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Characterization of two nuclear-encoded protein components of mitochondrial ribonucleoprotein complexes from *Leishmania tarentolae*

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Abstract

Two mitochondrial proteins with molecular masses of 18 and 51 kDa were isolated from *Leishmania tarentolae*, and N-terminal amino-acid sequences were obtained. The cDNAs and genes encoding these proteins were cloned using RT-PCR. The proteins were identified as components of the previously characterized mitochondrial ribonucleoprotein complexes, T-Ia and T-VI, by comigration in native gels. The p18 and p51 genes contain 17 and 9-amino-acid N-terminal sequences, which are not present in the mature proteins and may represent cleavable mitochondrial targeting sequences. There are two identical p18 genes separated by 1.7 kb in tandem array and both are transcribed. The p18 amino-acid sequence is not similar to any sequence in the database. Antiserum to p18 expressed in *Escherichia coli* reacts with the entire tubular mitochondrion. The p51 gene is single copy, and the amino-acid sequence is similar to mitochondrial aldehyde dehydrogenases from other organisms. The N-terminal amino-acid sequences of 71 and 62-kDa mitochondrial proteins which co-migrated in native gels with several other T-complexes were also obtained. The p71 sequence proved to be similar to hsp70 sequences from other organisms. The p62 sequence was identical to an hsp60 sequence from *Trypanosoma brucei*.

Keywords: *Leishmania tarentolae*; Mitochondrion; Aldehyde dehydrogenase; hsp70; hsp60; Targeting signal

1. Introduction

In the course of a study on mitochondrial proteins from *Leishmania tarentolae* which co-migrate in native gels with the ribonucleoprotein (RNP) complexes that become labeled when mitochondrial extract is incubated with [α-^{32}P]UTP (the so-called 'T-complexes') [1], we cloned and characterized in

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detail two genes which encode proteins that appear to contain cleavable N-terminal mitochondrial signal peptides. In addition, two other mitochondrial proteins comigrating with T-complexes in native gels were studied by N-terminal amino-acid sequence analysis, and shown to contain cleavable N-terminal amino-acid putative signal sequences. All but one of these proteins could be identified by homology with sequences in the database.

2. Materials and methods

2.1. Cell culture, mitochondrial isolation and preparation of mitochondrial extracts

L. tarentolae (UC strain) cells were grown to late log phase ($(1-2) \times 10^8$ cells ml^{-1}) [2] and the kinetoplast-mitochondrial fraction was isolated by flotation in Renografin density gradients [3,4]. The mitochondrial fraction was resuspended in 20 mM Hepes/KOH (pH 7.5)/20 mM KCl/1 mM EDTA/10% glycerol, at a concentration of approx. 5 mg protein ml^{-1}, and stored at -80°C in aliquots. Triton supernate extract (TS) was prepared by 15-s homogenization of the mitochondria in 0.3% Triton X-100 and clarification at $14\,000 \times g$ for 30 min [5].

2.2. Superose 6 gel filtration analysis of mitochondrial extracts

TS (200 μl) was filtered through a 0.22-μm filter and applied to an FPLC (Pharmacia) 10/30 Superose 6 column (Pharmacia) equilibrated with 20 mM Hepes/KOH (pH 7.5)/20 mM KCl/1 mM EDTA. The separation was performed using the same buffer with a 0.5 ml min^{-1} flow rate, and 25 1-ml fractions were collected. The fractions were micro-concentrated to 100 μl using Centricon 10 micro-concentrators (Amicon).

2.3. Native-polyacrylamide gradient gels and UV cross-linking

Aliquots (15 μl) of micro-concentrated fractions #10 to #16 were incubated for 40 min at 27°C in 1 mM ATP/1 mM GTP, 20 mM DTT/12.5 mM HEPES/KOH (pH 7.5)/40 mM KCl/6 mM $Mg(CH_3COO)_2$/3 mM K · phosphate (pH 7.5)/3×10^5 cpm of [α-^{32}P]ATP-labeled synthetic gND7-II gRNA. The samples were loaded on a native-polyacrylamide gradient (4–16%) gel, which contained 0.1% Tween 20 and was stabilized with a 10–30% glycerol gradient [6]. The 14 cm × 14 cm gels were run at 125 V for 24 h at 4°C in 40 mM Tris-acetate/1 mM EDTA (pH 8). The wet gels were exposed to X-ray film and the experimental lanes were excised. The excised bands containing labeled complexes were irradiated with 254 nm UV light for 5 min at 25°C at a distance of 5 cm. from the UV source (Stratalinker UV box, Stratagene), incubated with RNase A (10 μg ml^{-1}) for 30 min at 37°C, and then incubated with 50 mM Tris (pH 6.8)/100 mM DTT/2% SDS/0.1% bromophenol blue, for 30 min at 25°C. The gel blocks were layered on a 0.1% SDS-12% polyacrylamide gel, electrophoresis was performed as previously described [7], and the gels were dried before autoradiography.

2.4. Two-dimensional separation of proteins

An aliquot of [α-^{32}P]UTP-labeled TS mitochondrial extract was first separated in a native-gradient gel as described above. The lane was excised, and incubated with 50 mM Tris (pH 6.8)/100 mM DTT/2% SDS/0.1% bromophenol blue, for 30 min at 25°C. The gel slice was layered on a 0.1% SDS-10% polyacrylamide gel for electrophoresis and the gel was stained with Coomassie blue.

2.5. Purification and microsequencing of proteins

The p51 and p71 proteins were separated by native-SDS two-dimensional gel electrophoresis, while p18 was separated by SDS-PAGE. The proteins were electroblotted onto Immobilon-P^{SQ} filters (Millipore) as described before [8], the filters were Coomassie-stained and the N-terminal amino-acid sequencing of the eluted p18, p51 and p71 proteins was performed by the UCLA Microsequencing Facility.

2.6. Cloning and analysis of the genes encoding the p18 and p51 proteins

The overall strategy was as follows: (*i*) Single-stranded cDNA primed by random hexanucleotides

was synthesized from *L. tarentolae* total cell poly(A)$^+$RNA. (*ii*) This cDNA was then used as a substrate for PCR amplification of fragments of the p18 and p51 genes, using the Sp18-3 and the Sp51-4 degenerate oligonucleotides as 3′ primers and a 26-nt oligonucleotide corresponding to a portion of the common spliced leader ('mini-exon') of all *Leishmania* mRNAs [9] (5′-AACTAACGCTATATAAGTATCAGTTT-3′) as a 5′ primer.

Experimental details are as follows: Total cell RNA was prepared as described [10], and poly(A)$^+$RNA was purified using the poly(A) Quick kit from Stratagene. The Sp18-3 (5′-⟨TC⟩TC⟨GA⟩TA⟨GATC⟩CC⟨GA⟩AA⟨GATC⟩A⟨GA⟩⟨GA⟩TC⟨GA⟩TA-3′) and the Sp51–4 (5′-AC⟨GA⟩AA⟨TC⟩TT⟨GATC⟩CC⟨AG⟩TT⟨GAT⟩AT⟨GATC⟩A⟨GA⟩⟨GATC⟩A⟨GA⟩⟨CT⟩TT⟨TC⟩TC-3′) degenerate oligonucleotides were generated by reverse translation of a 7-amino-acid N-terminal sequence of the p18 protein (YDLFGYE) and a 10-amino-acid N-terminal sequence of the p51 protein (EKLLINGKFV), respectively. The cDNA and primers were denatured for 10 min at 95°C, and then 30 cycles of PCR (10 s at 95°C, 30 s at 60°C, 30 s at 72°C) were performed in 50 μl reaction volumes as described previously [11]. The amplified fragments were gel-isolated and cloned into the pT7blue T-vector (Novagene). Both strands of 4 independent clones were sequenced by dideoxy-chain termination (Sequenase kit, US Biochemical) using the U-19-mer primer and the T7 promoter primer (Novagene). The PCR clones, pPCR18-6 and pPCR51-14, which contain fragments of the p18 and p51 genes were used for further analysis.

Two cDNA clones and a clone containing a large genomic DNA fragment (cos18) encoding p18 were obtained, from a *L. tarentolae* (UC strain) λgt11 cDNA library (kindly provided by D.A. Campbell) and from a *L. tarentolae* (UC strain) cosmid genomic DNA library (also provided by D.A. Campbell) [12], by screening with a [α-^{32}P]ATP-labeled PCR fragment derived from the PCR clone pPCR18-6. The cDNA fragments were subcloned into the pUC18 vector (plt18a and plt18b).

The same cosmid DNA genomic library from the UC strain of *L. tarentolae* [12] was screened by hybridization with a labeled PCR fragment derived from the PCR clone pPCR51-14 to clone a large genomic DNA fragment containing the gene encoding the p51 protein (cos51). The complete gene encoding p51, contained in a 5-kb DNA fragment, was subcloned into the pUC18 vector (plt51-5). The cDNA fragments contained in the plt18a and plt18b plasmids and the genomic DNA fragment contained in the plt51-5 plasmid were sequenced by the dideoxy-chain termination method (Sequenase kit) using specific oligonucleotide primers.

DNA and amino-acid sequences were analyzed using the DNA STRIDER program and the GCG sequence analysis software package, and database searches were done with BLAST. The nucleotide sequences of the cDNAs and the genomic DNA fragments contained in the plasmids plt18a and plt51-5 have EMBL accession numbers Z31697 and Z31698, respectively.

2.7. Southern blot analyses

Genomic DNA (2.5 μg) isolated as described elsewhere [13] or the cos18 genomic DNA fragment (1 μg) was subjected to endonuclease digestion, electrophoresed in a 0.8% agarose gel, and blotted onto a Hybond N + membrane (Amersham). The membranes were prehybridized 3 h at 37°C in 6 × SSC (1 × SSC is 0.15 M NaCl, 15 mM Na_3·citrate)/1 mM EDTA/50 mM NaH_2PO_4 (pH 7.0)/0.2% SDS/100 μg ml^{-1} sonicated salmon sperm DNA/0.1% polyvinylpyrrolidone/0.1% bovine serum albumin (Fraction V, Sigma)/0.1% Ficoll (Type 400, Pharmacia)/50% formamide, and hybridized 15 h at 37°C with the same buffer containing as a probe α-^{32}P-labeled PCR fragments which were labeled by random priming. The last washings were performed at 65°C in 0.2 × SSC/0.5% SDS before autoradiography. To rehybridize blots, probes were removed from the membranes by washing in boiling water containing 0.5% SDS.

2.8. Production of immune serum against the p18 protein and Western blot analysis

In order to obtain a large amount of protein for immunization challenges, the p18 protein was overexpressed in *E. coli*. An initial construct was created using the pTC226 plasmid, which contained the 15

C-terminal amino acids of the lactose permease gene from *E. coli* against which a polyclonal antibody was available [14]. A PCR fragment containing the p18 gene was generated using the plt18a plasmid as template and the oligonucleotides 5′-GCGGTCGGATCCATTGAAGGTAGAGCGTCCGCCGGTGGCGCGAAGA-3′ and 5′-AAGCGGGCCCGGATGATGATGATGATGTGTCTACCTTCAATCTGAATCTTGATGTCGAA-3′ as 5′ and 3′ primers, respectively. After digestion with *Bam*HI and *Apa*I, the PCR fragment was inserted into the *Bam*HI-*Apa*I fragment of the pTC226 plasmid, to create the pTC18 plasmid. This recombinant plasmid encodes the mature p18 protein, the C-terminal extremity of which is fused with six histidine residues followed by the 15 C-terminal amino acids of the lactose permease. The second plasmid construct, termed pGEX18, was constructed by recombination of a *Bam*HI + *Eco*RI-digested PCR fragment containing the p18 gene with the pGEX-5X-1 vector (Pharmacia) linearized by *Bam*HI and *Eco*RI restriction enzymes. The PCR fragment was obtained using the pTC18 plasmid DNA as template and the oligonucleotides 5′-CAGTGGATCCATATTGAAGGTAGAGCGTCCGCCGGTGGCG-3′ and 5′-ATCGGAATTCTTAAGCGACTTCATTCACCTGACGACG-3′ as 5′ and 3′ primers, respectively. The recombinant p18 protein encoded by the pGEX18 plasmid initiates with glutathione S-transferase (GST) followed by two factor Xa recognition sites at the N-terminal extremity, plus six histidine residues followed by the 15 C-terminal amino acids of the lactose permease at the C-terminal extremity.

E. coli (DH5α) transformed with the pGEX18 plasmid was grown in Luria-Bertani medium at 37°C to an OD_{600} of 0.6, and expression of the recombinant p18 protein was induced by an additional 4 hours of growth in presence of 1 mM isopropyl β-D-thiogalactopyranoside. Cells were disrupted in a French Press and the lysate centrifuged at 4000 rpm for 10 min to collect the inclusion bodies which were solubilized in buffer S (7 M urea/50 mM sodium phosphate, pH 8). The solution was applied to a Ni^{2+} nitriloacetic acid-agarose column (Qiagen), which was then washed with 10 column volumes of buffer S followed by 2 column volumes of the same buffer containing 5 mM imidazole. Recombinant p18 protein was eluted into buffer S containing 250 mM imidazole and was dialyzed against 50 mM Tris · HCl (pH 8)/0.8 M urea/1 mM $CaCl_2$/100 mM NaCl. The peptides obtained after proteolysis for 15 h at 27°C with 1 μg ml^{-1} Factor Xa (Boehringer-Mannheim) were separated by SDS-PAGE and the 20-kDa peptide containing the p18 protein was electroeluted for 15 h at 50 V in 0.1% SDS/50 mM NH_4HCO_3 using the Little Blue Tank™ electroelutor (ISCO). A rabbit was initially injected with 150 μg of the 20-kDa peptide containing the p18 protein with Freund's complete adjuvant, and all the following injections (days 28, 35, 42, 49, 56 and 77 after the first injection) were 100 μg of antigen with Freund's incomplete adjuvant. Blood from the rabbit was collected 7 days after each injection and tested for its ability to recognize the p18 protein.

Immunoblotting from SDS-polyacrylamide gels was carried out by using procedures modified from Ref. 8. Filters were blocked 15 min with TPBS-milk (137 mM NaCl/2.7 mM KCl/4.3 mM $Na_2HPO_4 \cdot 7H_2O$/1.4 mM KH_2PO_4/0.05% Tween-20/5% low fat dry milk) incubated 2 h with serum against the p18 protein 1:10000 in TPBS-milk, washed with TPBS, blocked again 15 min with PTBS-milk and incubated 1 h with goat anti-rabbit IgG conjugated to horseradish peroxidase (BioRad) 1:1000 in TPBS-milk. After washing with buffer A (50 mM Tris · HCl pH 7.5/20 mM NaCl) the filters were incubated with buffer A containing 0.05% H_2O_2/2.8 mM 4-chloro-1-naphthol.

2.9. Immunofluorescence

The procedures were modified from Ferguson et al. [15] and Melendy et al. [16]. Log phase cells were fixed in 2% paraformaldehyde in 0.15 M NaCl/5 mM K · phosphate (pH 7.4) (PBS) for 6 min at 25°C. The solution was adjusted to 0.1% Triton X-100 and incubated for 10 min. Glycine (0.1 M) was added to neutralize active aldehyde groups and incubation continued for 10 min. The cells were washed by centrifugation, resuspended in PBS, and let adhere to polylysine-treated slides for 30 min. The slides were prepared by treating an area with 1 mg ml^{-1} poly(L-lysine) for 15 min, rinsing with water and drying. The slides were blocked with 20% filtered goat serum/0.1% Tween-20 in PBS (blocking buffer) for

30 min and washed with PBS. The slides were then incubated with the p18 antiserum diluted 1:1000 with blocking buffer for 60 min and washed. Pre-immune serum was used as a control. The FITC-conjugated goat anti-rabbit second antibody (500 μg ml^{-1} stock solution, diluted 1:50) was added for 30 min. The slides were washed with PBS-0.1% Tween 20 and stained with 0.4 μg ml^{-1} DAPI in PBS. The slides were mounted with anti-fade solution (10% glycerol/0.1 M NaCl/50 mM Tris · HCl (pH 9.0)/1 mg ml^{-1} *p*-phenylenediamine (Fisher)/4 μl ml^{-1} 1 M NaOH), and the cover slips sealed with clear nail polish. The cells were observed with a Nikon UV microscope using the appropriate excitation and barrier filters, and micrographs taken with T-max 400 ASA film.

3. Results

3.1. Characterization of mitochondrial RNA-binding proteins

In order to detect guide RNA (gRNA)-binding proteins, the TS mitochondrial extract from *L. tarentolae* was fractionated by gel filtration in Superose 6 and the fractions incubated with [α-^{32}P]ATP-labeled synthetic guide RNA for editing block II of NADH dehydrogenase subunit 7 (gND7-II). As shown in Fig. 1A, the labeled gND7-II RNA migrated as several retarded bands as compared to free gRNA, and the abundance and size of the retarded bands varied from fraction to fraction. Proteinase K digestion of TS prior to incubation with the labeled gRNA eliminated the appearance of the high-molecular-mass labeled bands, whereas micrococcal nuclease or DNase I digestion of TS did not affect these bands (data not shown), indicating that the gel shift is due mainly to the binding of gRNA to proteins. Peris et al. [1] previously showed that the mitochondrial terminal uridylyl transferase (TUTase) activity and the associated RNP T-complexes fractionate in Superose 6 with a peak in fraction 14.

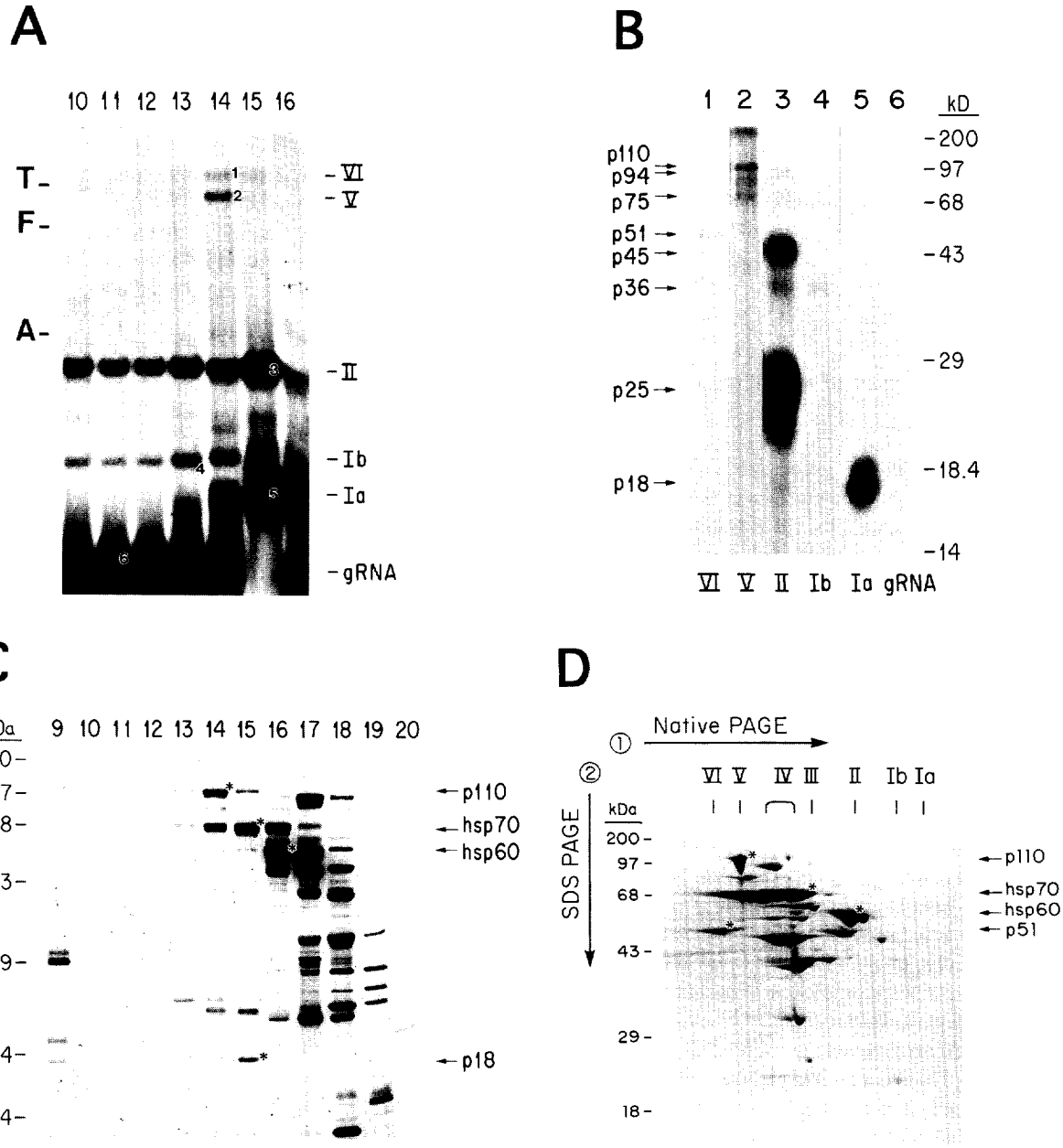
The indicated bands (numbered 1–5 in Fig. 1A) containing labeled specific gRNA-protein complexes were excised, and the gel slices irradiated with 254 nm UV light and then digested with RNase A. Each slice was then electrophoresed on a SDS-polyacrylamide gel (Fig. 1B, lanes 1–6 correspond to gel slices 1–6 in 1A) for identification of proteins which were UV-cross-linked to the labeled gRNA. A control experiment shown in Fig. 1B, lane 6, showed that UV irradiation and RNase A-treatment of labeled synthetic gRNA by itself did not generate any labeled bands. Several UV-cross-linked proteins were detected, including a 51-kDa protein from complex T-VI, a 110-kDa, a 94-kDa and a 75-kDa protein from T-V, 45, 36 and 25-kDa proteins from T-II, a 36-kDa protein from T-1b, and an 18-kDa protein from T-Ia.

Several specific UV-cross-linked proteins could be tentatively identified as stained bands on gels on the basis of the molecular mass. The p18 protein from T-Ia in fraction #15 from the Superose 6 column which interacts with exogenous gRNA (Fig. 1B, lane 5) was tentatively identified as the stained band indicated by an asterisk and arrow in Fig. 1C. In addition, the p110 and the p51 proteins were tentatively identified as Coomassie-stained spots in a two dimensional native-SDS gel of the TS extract, as shown in Fig. 1D. It should be stressed that these identifications of UV-cross-linked species as specific stained bands are tentative and must be confirmed by further work.

3.2. Cloning of the gene coding for the p18 protein

The p18 protein was isolated from Superose 6 fraction #15 by SDS-polyacrylamide gel electrophoresis as shown in Fig. 1C. The isolated band was used for microsequence analysis. A 17-amino-acid N-terminal sequence was obtained which lacked an N-terminal methionine residue. The 5′ portion of the gene for this protein was PCR-amplified from total cell cDNA (data not shown) by use of a 3′ degenerate oligonucleotide derived from back-translation of the amino-acid sequence and a 5′ oligonucleotide derived from the 39 nt miniexon sequence present at the 5′ end of all kinetoplastid mRNAs [17,18]. The PCR fragment was cloned and sequenced, and used as a probe to clone two cDNAs (plt18a and plt18b) from a *L. tarentolae* cDNA library. Although the two cDNAs show some sequence differences, both contain an identical open reading frame which encodes a protein of 187 amino acids, initiating with a methionine residue 17 amino acids upstream of the N-terminal amino acid of the

sequenced p18 protein (Fig. 2). The 17 N-terminal amino acids encoded by the cDNA but not present in the mature protein probably correspond to the mitochondrial signal sequence. The p18 protein is unrelated to any sequence in the current protein database by BLAST analysis, and contains no sequence motifs



recognized by the MOTIFS program (which uses the Prosite database) from the Genetics Computer Group package.

3.3. Genomic organization of the p18 genes

A cloned genomic DNA fragment which contains genes coding for the p18 protein was obtained by colony hybridization from a *L. tarentolae* UC strain cosmid library. A restriction map of the cloned genomic fragment was obtained by Southern analysis (Fig. 3A,B). Use of hybridization probe A, which is common to both cloned cDNAs, detected two p18 genes localized in tandem separated by a 1.7-kb intergenic sequence. Use of hybridization probes B and C, which are specific for the plt18a and plt18b cDNAs, respectively, localized the p18α and the p18β genes on the map as shown. Northern analysis (data not shown) indicated that an abundant 2.1-kb transcript is encoded by the p18β gene and a less abundant 2.0-kb transcript by the p18α gene.

3.4. Cellular localization of the p18 protein

Antibodies were raised against a recombinant p18 protein overexpressed in *E. coli*. Polyclonal antiserum was obtained by immunization of a rabbit with the 20-kDa recombinant p18 fusion protein (see Materials and methods). As shown in Fig. 4, lanes 3–5, this antiserum specifically recognized an 18-kDa protein in a mitochondrial fraction. No signal was obtained using a cytosolic fraction as shown in lane 2. These results are consistent with a mitochondrial localization of this protein.

In addition, the absence of degradation of the p18 protein in the TS extract and in the gel-filtration peak, as compared to the original TL extract, suggest strongly that N-terminal amino-acid sequence obtained is the N-terminal sequence of the mature imported protein and is not due to breakdown occurring during sample preparation.

3.5. Immunofluorescent localization of p18 protein

Cells were fixed and prepared for immunofluorescence as described in Materials and methods. The p18 antiserum yielded a bright fluorescence of the entire tubular mitochondrion (Fig. 5), whereas preimmune serum gave no fluorescence (data not shown). Careful analysis of the images showed that the kinetoplast nucleoid body did not show fluorescence, but that a region adjacent to the nucleoid body, which appears in the electron microscope to contain cristae, did fluoresce together with the remainder of the tubular mitochondrion extending throughout the cell, usually on one side of the nucleus. These results indicate that the p18 protein probably represents an inner membrane protein distributed throughout the entire unitary mitochondrion. A membrane localization of the p18 protein is also indicated by the presence of most of the protein in the membrane fraction after Triton clarification of the mitochondrial extract (Fig. 4, lanes 3 and 4).

Fig. 1. Detection of gRNA-binding proteins in mitochondrial extracts. (A) Aliquots (10 μl) of microconcentrated fractions #10 to #16 from a Superose 6 gel filtration of TS were incubated for 40 min at 27°C with 3×10^5 cpm of [^{32}P]ATP-labeled synthetic gRNA (gND7-II). The samples were electrophoresed in a native polyacrylamide gel. The locations of RNP complexes T-I to T-VI and the unbound gRNA are indicated on the right and the positions of molecular mass standards are indicated on the left: T, thyroglobulin (669 kDa); F, ferritin (443 kDa); A, β-amylase (200 kDa). (B) Several bands containing labeled gRNA/protein complexes were excised from the native polyacrylamide gradient gel in panel A. The specific bands which were excised and analyzed by UV cross-linking are labeled 1 to 6 in panels A and B. The bands were subjected to UV irradiation and RNase A digestion as described in Materials and methods. After electrophoresis (0.1% SDS-12% polyacrylamide), the gel was vacuum-dried and exposed to X-ray film. Molecular mass markers are indicated (kDa), and the molecular masses of the characterized proteins shown. The specific T-complexes (Ia, Ib, II, V and VI) and the free gRNA (gRNA) are indicated at the base of the lane. Panels C and D show silver (C) and Coomassie blue (D) staining of mitochondrial proteins from *L. tarentolae* in SDS polyacrylamide gels. In panel C, aliquots of microconcentrated fractions #9 to #20 from the Superose 6 gel filtration of TS were analyzed on a 0.1% SDS-12% polyacrylamide gel. Panel D shows a two-dimensional native-SDS gel: 50 μl (200 μg protein) of [α-^{32}P]UTP-labeled TS were electrophoresed in a native polyacrylamide gel (first dimension) and the proteins in the lane were separated by electrophoresis in a 0.1% SDS-10% polyacrylamide gel (second dimension). Molecular mass markers (kDa) are indicated on the left of both panels and the bands eluted for microsequence analysis are indicated by stars, with the identities of the proteins shown on the right. In panel D, the positions of the T-complexes in the native gel are indicated across the top.

3.6. Characterization of the p51 protein

The p51 protein, which migrates in native gels in the T-VI position, was isolated for microsequence analysis by two dimensional native-SDS gel electrophoresis, as shown in Fig. 1D. This analysis yielded a 22-amino-acid N-terminal sequence (Fig. 6, sequence underlined). A cloned PCR product corresponding to the 5′ extremity of the gene was obtained using the strategy described above for the p18 protein. The cloned fragment was used as a probe to clone a large genomic fragment, which was partially sequenced. The DNA sequence, shown in Fig. 6, contains an open reading frame coding for

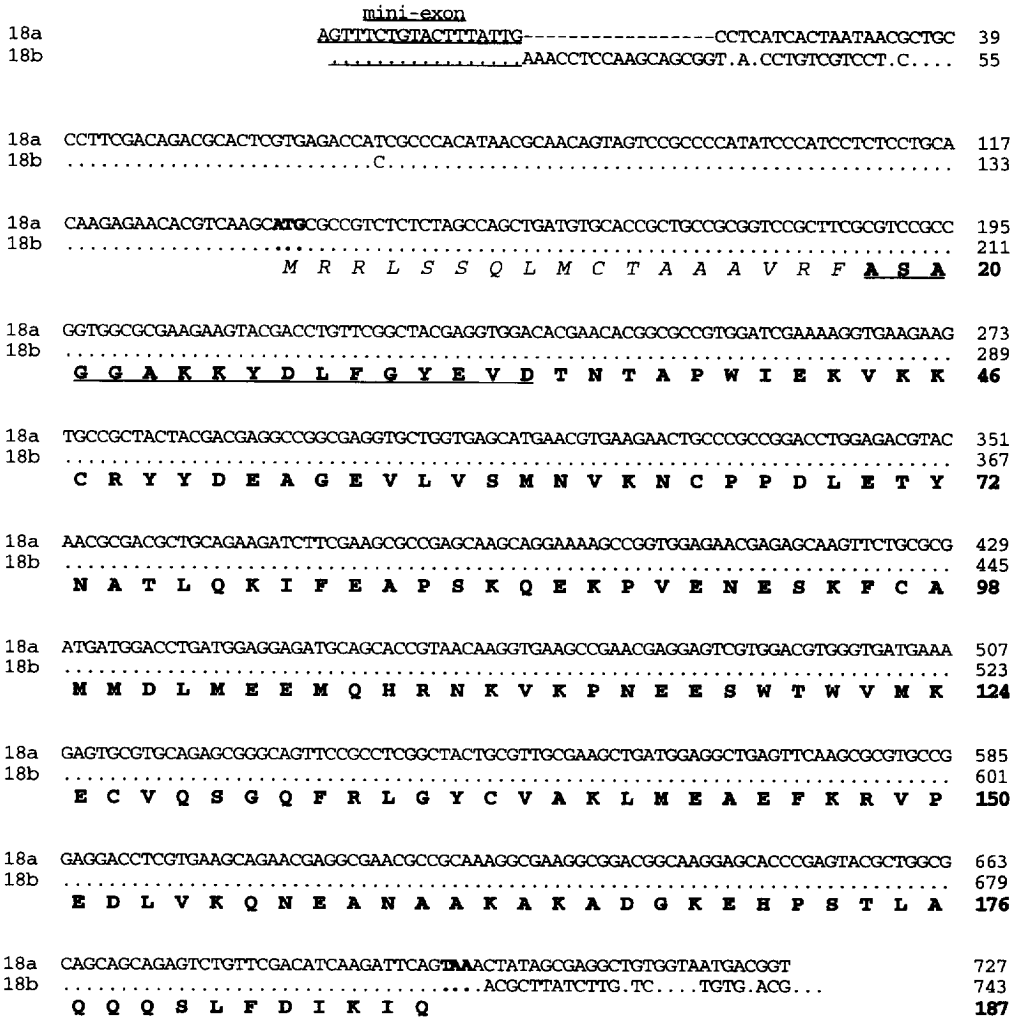


Fig. 2. DNA sequence and predicted amino-acid sequence of the cDNAs encoding the p18 protein. Sequence identity between the cDNAs contained in the plt18a (18a) and plt18b (18b) plasmids are represented by dots (·) in the plt18b sequence. Gaps (-) are introduced to maximize the alignment. The underlined nucleotide sequences at the 5′ extremity of both cDNAs correspond to partial mini-exon sequences used for PCR amplification. The bold-faced nucleotides represent the first ATG codon and the stop codon of the open reading frame encoding the p18 protein, the sequence of which is shown on the third lane of the alignment. The underlined amino-acid sequence was previously determined by microsequencing the N-terminal extremity of the p18 protein, and the amino acids in italics constitute the putative cleavable mitochondrial targeting sequence.

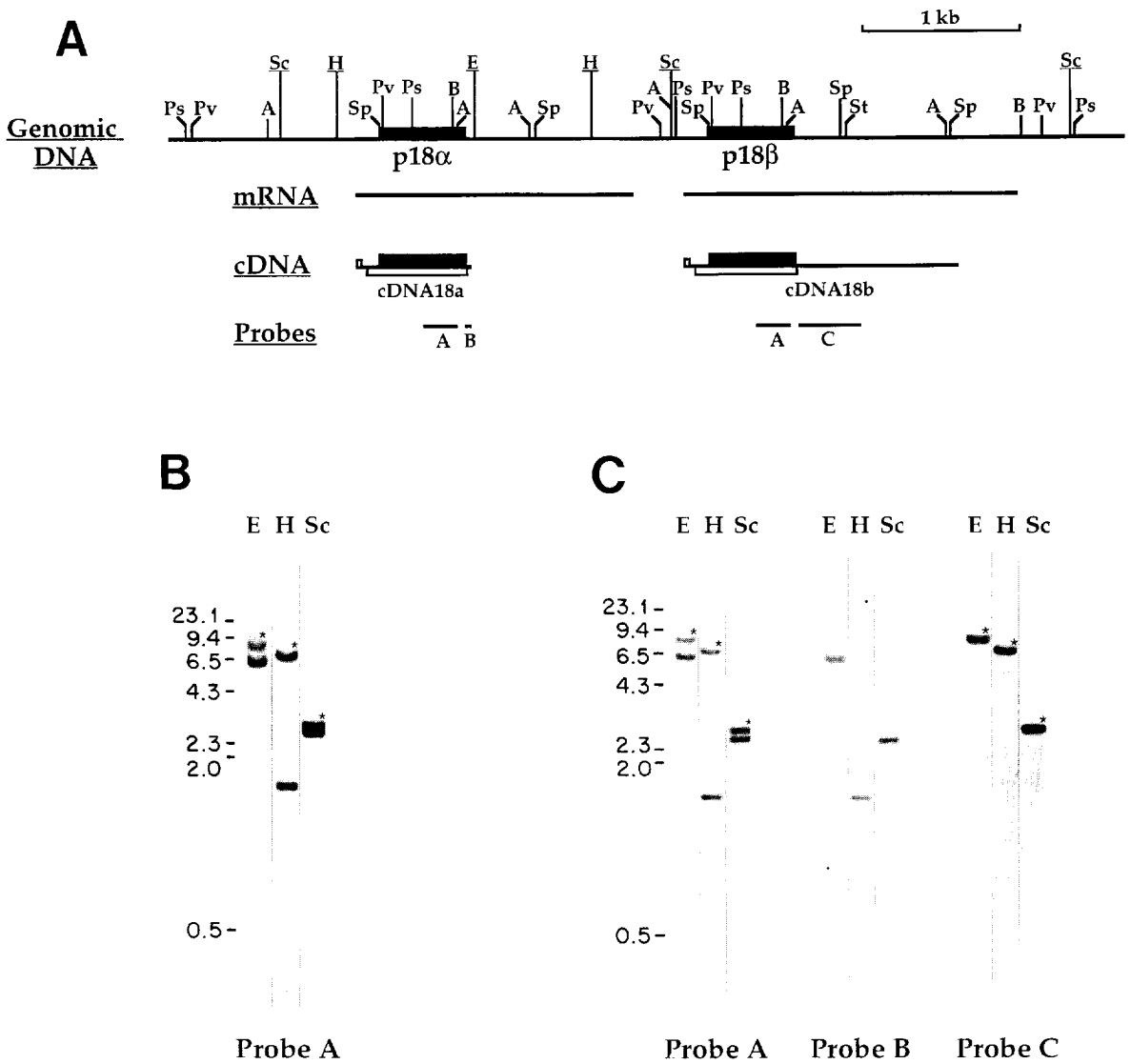


Fig. 3. Genomic organization of the p18 encoded genes. (A) The restriction map of the 6-kb genomic DNA fragment containing the p18 genes was constructed from the cos18 DNA fragment analysis (partially shown in panel C). The cleavage sites of the restriction enzymes used in panels B and C are underlined. The position of the two steady-state mRNAs detected by Northern blotting (data not shown) and the two cloned cDNA fragments encoding the p18 proteins are shown under the map. The black boxes represent the open reading frames encoding the p18α and p18β proteins, the open boxes under the cDNAs show the identical sequences between the cDNA18a and 18b, and the open squares at the 5′ end of the cDNAs represent the mini-exons. The position of the probes A, B and C, used in the panels B and C, is also shown. (B and C) Restriction endonuclease digestion of *L. tarentolae* (UC strain) genomic DNA (B) or of the cos18 genomic DNA fragment (C) were separated, blotted and hybridized with the probe common to both p18 genes (probe A) as described in Materials and methods. The blot in panel C was hybridized successively with the probes B and C which are specific to the 3′ untranslated region of the cDNAs encoding p18α and p18β, respectively. The DNA fragments containing the p18β gene are indicated by asterisks (*), and the size of the molecular mass markers is indicated in kb. Abbreviations for restriction sites: A, *Ava*I; B, *Bgl*I; E, *Eco*RI; H, *Hin*dIII; Ps, *Pst*I; Pv, *Pvu*II; Sc, *Sac*I; Sp, *Sph*I; St, *Stu*I.

498 amino acids. The open reading frame initiates with a methionine residue located 9 amino acids upstream of the N-terminal amino acid of the p51 protein determined by direct amino-acid sequencing. The 9-amino-acid N-terminal sequence probably represents a cleavable signal peptide for mitochondrial targeting.

A BLAST computer search of this sequence against the Swiss protein database showed this sequence to have significant similarity with mitochondrial aldehyde dehydrogenases (EC 1.2.1.3), with the best alignment occurring with the human enzyme (ALDH2) [19] which exhibited 51% identity with the p51 sequence (Fig. 6). In addition, 37 of the 41 amino acids conserved between seven aldehyde dehydrogenases from different organisms are present at the same relative positions in the p51 protein. These data indicate that the p51 protein is an aldehyde dehydrogenase, and it will therefore be referred to as LtALDH2.

The Southern blot analysis shown in Fig. 7 indicates that the LtALDH2 gene is single copy, since the probe only detected a single restriction fragment of variable size when genomic DNA was digested with several enzymes.

3.7. Identification of the mitochondrial hsp60 and hsp70 proteins

A monoclonal antibody against the mitochondrial hsp60 protein from *Trypanosoma brucei* (F. Bringaud, S. Maillet, D. Baltz, C. Giroud, L. Simpson and T. Baltz, unpublished results) recognized a 61-kDa protein from *L. tarentolae* (data not shown). This protein migrated in a native gel in the vicinity of the T-II and T-III RNP complexes [1], as shown in the stained two dimensional native-SDS gel in Fig. 1D. This band (labeled with * in Fig. 1D) was eluted and subjected to microsequence analysis. The N-terminal sequence obtained (AGKDVRFG) was identical to the sequence 8 amino acids downstream of the initiating methionine of the hsp60 protein from *T. brucei* (F. Bringaud, S. Maillet, D. Baltz, C. Giroud, L. Simpson and T. Baltz, unpublished results). The first 8 amino acids of the *T. brucei* hsp60 gene are believed to constitute the cleavable mitochondrial targeting sequence.

An abundant 71-kDa mitochondrial protein migrated in the stained native SDS gel as a smear from the T-III to the T-VI region, as shown in Fig. 1D. A portion of this band (labeled with *) was subjected to micro-sequence analysis. The 22-amino-acid N-terminal sequence obtained (ESQKVOGDVIGVDLGTTYSCVA) proved to be identical to the sequence of the encoded hsp70 from *L. major* 20 amino-acids downstream of the initiating methionine, and differed by two amino acids from the sequence of the hsp70 protein from *C. fasciculata* (**AS**DKVQGDVIGVDLGTTYSCVA) at the same

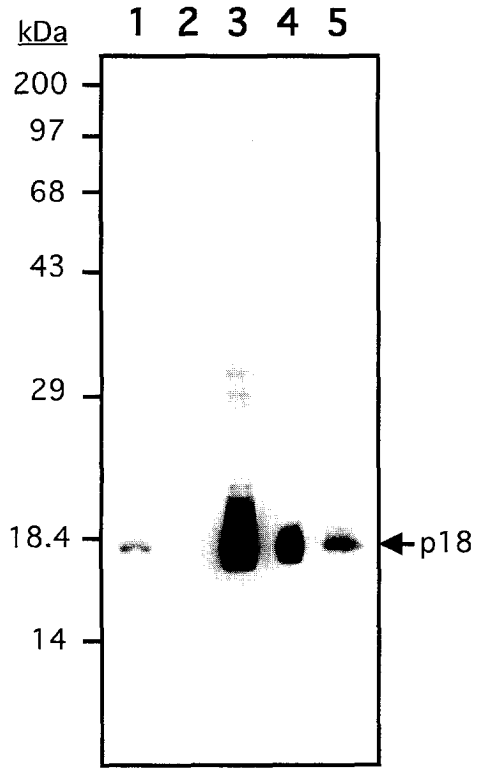


Fig. 4. Immunodetection of the p18 protein. Cell extracts from *L. tarentolae*, after SDS-PAGE and transfer to a nitrocellulose membrane, were probed with antibodies raised against the p18 protein as described in Materials and methods. Lane 1, total cell extract; lane 2, cytosolic fraction; lane 3, mitochondrial fraction; lane 4, mitochondrial TS extract; lane 5, fraction #15 of the Superose 6 gel-filtration separation of the proteins from the mitochondrial TS extract. Lanes 1 to 4 contain 150 μg protein, while lane 5 contains 10 μg protein. The standard protein markers (kDa) are indicated on the left hand side of the gel, and the position of the p18 protein is shown.

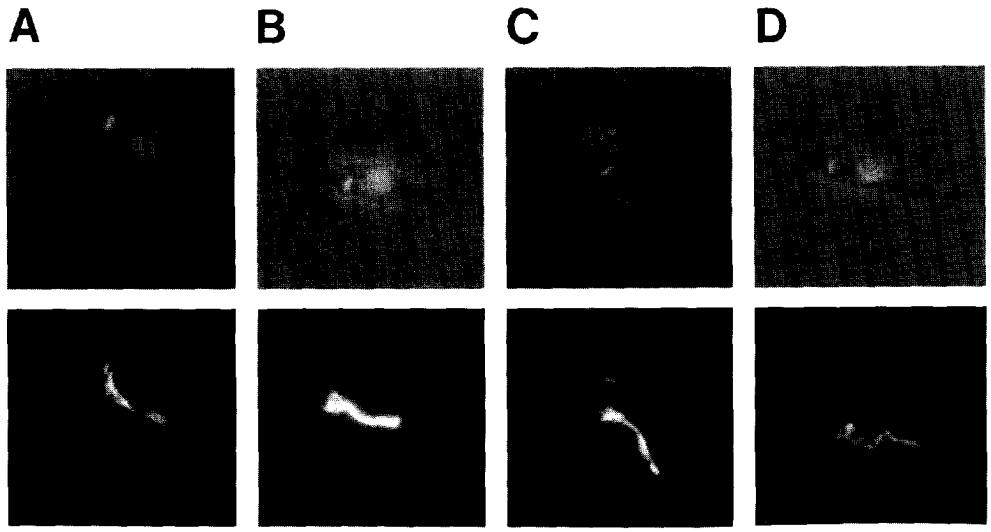


Fig. 5. Immunofluorescence localization of p18 protein in *L. tarentolae* cells. Cells were fixed and processed for immunofluorescence as described in Materials and methods. Several examples of cells stained with the p18 antiserum and FITC-conjugated second antibody are shown (bottom panels), together with the same cells showing the DAPI-stained kinetoplast and nucleus and the cell in phase contrast (top panels).

relative location. This evidence identifies the 71-kDa *L. tarentolae* mitochondrial protein as an hsp70 homologue, and suggests that an N-terminal signal peptide had been cleaved upon transport into the mitochondrion.

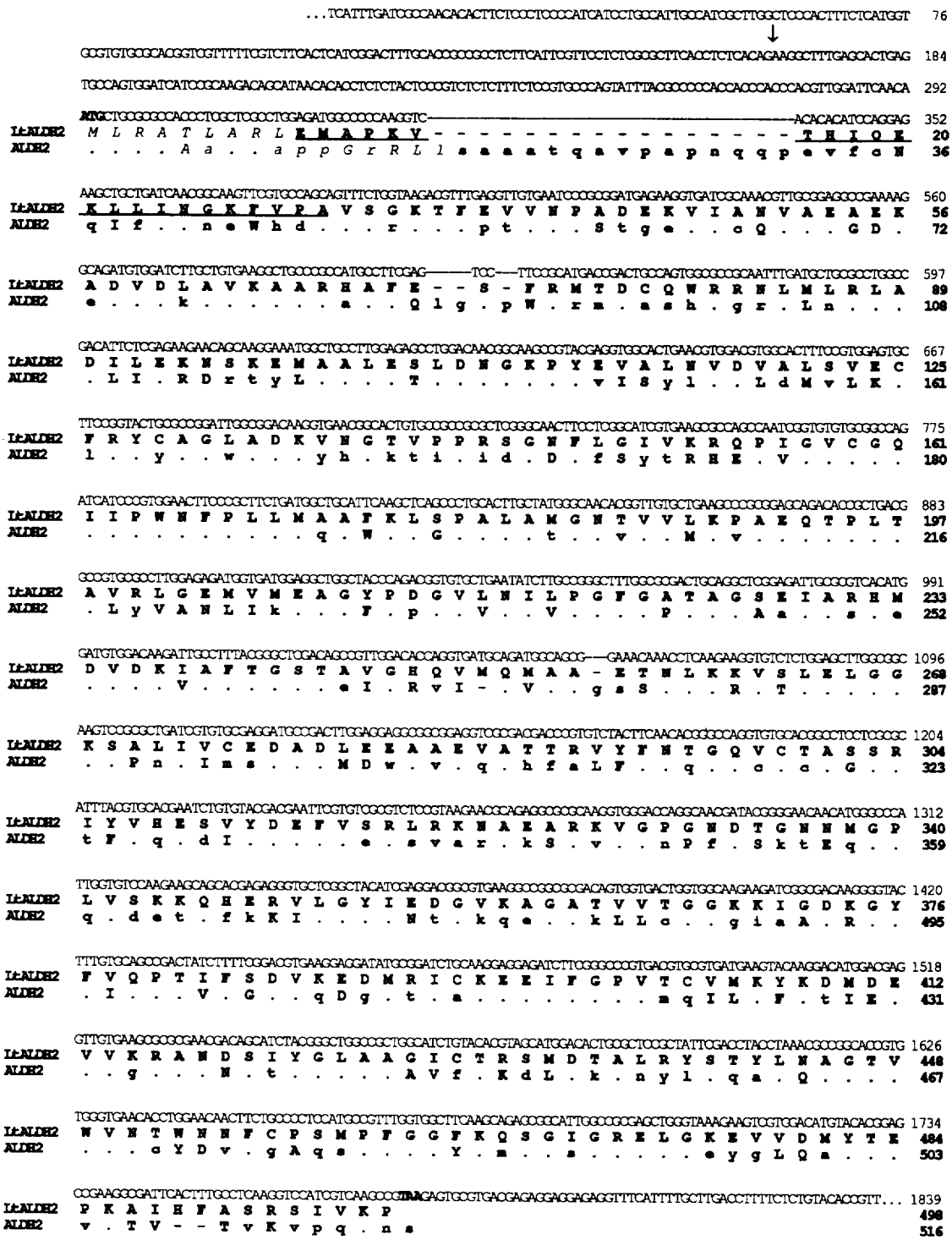
It is of some interest that a heterologous monoclonal antibody against an epitope in mammalian hnRNP C proteins [20] reacted with the mitochondrial hsp70 from *L. tarentolae* (data not shown). The reason for this cross-reactivity is not known.

4. Discussion

Two proteins with molecular masses of 18 and 51 kDa, as analyzed by SDS-PAGE, were isolated from a *L. tarentolae* mitochondrial extract and the genes cloned and characterized. These proteins co-migrate in native gels with specific RNP T-complexes: p18 is a possible component of complex T-Ia and p51 a possible component of complex T-VI.

By gel-retardation experiments, it was shown that labeled synthetic gRNA binds to several of the T-complexes, and from UV cross-linking experiments it was shown that an 18-kDa component of T-Ia and a 51-kDa component of T-VI interacted closely with the labeled gRNA. The identified p18 and p51 proteins may correspond to the proteins which were labeled by UV cross-linking to gRNA. In the case of the p18 protein, however, the immunofluorescence results indicate a lack of localization of the protein with the kinetoplast DNA nucleoid body, but rather a distribution throughout the entire mitochondrion, making it less likely that this cloned protein is the UV cross-linked 18-kDa protein, since a kDNA localization might have been expected for a gRNA-binding protein. A definitive answer to the question whether the cloned p18 and p51 proteins have gRNA-binding activity awaits direct analysis of RNA-binding activity of the expressed proteins.

The cDNAs encoding the p18 and p51 proteins both contain at their 5′ extremity the mini-exon sequence which is located at the 5′ end of all nuclear-encoded mRNAs in trypanosomatid protozoa [17,18]. Analysis of genomic sequences showed that the p51 protein is encoded by a single copy gene, whereas the gene for the p18 protein is present as two identical copies separated by a 1.7-kb intergenic region. Both p18 genes are transcribed, with the mRNA encoding p18β being more abundant than the mRNA encoding p18α. This differential expression may be due to the observed sequence differ-


...TCATTTGATCGCCAACACACTTCTCCCTCCCCATCATCCTGCCATTGCCATCGCTTGGCTCCCACTTTCTCATGGT 76
GCGTGTGCGCACGGTCGTTTTTCGTCTTCACTCATCGGACTTTGCACCCCCCGCCTCTTCATTCGTTCCTCTCGCGCTTCACCTCTCACAGAAGGCTTTGAGCACTGAG 184
TGCCAGTGGATCATCCGCAAGACAGCATAACACACCTCTCTACTCCCGTCTCTCTTTCTCCGTGCCCAGTATTTACGCCCCCACCACCCACCCACGTTGGATTCAACA 292
ATGCTGCGCGCCACCCTCGCTCCCCTGGAGATGCCCCCCAAGGTC----------ACACACATCCAGGAG 352
IcALDH2 M L R A T L A R L K M A P K V - - - - - - - - - - - - - - - - - - - T H I Q K 20
ALDH2 . . . . A a . . a p p G r R L l s a a a t q a v p a p n q q p e v f c N 36
AAGCTGCTGATCAACGGCAAGTTCGTGCCAGCAGTTTCTCGTAAGACGTTTGAGGTTGTGAATCCCGCGGATGAGAAGGTGATCGCAAACGTTGCGGAGCCCGAAAAG 560
IcALDH2 K L L I N G K F V P A V S G K T F E V V N P A D E K V I A N V A E A E K 56
ALDH2 q I f . . n e W h d . . . r . . . p t . . . S t g e . . c Q . . . G D . 72
GCAGATGTGGATCTTGCTGTGAAGGCTGCCCCCCATGGCTTCGAG-------TCC---TTCCGCATGACGGACTGCCAGTGGCGCCGCAATTTGATGCTGCGCCTGCCC 597
IcALDH2 A D V D L A V K A A R H A F E - - S - F R M T D C Q W R R W L M L R L A 89
ALDH2 e . . . k . . . . . . a . . Q l g . p W . r m . a s h . g r . L n . . . 108
GACATTCTCGAGAAGAACAGCAAGGAAATGGCTGCCTTGGAGAGCCTGGACAACGGCAAGCCGTACGAGGTGGCACTGAACGTGGACGTGGCACTTTCCGTGGAGTGC 667
IcALDH2 D I L E K N S K E M A A L E S L D N G K P Y E V A L N V D V A L S V E C 125
ALDH2 . L I . R D r t y L . . . . T . . . . . v I S y l . . L d M v L K . 161
TTCCGGTACTGCGGCCGGATTGGCGGACAAGGTGAACGGCACTGTGCCGCCGGCTCGGGCAACTTCCTCGGCATCGTGAAGCGCCAGCCAATCGGTGTGTGCGGCCAG 775
IcALDH2 F R Y C A G L A D K V N G T V P P R S G N F L G I V K R Q P I G V C G Q 161
ALDH2 l . . y . . w . . . y h . k t i . i d . D . f S y t R H E . V 180
ATCATCCCGTGGAACTTCCCGCTTCTGATGGCTGCATTCAAGCTCAGCCCTGCACTTGCTATGGGCAACACGGTTGTGCTGAAGCCCCCGGAGCAGACACCGCTGACG 883
IcALDH2 I I P W N F P L L M A A F K L S P A L A M G N T V V L K P A E Q T P L T 197
ALDH2 . . . . . q . W . . G . . . . t . . v . . M . v 216
GCCGTGCGCCTTGGAGAGATGGTGATGGAGGCTGGCTACCCAGACGGTGTGCTGAATATCTTGCCCGGCTTTGGCCCGACTGCAGGCTCGGAGATTGCCGGTCACATG 991
IcALDH2 A V R L G E M V M E A G Y P D G V L N I L P G F G A T A G S E I A R H M 233
ALDH2 . L y V A N L I k . . . F . p . . V . . V . . . . . P . . . A a . . s . e 252
GATGTGGACAAGATTGCCTTTACGGGCTCGACAGCCGTTGGACACCAGGTGATGCAGATGGCAGCG--GAAACAAACCTCAAGAAGGTGTCTCTGGAGCTTGGCGGC 1096
IcALDH2 D V D K I A F T G S T A V G H Q V M Q M A A - E T N L K K V S L E L G G 268
ALDH2 . . . . V . . . . . e I . R v I - . V . . g s S . . . R . T 287
AAGTCCGCGCTGATCGTGTGCGAGGATGCCGACTTGGACGACGCGCCGCGAGGTCGCCGACGACCCGTGTCTACTTCAACACGGCCAGGTGTGCACCGCCTCCTCGCGC 1204
IcALDH2 K S A L I V C E D A D L E E A A E V A T T R V Y F N T G Q V C T A S S R 304
ALDH2 . . P n . I m s . . . M D w . v . q . h f a L F . . q . . c . c . G . . 323
ATTTACGTGCACGAATCTGTGTACGACGAATTCGTGTCGCGTCTCCGTAAGAACCCACAGGCGCGCAAGGTGGGACCAGCCAACGATACGGGGAACAACATGGGCCCA 1312
IcALDH2 I Y V H E S V Y D E F V S R L R K N A E A R K V G P G N D T G N N M G P 340
ALDH2 t F . q . d I . . . . . e . s v a r . k S . v . . n P f . S k t E q . . 359
TTGGTGTCCAAGAAGCAGCACGAGAGGGTGCTCGGCTACATCGAGGACGGCGTGAAGGCCGGCGGCACAGTGGTGACTGGTGGCAAGAAGATCGGCGACAAGGGTAC 1420
IcALDH2 L V S K K Q H E R V L G Y I E D G V K A G A T V V T G G K K I G D K G Y 376
ALDH2 q . d e t . f k K I . . . . N t . k q e . . k L L c . . g i e A . R . . 395
TTTGTCCAGCCGACTATCTTTTCGGACGTGAAGGAGGATATGCGGATCTGCAAGGAGGAGATCTTCGGGCCCGTGACGTGCGTGATGAAGTACAAGGACATGGACGAG 1518
IcALDH2 F V Q P T I F S D V K E D M R I C K E E I F G P V T C V M K Y K D M D E 412
ALDH2 . I . . . V . G . . q D g . t . a . . . . . m q I L . F . t I E . 431
GTTGTGAAGCGCGGCAACGACAGCATCTACGGCCTGGCCGCTGGCATCTGTACACGTAGCATGGACACTGCGCTCCGCTATTCGACCTACCTAAACGCCGGCACCGTG 1626
IcALDH2 V V K R A N D S I Y G L A A G I C T R S M D T A L R Y S T Y L N A G T V 448
ALDH2 . . g . . . N . t . . . . . A V f . K d L . k . n y l . q a . Q 467
TGGGTGAACACCTGGAACAACTTCTGCCCCTCCATCCCGTTTGGTGGCTTCAAGCAGAGCGGCATTGGCCGCGAGCTGGGTAAAGAAGTCGTCGACATGTACACCGAG 1734
IcALDH2 N V N T W N N F C P S M P F G G F K Q S G I G R E L G K E V V D M Y T E 484
ALDH2 . . . c Y D v . g A q s . . . . Y . m . . s . . . . . e y g L Q a . . . 503
CCGAAGCCGATTCACTTTGCCTCAAGGTCCATCGTCAAGCCGTAAGAGTGCGTGACGAGAGGAGGAGAGGTTTCATTTTGCTTGACCTTTTCTCTGTACACCGTT... 1839
IcALDH2 P K A I H F A S R S I V K P 498
ALDH2 v . T V - - T v K v p q . n s 516

ences in the 5′ and 3′ untranslated regions of the p18α and p18β genes which could affect the efficiency of splicing, polyadenylation, or turnover.

The p18 protein has no similarity with any sequence in the database. The p51 protein, however, was identified by computer analysis as a mitochondrial aldehyde dehydrogenase, LtALDH2. This does not completely eliminate the possibility that the LtALDH2 protein also has RNA-binding capacity, since there is evidence that several metabolic enzymes including glutamate dehydrogenase [21], isocitrate dehydrogenase [22], glyceraldehyde-3-phosphate dehydrogenase [23], 3-oxoacyl-CoA thiolase [24] and aconitase [25] also exhibit RNA-binding properties which may or may not have a biological significance.

Several lines of evidence indicate that LtALDH2 and the p18 protein are located in the mitochondrion of *L. tarentolae*. Both proteins were isolated from mitochondrial extracts which had very little cytosolic contamination (data not shown), and polyclonal antibodies raised against a recombinant p18 protein recognized an 18-kDa protein which is enriched in the mitochondrial fraction and, by immunofluorescence is specifically localized throughout the entire tubular mitochondrion. Furthermore, the LtALDH2 sequence shows a greater similarity in a BLAST database search to mitochondrial aldehyde dehydrogenases than to cytosolic aldehyde dehydrogenases from other organisms (data not shown). Finally, the genes encoding LtALDH2 and p18 contain short N-terminal peptides which have the characteristics of cleavable mitochondrial targeting sequences.

Two additional *L. tarentolae* mitochondrial proteins which co-migrate with T-complexes in native gels were gel-isolated and N-terminal sequences obtained. These proved to be homologues of mitochondrial hsp60 and hsp70 proteins, respectively, and

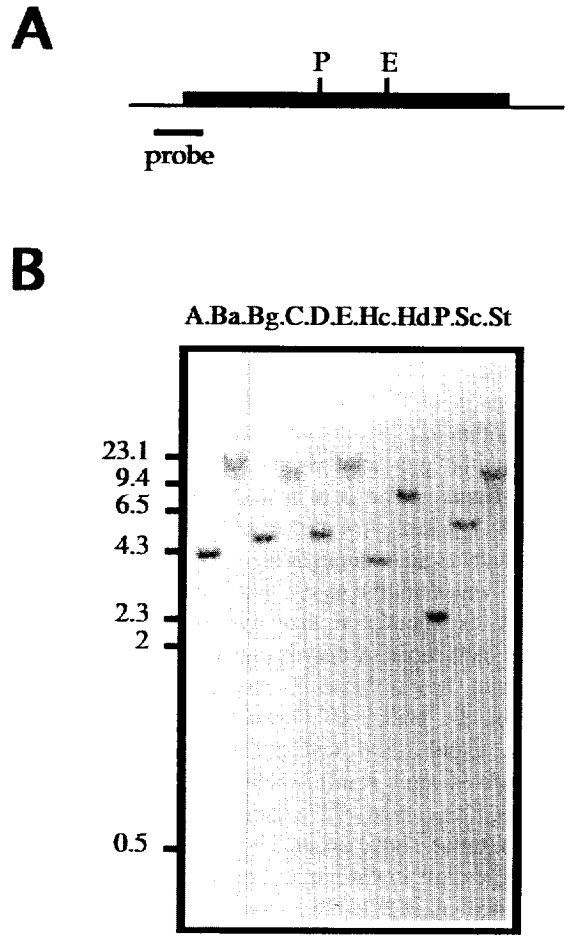


Fig. 7. Genomic organization of the p51 gene. (A) Restriction map of the sequenced genomic DNA fragment containing the p51 gene. The black box represents the open reading frame encoding the p51 protein and the position of the probe used in panel B is indicated under the map. (B) Restriction enzyme-digested DNA from *L. tarentolae* (UC strain) was fractionated by gel electrophoresis, blotted and hybridized with a probe specific to the p51 gene (panel A), as described in Materials and methods. The sizes of the molecular mass markers are indicated in kb. A, *Ava*I; Ba, *Bam*HI; Bg, *Bgl*I; C, *Cla*I; D, *Dra*I; E, *Eco*RI; Hc, *Hin*cII; Hd, *Hin*dIII; P, *Pst*I; Sc, *Sac*I; Sp, *Sph*I.

Fig. 6. Alignment of the translated amino-acid sequence of the p51 protein with the human mitochondrial aldehyde dehydrogenase sequence. A partial sequence of the plt51-5 genomic DNA fragment showing the p51 gene and the translated amino-acid sequence aligned with the amino-acid sequence of the human mitochondrial aldehyde dehydrogenase protein (ALDH2) [19]. Amino-acid matches are indicated by dots, and conservative and nonconservative amino-acid substitutions are shown by capital and lower-case letters, respectively. Gaps introduced into the LtALDH2 nucleotide and amino-acid sequences to maximize the alignment are shown as dashes. The arrow indicates the trans-splicing site deduced from the position of the miniexon in the 253-bp PCR fragment (pPCR51-14) encoding the N-terminal extremity of p51 (data not shown). The first ATG codon corresponding to the probable translation initiation codon and the stop codon of the p51 gene are in bold face. The nucleotide and amino-acid numbers are shown on the right. The underlined amino acids indicate the N-terminal sequence obtained by sequencing the electroblotted p51 protein, and the putative mitochondrial targeting sequence is in italics.

circumstantial evidence was presented that these proteins also contain cleavable N-terminal sequences. Preliminary UV cross-linking evidence was obtained for a tentative identification of hsp70 as possessing gRNA-binding activity (F. Bringaud and L. Simpson, unpublished results), but this must be confirmed by further work.

It should be stressed that the role of an amino-acid sequence in protein targeting must be experimentally established by transfection or in vitro importation studies, and such studies remain to be performed in the case of the p18, LtALDH2, hsp60 and hsp70 proteins from *L. tarentolae*. Nevertheless, the observation that the N-terminal amino-acid sequences of the mature, gel-isolated proteins lack several amino acids found in the gene sequences is strong circumstantial evidence for the existence of cleavable N-terminal signal sequences. Furthermore, the 9-amino-acid putative signal sequence of the LtALDH2 protein is similar in size and composition to the signal sequences of dihydrolipoamide dehydrogenase [26] and hsp60 from *T. brucei* (F. Bringaud, S. Maillet, D. Baltz, C. Giroud, L. Simpson and T. Baltz, unpublished results), and to the signal sequences of three small kDNA-binding basic proteins from *C. fasciculata* [27]. In addition, the 17-amino-acid putative signal sequence of the p18 protein from *L. tarentolae* is similar to the 20-amino-acid signal sequence of the hsp70 protein from *C. fasciculata* [28], and probably also the hsp70 from *L. tarentolae*, as shown in this paper.

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