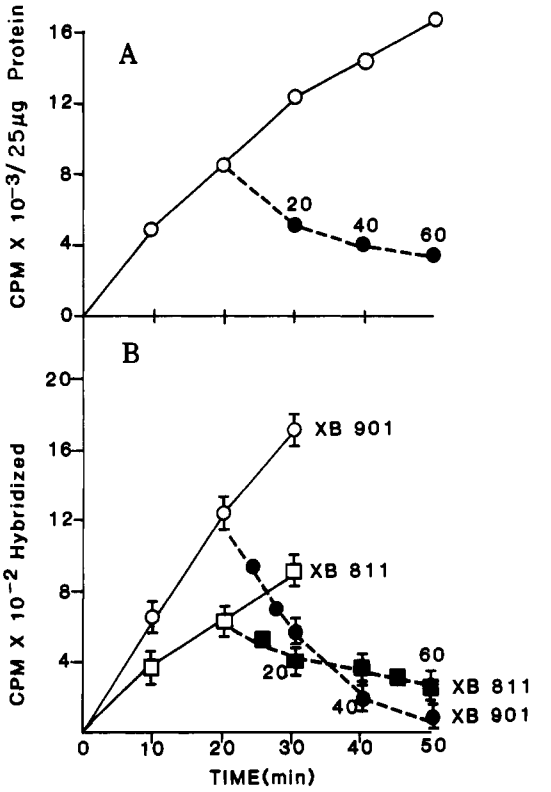

FIGURE 3: Rates of synthesis and decay of cyt *b* and URF6 mRNAs. Freshly isolated mitoplasts from Ehrlich ascites cells were labeled with 200 μCi/mL each of [^{32}P]UTP and CTP. Pulse-labeled mitoplasts were chased with 0.5 mM each of unlabeled UTP and CTP and 1 μM cordycepin triphosphate as described under Materials and Methods. (A) Aliquots (2.5 μL each) of samples at various time intervals of pulse-labeling (solid lines) and chasing (dashed lines) were used for determining the cold Cl_3CCOOH-insoluble cpm. (B) Two micrograms of each RNA from mitoplasts pulse-labeled for various time intervals (solid line) and chased for various times following a 20-min pulse-labeling (dashed line) was hybridized to 1 mg each of M13 DNA clones covalently linked to epoxy-activated cellulose as described before (Kantharaj et al., 1983). The DNA clones used were XB 811 (□) and XB 901 (○). The numbers along the dashed lines indicate the time of chasing following 20-min pulse-labeling. The mean and standard deviations were calculated from three separate hybridization values.

putative URF6 mRNA, and the basis for the difference in their relative steady-state levels were studied by using the mitoplast system active in in vitro transcription (Kantharaj et al., 1983; Bhat et al., 1984). The pulse–chase kinetics of RNA labeling in vitro in the Ehrlich ascites mitoplast system are presented in Figure 3A. It is seen that labeling continues linearly up to about 30 min of incubation. When pulse-labeled mitoplasts are chased in a medium containing unlabeled UTP and CTP (0.5 mM each) and 1 nM cordycepin triphosphate, the ^{32}P-labeled RNA decays with a biphasic curve similar to the decay of mitochondrial poly(A) RNA as shown before (Avadhani, 1979). Cordycepin triphosphate (0.5–1.0 nM) inhibits RNA synthesis in the mitoplast system by about 90% (Kantharaj et al., 1983). Electrophoretic analysis of RNA chased in the presence of varied concentrations of cordycepin triphosphate (G. R. Kantharaj and N. G. Avadhani, unpublished results) has revealed no detectable effects of this inhibitor on RNA processing or decay. The rates of synthesis and decay of transcripts coded by cyt *b* and URF6 reading frames were studied by hybridization of pulse-labeled and pulse–chased RNA to over 1000-fold molar excess of single-stranded DNA probes XB 811 and XB 901 covalently linked to epoxy-activated cellulose. The hybridization kinetics presented in Figure

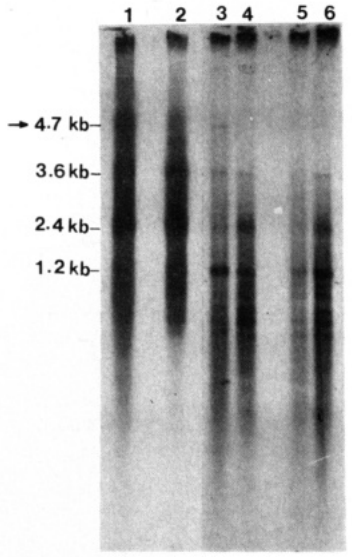

FIGURE 4: Nature of pulse-labeled and pulse–chased RNA hybridizing to H- and L-strand probes. Duplicate samples of Ehrlich ascites mitoplasts (20 mg each) were pulse-labeled for 10 min with [^{32}P]UTP and [^{32}P]CTP. One sample was subsequently chased for 20 min in the presence of 1 μM cordycepin triphosphate, and RNA was isolated from both mitoplast samples. Poly(A) RNA was prepared by poly(U)–Sepharose chromatography. About 6 μg each of poly(A) RNA from both pulse-labeled and pulse–chased mitoplasts (3.5×10^4 cpm/μg) was hybridized to 7 mg of cellulose-linked XB 811 and XB 901 as described by Moss et al. (1981). The bound RNA was eluted with 80% formamide, precipitated with ethanol in the presence of 10 μg of carrier tRNA, and electrophoresed on 1.7% agarose–methylmercury gels. Lane 1, pulse-labeled poly(A) RNA (1.5×10^5 cpm); lane 2, pulse–chased poly(A) RNA (1.2×10^5 cpm). Lanes 3 and 4 represent pulse-labeled poly(A) RNA hybridized to XB 811 and XB 901 DNA, respectively. Lanes 5 and 6 represent pulse–chased poly(A) RNA hybridized to XB 901 DNA and XB 811 DNA, respectively. Mitochondrial 12S and 16S rRNAs and cytoplasmic 18S and 28S rRNAs were run as molecular weight markers.

3B show that the URF6 region is transcribed at about 1.5–1.7 times more profusely than the H-strand region containing the cyt *b* reading frame. In contrast to the increased rate of synthesis, the transcripts hybridizing to URF6 probe XB 901 decay with a short half-life of about 16–18 min vs. 58–60 min for the decay of cyt *b* region coded transcripts. A similar half-life (56 min) for cyt *b* mRNA was reported in the human mitochondrial system (Gelfand & Attardi, 1981).

The nature of the transcripts coded by the H and L strands of the cyt *b* region was further studied by electrophoretic analysis of poly(A) RNA absorbed to cellulose-linked DNA probes. The electrophoretic patterns of poly(A) RNA from mitoplasts pulse-labeled with ^{32}P-nucleotides and subsequently chased for 20 min in the presence of 1 nM cordycepin triphosphate are shown in Figure 4 (lanes 1 and 2). It is seen that some of the high molecular weight RNAs, such as a 4.7-kb species shown by the arrows in the pulse-labeled RNA (lane 1), are not present in detectable levels after 20 min of chase (lane 2). In confirmation with the results of Northern blot analysis (Figure 2A), pulse-labeled RNA, hybridizing to cyt *b* probe XB 811, consists of 3.6- and 2.4-kb RNAs and relatively more abundant 1.2-kb RNAs (lane 3). In addition, a minor species of 4.7-kb RNA is also detected. Similarly, the pulse-labeled RNA, hybridizing with the URF6 probe XB 901, consists of 3.6-, 2.4-, and a 1.15-kb RNA (lane 6). The pulse–chased RNA, hybridizing with XB 811, contains the 1.2-kb cyt *b* mRNA of comparable intensity, the 2.4-kb RNA with increased intensity, the 3.6-kb RNA with reduced intensity, and no detectable 4.7-kb RNA (lane 4). The URF6-specific 1.15-kb RNA, on the other hand, shows over 50% reduced intensity after 20 min of chase (lane 5), suggesting a higher relative turnover rate. Furthermore, in support of the hybridization kinetics presented in Figure 3B, ^{32}P cem in the 1.2-kb (XB 811 DNA bound) and 1.15-kb (XB 901 DNA bound) RNA bands decay with $t_{1/2}$ values of about 56–60 and 16–18 min, respectively (results not presented). In addition to the RNA species described above, both XB 811 and XB 901 bound RNAs show a number of smaller transcripts, many of which are not detected on the Northern blots of total poly(A) RNAs. It is therefore likely that they represent nicked RNA generated during hybridization and elution. These results on the distinctive turnover rates of the 1.2- and 1.15-kb RNAs provide further support to the notion that they indeed represent independent transcripts.

Mapping of URF6 mRNA by S_1 Nuclease Protection. Although the results of Northern blot analysis suggest that the 1.15-kb RNA may be putative URF6 mRNA, it is over twice as large as the actual reading frame predicted by the DNA sequence analysis (Bibb et al., 1981). The precise map position of this RNA was therefore determined by the S_1 nuclease protection of DNA–RNA hybrids (Berk & Sharp, 1977). Three different single-stranded DNA probes labeled with ^{32}P at 3′ or 5′ ends were hybridized with mitochondrial poly(A) RNA. The details of the probes used are presented in Figure 5A. Probe 1 is the L strand of the *Bgl*II–*Xho*I fragment labeled at the 5′ end with [γ-^{32}P]ATP by using polynucleotide kinase following dephosphorylation. Probe 2 represents the L strand of the *Hin*dIII–*Xho*I fragment labeled at the 3′ end with [α-^{32}P]dCTP by gap filling (O'Farrel, 1981). Probe 3 represents the H strand of the *Hin*dIII–*Xho*I fragment 5′ end labeled with [γ-^{32}P]ATP. As shown in Figure 5B (lane 1), the use of probe 1 yields an S_1 fragment of about 515 nucleotides, whereas probe 2 yields a slightly larger fragment of about 590–600 nucleotides (lane 4). Small molecular weight fragments (200–300 nucleotides) in both of these lanes are probably generated due to nicking of RNAs. As expected, use of ^{32}P-labeled DNA probes alone without added RNA does not afford protection against S_1 nuclease (see lanes 2 and 3). On the other hand, nearly the complete length of probe 3 is protected by mitochondrial poly(A) RNA against S_1 nuclease action (Figure 5C, lane 2), demonstrating that it hybridizes to RNA species distinct from those hybridizing with probes 1 and 2. To ensure that 1.15-kb RNA is involved in the S_1 protection of probes 1 and 2, experiments were carried out with in vitro pulse-labeled RNA. In these experiments, ^{32}P-labeled RNA adsorbed to the L-strand DNA XB 901 linked to cellulose was electrophoresed under denaturing conditions as shown in Figure 4. The 1.15-kb putative URF6 RNA was eluted out of the gel and hybridized to the M13 clones. As shown in Figure 5B (lane 5), a 590–600-nucleotide stretch of the 1.15-kb RNA is rendered resistant to S_1 nuclease after hybridization with XB 905 DNA. This length is nearly identical with the size of the 3′ end of probe 2 protected by the unlabeled mitochondrial poly(A) RNA (lane 4). Although not shown, hybridization of ^{32}P-labeled 1.15-kb RNA to *Xho*I–*Hin*dIII and *Xho*I–*Bgl*II fragments under R-loop conditions (Casey & Davidson, 1977) or to single-strand DNA probes XB 901 and XH 905 yields nearly identical S_1 fragments. Thus, as shown in Figure 1, the 1.15-kb RNA seems to map between the start of the URF6 reading frame and a region located 590–600 nucleotides to the 5′ end of this reading frame.

Since the secondary structure appears to be an important factor in mitochondrial RNA processing (Clayton, 1984), the L-strand region corresponding to the URF6 mRNA termi-

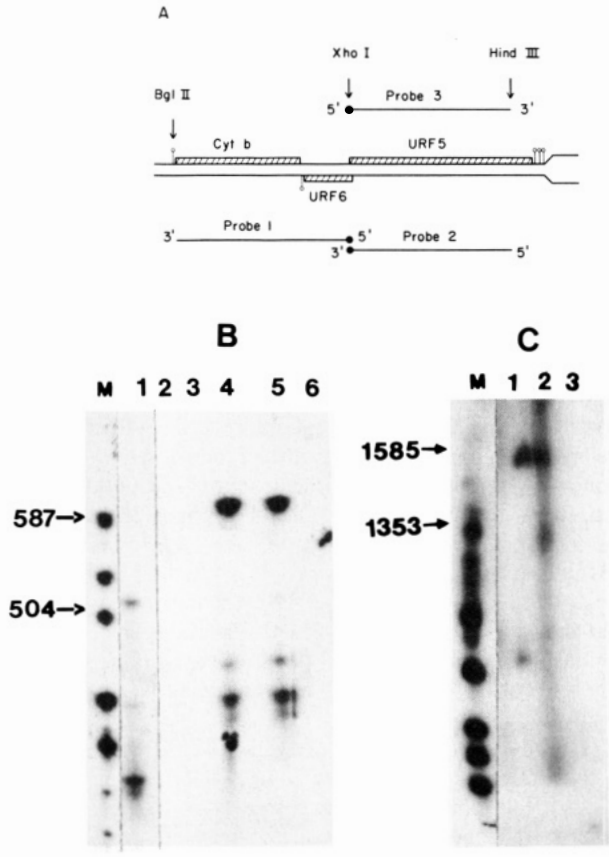

FIGURE 5: S_1 nuclease mapping of 1.15-kb RNA. (A) The three single-stranded DNA probes used in the S_1 nuclease protection analysis were derived from the *Hin*dIII–*Xho*I and *Xho*I–*Bgl*II fragments of the cyt *b*–URF6–URF5 region as indicated. The rounded pinhead on each probe indicates the position of ^{32}P end labeling. pAM1 DNA was linearized by digestion with *Xho*I. About 1 μg of DNA was 3′ end labeled by gap filling using unlabeled TTP and [^{32}P]dCTP as described under Materials and Methods. The labeled DNA was cut with *Hin*dIII and separated on a 1.6% agarose gel under denaturing conditions (Hayward, 1972), and the 1.58-kb DNA fragment was recovered and used as probe 2. For preparation of probes 1 and 3, the *Xho*I-linearized DNA was 5′ end labeled with [^{32}P]ATP following dephosphorylation and digested either with *Bgl*II or with *Hin*dIII. The 1.78-kb (probe 1) and the 1.58-kb (probe 3) 5′-end-labeled DNA strands were separated as above. (B) The DNA probes (10 ng) were hybridized with poly(A) RNA (12–15 μg), and the S_1 nuclease resistant DNA fragments were analyzed on 5% polyacrylamide–urea gels: lanes 1 and 4, S_1 nuclease protection of probes 1 and 2, respectively, hybridized to poly(A) RNA; lanes 2 and 3; S_1 nuclease reactions run without added RNA using probes 1 and 2, respectively; lane 5, ^{32}P-labeled 1.15-kb RNA (0.5 μg, 15000 cpm) hybridized with 1.5 μg of HX 905 DNA; lane 6, same as lane 5, but no DNA added. In the lane marked M, the 5′-end-labeled *Hae*III digest of pBR 322 DNA was run as a marker. Electrophoresis was run on a 5% polyacrylamide–urea gel. (C) The 5′-end-labeled probe 3 was hybridized with poly(A) RNA: lane 1, about 1000 cpm of probe 3; lanes 2 and 3, S_1 nuclease resistant fractions of probe 3 hybridized with and without RNA, respectively. In the lane marked M, the 3′-end-labeled *Hae*III digest of φX174 DNA was run as a marker. The arrows in (C) indicate the positions of probe 3 (1585 nucleotides) and the largest fragment of marker DNA (1353 nucleotides). Electrophoresis was run on a 4% polyacrylamide–urea gel.

nation site (around nucleotide 12950) was analyzed for the presence of palindrome-like secondary structure by using an IBM/370 computer and a CP/CMS operation system. As shown in Figure 6, a palindromic structure exists in the nucleotide region 12921–12976 of the mouse mitochondrial DNA. There is no other secondary structure of significance in the 300–350-nucleotide region flanking the 3′ and 5′ ends of this palindromic structure. This structure consists of a 16-nucleotide-long base-paired region (11 nucleotides out of

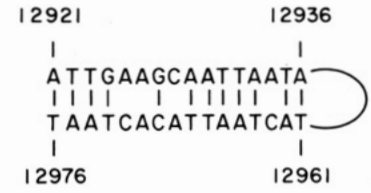

FIGURE 6: Secondary structure characteristics of the L-strand DNA sequence near the URF6 mRNA termination site.

16 are homologous) and a 25-nucleotide loop-out structure (Figure 6). Although not shown here, both human and bovine mitochondrial genomes contain similar palindromic structures at about 1100 nucleotides downstream from the start of the URF6 reading frame. Particularly in the bovine mitochondrial DNA, this appears to be the only significant palindromic structure (16 out of 20 nucleotides are homologous) in the 500-nucleotide stretch on each side of the putative URF6 mRNA processing site.

Discussion

An interesting feature of mitochondrial gene organization in mouse, bovine, and human cell systems is that the genes coding for the two rRNAs and the majority of reading frames for putative mRNAs are punctuated by tRNA genes (Anderson et al., 1981, 1982; Bibb et al., 1981). The only exceptions are the two genomic regions coding for cytox III–URFA6L-6ATPase and URF5–cyt *b* (Anderson et al., 1981, 1982; Bibb et al., 1981). It has been postulated that mRNAs encoded by reading frames containing an upstream tRNA gene may be processed from the nascent transcripts by using the tRNA secondary structure as a processing signal (Van Etten et al., 1980; Montoya et al., 1981; Ojala et al., 1981). Recent results in our laboratory have indicated that mRNAs for the above two sets of reading frames, which do not contain interspersed tRNA coding sequences, are processed from relatively long-lived precursors (Bhat et al., 1984). In the present study, we have further characterized the transcripts coded by both the H and L strands of the URF5–cyt *b* region of mouse mitochondrial DNA with a view to identify the mRNA coded by the only L-strand open-reading frame URF6 and also to characterize their maturation pathways.

The results of Northern blot experiments (Figure 3A, Table I) show that the H strand of this DNA region codes for 3.6- and 2.4-kb RNAs of relatively lower abundance and an 8–10 times more abundant 1.2-kb RNA. In agreement with the DNA sequence analysis predicting the size of various reading frames (Anderson et al., 1981; Bibb et al., 1981), S_1 nuclease mapping of mitochondrial transcripts (Battey & Clayton, 1978, 1980; Van Etten et al., 1982), and partial sequencing of mitochondrial poly(A) RNA termini (Ojala et al., 1981; Montoya et al., 1981), the 1.2-kb RNA appears to be the matured cyt *b* mRNA. In a previous report from this laboratory, using a nick-translated *Hin*dIII–*Apa*I restriction fragment corresponding to this region, we detected a minor species of 1.85-kb RNA in the total mitochondrial RNA fraction (Bhat et al., 1984). However, in the present study, both duplex DNA probes and single-stranded M13 DNA probes detected insignificant levels of 1.85-kb transcripts in the poly(A) RNA fraction (Figure 2A). The nature of 1.85-kb RNA in the total mitochondrial RNA remains unknown at the present time. Thus, in agreement with previous reports on mouse and human mitochondrial systems (Montoya et al., 1981; Van Etten et al., 1982), the 2.4-kb poly(A) RNA may be putative URF5 mRNA. A surprising observation of this study was that the L-strand-specific probes XB 901 and XB 905, which together correspond to the complete length of the genomic region under

study, identify three transcripts of nearly identical size class of 8–10 times lower abundance. The only notable difference is that the smallest RNA identified by these probes is about 50 nucleotides shorter than the cyt *b* mRNA. The difference in the size of these two RNA species was conclusively established by sequential hybridization of the same RNA blot with ^{32}P-labeled XB 811 and XB 901 (see Figure 2B, lane 2).

The 1.15-kb RNA detected by both of the L-strand probes is over 2 times larger than the 519-nucleotide-long URF6 reading frame predicted by the DNA sequence analysis (Bibb et al., 1981; Anderson et al., 1981). It was, therefore, necessary to determine the precise map position to understand its functional role. The URF6 reading frame of the mouse mitochondrial genome starts immediately following the termination of tRNA Glu (Bibb et al., 1981) without any punctuating sequence in between. It is therefore apparent that, as in the case of almost all the mammalian mitochondrial mRNAs (Montoya et al., 1981; Ojala et al., 1981; Van Etten et al., 1982), the URF6 mRNA may lack the 5′ leader sequence. If indeed the 1.15-kb RNA is the putative mRNA coding for the URF6 gene product, the 5′ end of this mRNA should precisely map at the start (3′ end) of this reading frame. With this assumption, it is possible to predict that the 1.15-kb RNA should protect a 514-nucleotide stretch from the 5′ end of probe 1 (L strand of the *Xho*I–*Bgl*II fragment labeled at the 5′ end) against S_1 nuclease. Similarly, it should also protect a 590–603-nucleotide fragment at the 3′ end of probe 2 (L strand of the *Xho*I–*Hin*dIII fragment labeled at the 3′ end; see Figure 5A). The S_1 mapping experiments presented in Figure 5B indeed show that hybridization with mitochondrial poly(A) RNA renders a 510–515-nucleotide fragment from the 5′ end of probe 1 (lane 1) and a 590–600-nucleotide fragment from the 3′ end of probe 2 (lane 4) resistant to S_1 nuclease. These two fragments add up to the full length of the RNA less an average of the 50-nucleotide poly(A) tail (i.e., 1100 nucleotides). Furthermore, affinity-purified 1.15-kb ^{32}P-labeled RNA when hybridized to HX 905 DNA yields an S_1 product (lane 5) nearly identical in size with those obtained with ^{32}P-labeled DNA probe 2 (lane 4). The results of S_1 protection studies therefore indicate that the 1.15-kb RNA maps between the 3′ end of the URF6 reading frame and a region located about 600 nucleotides 5′ to this reading frame (see Figure 1). Although not precisely mapped, the termination site of the 1.15-kb RNA is estimated to be around nucleotide 12 950 of the mouse mitochondrial genome. Thus, on the basis of the relative abundance in relation to other transcripts coded by the L strand of this region, its size, and the map position, the 1.15-kb poly(A) RNA appears to be the putative URF6 mRNA.

A notable difference between the 1.2-kb cyt *b* mRNA and the 1.15-kb URF6 mRNA is their relative molar abundance in steady-state RNA (Table I). The URF6-specific 1.15-kb RNA is only about one-tenth the level of cyt *b* mRNA. The basis for this vast difference was studied by using an in vitro mitoplast system. Recent experiments in our laboratory showed that isolated mitoplasts from Ehrlich ascites cells can synthesize both of the rRNAs (Kantharaj et al., 1983) and almost all of the poly(A) RNA species detected in steady-state mitochondrial RNA (Bhat et al., 1984). Results presented in the present paper (Figures 2 and 4) show that the in vitro mitoplast system transcribes both H- and L-strand-specific RNAs detectable in the steady-state RNA. The relative levels of hybridization to a large excess (>1000-fold) of single-strand DNA probes show that the URF6 region is transcribed at about 1.7 times faster than the cyt *b* region. A similar faster rate of L-strand transcription of human mitochondrial DNA was reported before (Gelfand & Attardi, 1981; Attardi et al., 1982). Nevertheless, as estimated by hybridization kinetics, the URF6 region (L strand) coded RNAs decay about 3–4 times faster than the cyt *b* region (H strand) coded RNA species. Furthermore, the ^{32}P label in the affinity-purified and electrophoretically separated 1.15-kb URF6 mRNA decays with a $t_{1/2}$ of 16–18 min as against a $t_{1/2}$ of 56–60 min for the 1.2-kb cyt *b* mRNA. These differential turnover rates may be responsible for the observed differences in the relative abundance of these two mRNAs.

The results presented in this study demonstrate that the 1.2-kb cyt *b* mRNA and the 2.4-kb URF5 mRNA are derived from a relatively long-lived common precursor of 3.6-kb RNA. Consistent with the model proposed by Attardi et al. (1982), the processing appears to occur at the 5′ end of the cyt *b* coding sequence possibly involving the secondary structure of the antisense sequence of tRNA Glu. An important finding of the present study is the identification of a 1.15-kb RNA coded by the L-strand reading frame URF6. Although this RNA is only one-tenth as abundant as the cyt *b* mRNA, experiments (not shown here) using L-strand-specific clones from different regions of the mitochondrial genome have shown that the 1.15-kb RNA is about 10–20 times more abundant than the large L-strand transcripts such as those coded by the URF4–URF4L and cyt ox III regions. Furthermore, the 1.15-kb URF6-specific RNA is nearly as abundant as the 2.4-kb URF5 mRNA. Thus, it may represent a low-abundance mitochondrial mRNA species. Although the precise maturation pathway of the 1.15-kb RNA could not be elucidated due to low abundance of the precursor species, it appears to involve a two-step processing of the 3.6-kb transcript. One of the processing signals may involve the secondary structure of tRNA Glu (Figure 1). Although the nature of molecular signal(s) for the processing at the 3′ end of 1.15-kb RNA remains unclear, distinct palindromic structures of the type shown in Figure 6 occur in this region. This is the most significant secondary structure in the 600-nucleotide stretch (nucleotide residues 12 500–13 400) of the mouse mitochondrial genome housing the putative URF6 mRNA processing site. Similar palindromic structures are also seen in the putative URF6 mRNA termination regions of both human (nucleotides 13 534–13 569) and bovine (nucleotides 13 310–13 346) mitochondrial genomes, suggesting that it is heavily conserved through evolution. It remains to be seen if the palindromic structures are indeed involved in the processing of mitochondrial mRNA.

In conclusion, single-strand-specific M13 clones have enabled the identification and resolution of URF6 mRNA from about 10-fold more abundant cyt *b* mRNA of nearly identical size. This approach may prove helpful in further characterization of the mitochondrial transcription process.

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