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An endoribonuclease activity associated with Lettre Ehrlich ascites tumor cell mitochondria

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The splicing of 3.36kb mouse mitochondrial mRNA precursor(L-strand) to 69bp RNA component is shown to be mediated by an endoribonuclease activity associated with Lettre Ehrlich ascites tumor cell mitochondria. The enzymic activity was partially purified by column chromatography and the in vitro reconstituted RNA processing assay was performed to document this finding.

The URF5-URF6-tRNA^{Glu}-Cytb coding region(nucleotide 11970 to nucleotide 15330) of the mouse mitochondrial genome[4] is unique in that both H and L strands of this 3.36Kb region(BglII-HndIII) contain potential mRNA reading frames and are symmetrically transcribed and proposed into polyA mRNAs of comparable size[1]. The 1.2Kb and 2.4Kb mRNAs coded by the H-strand, putative RNAs for Cytb and URF5 respectively, are derived from a precursor 3.5Kb RNA[1]. The L-strand coded 1.15Kb URF6 mRNA is derived from a short-lived precursor of 3.6Kb mRNA by a multiple step-processing involving a 2.4Kb intermediate mRNA[1]. It was proposed earlier[1-3] that the processing appeared to occur at

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the 5'-end of the Cytb mRNA and URF6 mRNA possibly involving the secondary structure of antisense and sense sequencs of tRNA^{Glu}. Recently, Chung and Clayton(1989) have shown that a MRNaseP in mouse mitochondria has a capacity to cleave mouse mitochondrial RNA complementary to the light strand of the displacement loop at a unique site. This report presents the evidence that a RNA processing endoribonuclease in Ehrlich ascites tumor cell mitochondria can cleave the mouse mitochondrial tRNA mRNA precursor to 69bp RNA component(tRNA^{Glu}).

MATERIALS AND METHODS

Materials

Guanidium thiocyanate, formamide and formaldehyde were purchased from Fluka Chemical Corp. Restriction endonucleases, the nick-translation kit, Klenow fragment of DNA polymerases I were purchased from Bethesda Research Laboratory. Escheria K12 recipient strain and T_4 DNA ligase were purchased from Promega Biotech and New Englano Biological Laboratories. ^{32}P-dCTP(> 3000 Ci/mmol), ^{32}P-UTP(>600 Ci/mmol), ^{32}P-CTP(<600 Ci/mmol) were purchased from New England Nuclear. Nitrocellulose membranes for RNA blot transfer were purchased from Schlier & Schuell. Other chemicals and biochemicals were purchased from Pierce Chemical Co., Sigma Chemical CO, Pharmacia, Boheringer Manheim, Promega Biotech and Bethesda Research Laboratory.

Isolation of mitochondria

The maintenance and growth of Lettre Ehlich ascites cell(LES) in the peritoneal cavity of Swiss mice were as described before(1,3,4). Freshly harvested LES

cells from animals bearing 7-day-old tumors were washed free of blood clots and were a source of mitochondria. Mitochondria were isolated by differential centrifugation and further purified by sedimentation through a discontinuous sucrose gradient.

Chromatographic isolation of endoribonuclease activity

The enzyme was isolated by DEAE-cellulose and octyl-sepharose chromatography as described for Hela cell mt RNaseP(5). The enzyme was concentrated and the protein concentration was estimated by Bradford method(6).

Assay of the enzyme

The enzyme was assayed as described before(5).

Run-off transcription

For the run-off transcription, p3369 plasmid was linearised with HindIII and XhoI and transcribed in vitro by T7 RNA polymerase(Promega Biotech) in 40mM Tris buffer,pH 7.9 containing 6mM $MgCl_2$, 10mM DTT, 2mM spermidine, 0.5mM ATP, GTP,CTP and UTP, 0.5 µCi ^{32}P-UTP, 1µg DNA and 0.5-1 unit µl Rnasin (5). After incubation at 37 ^{o}C for 1hour , the mRNAs were purified by digestion with DNase I(RNase-free) followed by passing through sephadex-G50 and extracted with phenol,chloroform and ether and precipitited with ethanol. The mRNAs were dissolved in water.

Substrate of the enzyme

The substrate of the enzyme were the $tRNA^{Glu}$ precursor mRNAs(3.36kb and 1.78kb, L-strand). 3.36kb

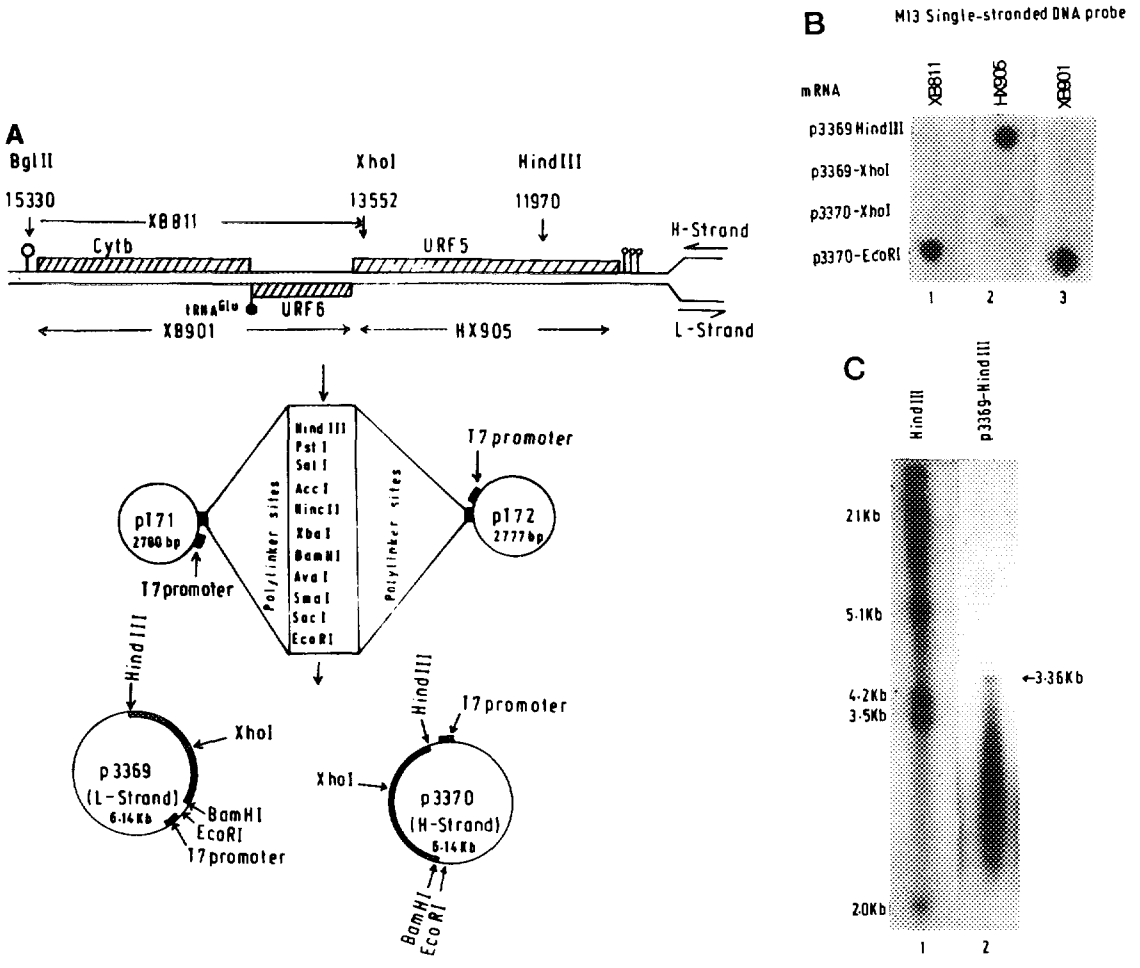

Fig.1.(A) Cloning of 3.36kb DNA of the mouse mitochondrial tRNA^{Glu} genome The 3.36kb fragment was described before(1).(B) Strand specificity by DNA-mRNA slot-blot hybridization. Lane 1, XB811; lane 2, HX905; lane 3, XB901. (C) Size analysis of 3.36kb mRNA in 1.2% agarose-2.2M formaldehyde gel. Lane 1, MW markers(A-Hind III); lane 2, 3.36kb mRNA.

DNA(HindIII-BglII fragment, nucleotides 11970 to nucleotides 15330) containing tRNA^{Glu} coding sequence of the mouse mitochondrial genome(7) was isolated by cleaving pHB336(1). with HindIII and BglII and cloned in the polylinker sites(BamHI and HindIII) of pT71 and pT72 expression vectors(United States Biochemical Corporation) in opposite

orientations(Fig.1). The recombinant plasmids were selected by antibiotic markers(8) designated as p3369(L-strand) and p3370(H-strand) and further analysed by HindIII, EcoRI, SmaI, XhoI and HinfI. The mitochondrial-specific mRNAs were synthesized with T7 RNA polymerase in vitro. The strand-specificities of these mRNAs were analysed by DNA-mRNA slot-blot hybridization using single-stranded strand-specific M13 DNA clones HX905(containing 1.58kb XhoI-HindIII L-strand, nucleotides 11970 to nucleotides 13552), X8901(containing 1.78kb XhoI-BglII L-strand, nucleotides 13552 to nucleotides 15330), XB811(containing 1.78kb XhoI-BglII H-strand, nucleotides 13442 to nucleotides 15330), p622(containing 3′-flanking sequence of cytb, nucleotides 15330 to nucleotides 15973 cloned into pur250; reference 1) and ΦX174 and pT70 as control(Fig.1B). The molecular sizes of 3.36kb mRNA(L-strand) and 1.78kb(L-strand) were verified by gel analysis(Fig.1C).

Purification of plasmid

The plasmids were purified by alkaline lysis method followed by ethidium bromide-CsCl density gradient centrifugation(9) and biogel A150 column chromatography (for run-on transcription) in TE buffer,pH 8.0. The later method was advantageous because the remaining ethidium bromide(not removed by n-butanol) trapped in the DNA impaired transcription efficiency.

Hybridization

The DNA samples were blotted on nylon membrane(Cat. No. NO4HY3MTFO, Fischer Scientific Company, USA) in a blot-blot apparatus and hybridized with ^{32}P-labelled mRNA(10 cpm/ ml) at 42 °C for 16 hours in 0.12M Tris buffer,pH 8.0 containing 50% formamide, 2X Denhardts, 0.6M NaCl, 0.1% SDS, 100µg/ ml yeast tRNA and 125µg/ ml polyA. The blots were washed with 4X, 3X, 1X, 0.5X, 0.2X SET buffer,pH 8.0 containing 0.1% SDS at 42 °C and 0.1XSET at room temperature.

RESULTS AND DISCUSSION

A RNA processing endoribonuclease was purified from Lettre Ehrlich ascites tumor cell mitochondria by DEAE-cellulose and octyl-sepharose chromatography. The enzyme was eluted between 90mM KCl and 280mM KCl concentrations by DEAE-cellulose chromatography and between 0.42M and 0.3M $(NH_4)_2SO_4$ by octyl-sepharose chromatography. The activity of this enzyme was observed with the specified concentrations of Mg^+ and K^+ ions for Hela cell mt RNaseP(5). As a control of mitochondrial specific enzyme, cytochrome c oxidase was purified by DEAE-cellulose chromatography from mouse liver mitochondria(10). The tRNA precursor mRNAs(L-strand) synthesized in vitro by T7 RNA polymerase from p3369 plasmid after HindIII and XhoI restriction enzymes cleavage was cleaved by RNA processing endoribonuclease to 69bp RNA comonent(tRNA) as shown in Fig.2. No 69bp tRNA RNA component was detected without this enzyme(lanes 2 & 5 in Fig.2). The self-splicing of 3.36kb

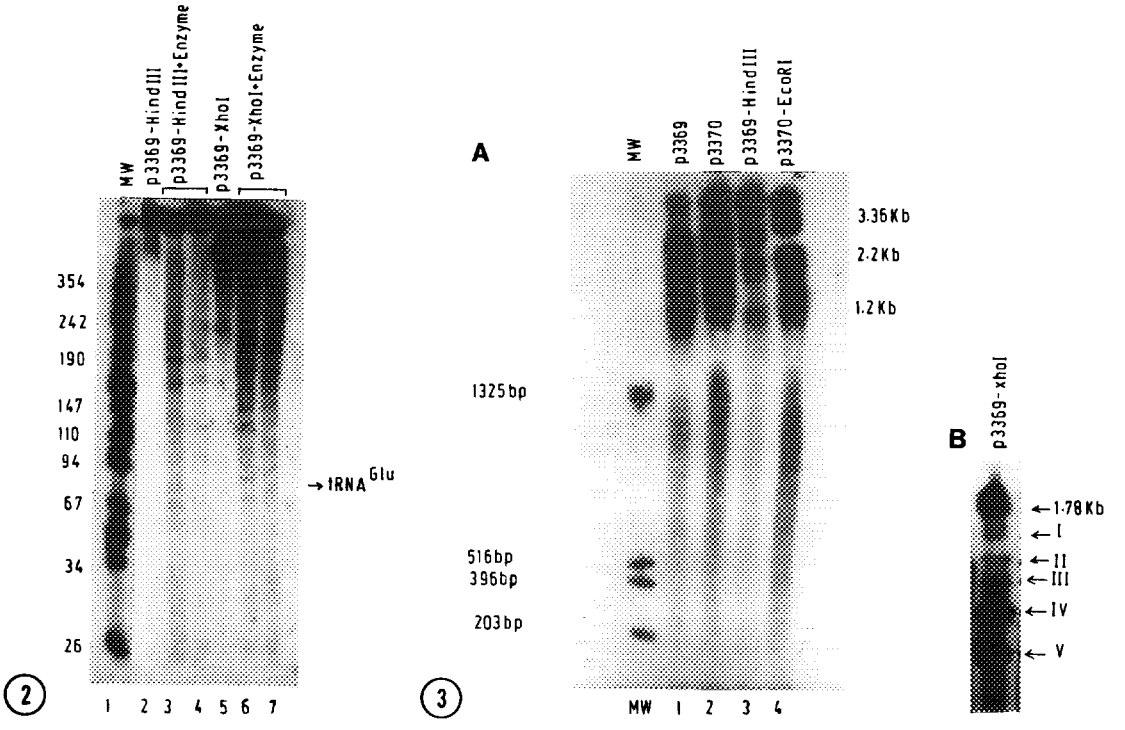

Fig.2. Analysis of RNA cleavage products of $tRNA^{Glu}$ precursor mRNA(L-strand) by purified RNA processing endoribonuclease in 8% sequencing gel. Lane 1, MW markers; lane 2, 3.36kb mRNA; lanes 3-4, 3.36kb mRNA + enzyme; lane 5, 1.78kb mRNA; lanes 6-7, 1.78kb mRNA + enzyme.

Fig.3. (A) Self-splicing of 3.36kb mRNA(L-strand) in 3.5% sequencing gel. Lane 1, MW markers; lane 2, p3369 mRNA(vector-read-through transcript without restriction enzyme digestion) and lane 3, 3.36kb mRNA.(B) Analysis of 1.78kb mRNA cleavage products by enzyme fraction.

mRNA(L-strand) as shown in Fig.3A and 3.36kb mRNA(H-strand)(Figure not shown) yielded 2.2kb and 2.8kb RNA components ^{Glu} excluding 69bp tRNA RNA component. 2.2kb RNA component produced from 3.36kb mRNA is comparable to 2.4kb RNA intermediate produced from 3.6kb mRNA(1), because HindIII site(11970) deleted about 200bp from URF5 sequence. The processing

of 1.78kb mRNA(L-strand) by this enzyme(column fraction) as shown in Fig.3B might reflect on the multiprocessing events of 3.6kb mRNA(L-strand) as discussed before(1). The 2.8kb RNA component of self-splicing was not detected and discussed earlier(1). 69bp RNA component was $tRNA^{Glu}$ (N. G. Avadhani, unpublished). The chromatographic profiles and the degree of enrichment(results not shown) may be discussed elsewhere along with the detailed properties of the enzyme.

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