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Native gel analysis of ribonucleoprotein complexes from a *Leishmania tarentolae* mitochondrial extract

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Abstract

Two polypeptides of 50 and 45 kDa were adenylated by incubation of a mitochondrial extract from *Leishmania tarentolae* with [α-^{32}P]ATP. These proteins were components of a complex that sedimented at 20S in glycerol gradients and migrated as a single band of approximately 1800 kDa in a native gel. The facts that RNA ligase activity cosedimented at 20S and that the ATP-labeled p45 and p50 polypeptides were deadenylated upon incubation with a ligatable RNA substrate suggested that these proteins may represent charged intermediates of a mitochondrial RNA ligase. Hybridization of native gel blots with guide RNA (gRNA) probes showed the presence of gRNA in the previously identified T-IV complexes that sedimented in glycerol at 10S and contained terminal uridylyl transferase (TUTase) activity, and also in a previously unidentified class of heterodisperse complexes that sedimented throughout the gradient. gRNAs were not detected in the p45 + p50-containing 1800 kDa complex. The heterodisperse gRNA-containing complexes were sensitive to incubation at 27°C and appear to represent complexes of T-IV subunits with mRNA. Polyclonal antiserum to a 70 kDa protein that purified with terminal uridylyl transferase activity was generated, and the antiserum was used to show that this p70 polypeptide was a component of both the T-IV and the heterodisperse gRNA-containing complexes. We propose that the p45 + p50-containing 1800 kDa complex and the p70 + gRNA-containing heterodisperse complexes interact in the editing process. Further characterization of these various complexes should increase our knowledge of the biochemical mechanisms involved in RNA editing. © 1997 Elsevier Science B.V.

Keywords: Leishmania; RNA editing; TUTase; RNA ligase; p70; gRNA; Kinetoplastid

Abbreviations: TUTase, terminal uridylyl transferase; gRNA, guide RNA; Cyb, cytochrome B; A6, ATPase, subunit 6; RPS12, ribosomal protein S12; COIII, cytochrome oxidase, subunit III; gCyb-II, gRNA that edits block II of cytochrome b; TS, cleared supernatant of a Triton X-100 lysed mitochondrial extract; p70, 70 kDa protein; nt, nucleotide.

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1. Introduction

The sequence information to insert and delete uridine (U) residues in mitochondrial mRNAs from kinetoplastid protozoa is provided by small guide RNA (gRNA) transcripts encoded in both the maxicircle and the minicircle components of the kinetoplast mitochondrial DNA. The gRNAs form an anchor duplex with the preedited mRNA just downstream of the sequence block to be edited and mediate the addition (and deletion) of U's by base-pairing with guide A or G nucleotides (nt), resulting in an extended gRNA-mRNA duplex. Several models have been proposed for this process and recent evidence suggests that the original enzyme cascade cleavage-ligation model [1] is the most likely mechanism [2–8]. In this model, several enzymatic activities which had been identified in mitochondrial extracts from trypanosomatids were proposed to sequentially participate in the insertion or deletion of uridines into the preedited region of the mRNA: an editing site-specific endonuclease [9–11], a terminal uridylyl transferase (TUTase) [12], a 3′ U-specific exonuclease [1] and an RNA ligase [12,13].

The presence in mitochondrial extracts of ribonucleoprotein (RNP) complexes containing various putative enzymatic and RNA components of the editing machinery has also been reported. In *Trypanosoma brucei*, two RNP complexes have been proposed to exist on the basis of a cosedimentation of several activities in glycerol gradients: Complex I which migrates at 19S and contains gRNA, RNA ligase, TUTase, and chimera-forming activity, and Complex II which migrates at 35–40S and contains the same components but lacks tightly bound TUTase and contains preedited mRNA [14]. Two adenylated proteins (57 and 50 kDa) were shown to cosediment with these complexes. Addition of a ligatable RNA substrate to gradient fractions containing these adenylated proteins resulted in deadenylation, suggesting that these proteins represent the AMP-charged intermediates of an RNA ligase [13]. Piller et al. [11] also reported a cosedimentation of TUTase, RNA ligase, an editing domain-specific endonuclease [9,10], and chimera-forming activity at approximately 20S, but did not detect a 35S complex. Four RNP complexes, labeled G1–G4, have been visualized by gel retardation analysis of labeled gRNA, using *T. brucei* mitochondrial extracts [15–18]. G1, G2 and G3 sedimented in glycerol gradients ranging from 10–20S, well separated from the peak of TUTase, RNA ligase, and RNA helicase activities that sedimented at 35–40S [19]. The G4 complex sedimented at 35-40S [19].

In *L. tarentolae*, sedimentation analysis and native gel electrophoresis of a mitochondrial extract has been used to detect several classes of

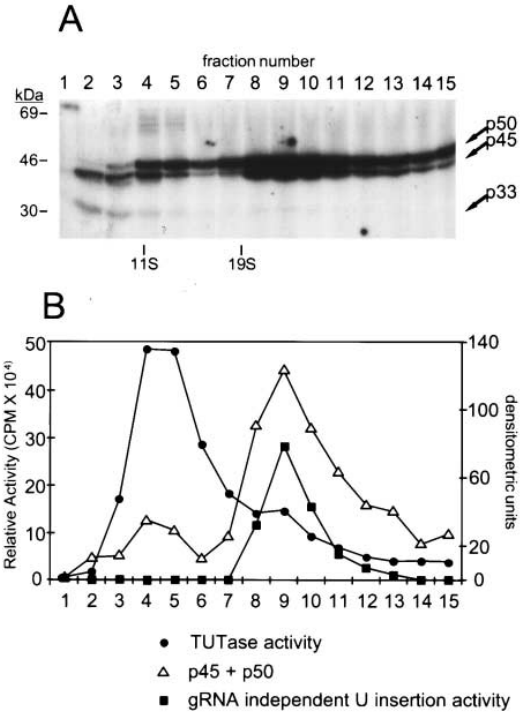


Fig. 1. Sedimentation analysis of *L. tarentolae* mitochondrial extract in terms of [α-^{32}P]ATP-labeled proteins, TUTase activity and gRNA-independent U-insertion activity. (A) Aliquots of glycerol gradient fractions were incubated with [α-^{32}P]ATP and the labeled proteins fractionated on a 10% SDS acrylamide gel. The positions of gel molecular weight standards are shown on the left and the S values of cosedimenting standards are shown at the bottom. The p45, p50 and p33 bands are indicated by arrows. (B) Quantitation of TUTase activity, p45 and p50 adenylated proteins, and gRNA-independent U-insertion activity. The values for the labeled p45 and p50 proteins and the U-insertion activity were obtained by densitometry of the scanned X-ray films.

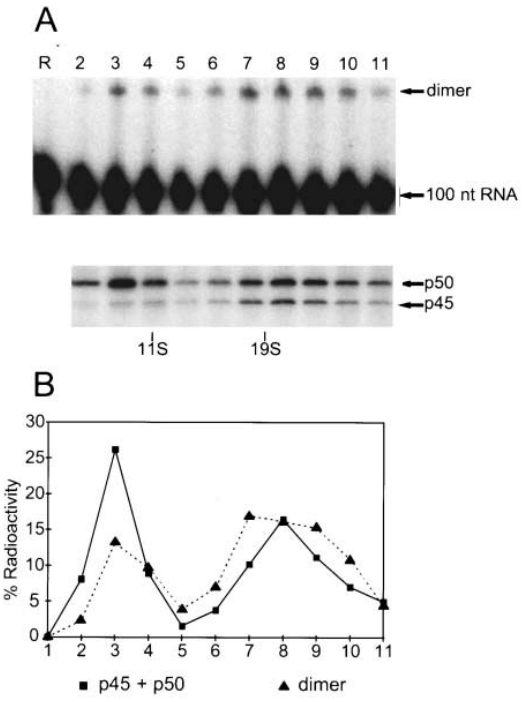


Fig. 2. RNA ligase activity and the adenylated p45 and p50 proteins from *L. tarentolae* mitochondrial extract cosediment at 20S in glycerol gradients. (A) The mitochondrial extract was fractionated on a 10–30% glycerol gradient and the fractions were assayed for RNA ligase activity by the dimerization of a 5′ end-labeled 100 nt substrate RNA and 8% acrylamide-urea gel electrophoresis. The positions of the 100 nt monomer and the dimer are indicated. R, untreated input RNA. The same fractions were labeled with [α-^{32}P]ATP and the p45 and p50 polypeptides separated by gel electrophoresis. (B) PhosphoImager quantitation of radioactivity in labeled p45 + p50 bands and the dimer RNA band in (A).

RNP complexes, some of which may possibly be involved in RNA editing [20,21]. Several [α-^{32}P]UTP metabolically-labeled complexes, which were separable on a native gel and were operationally termed 'T-complexes', were shown to comigrate with TUTase activity at approximately 10S. Two dimensional gel electrophoresis showed that the T-IV complex contained gRNA and complexes T-II–T-VI contained [α-^{32}P]UTP-labeled mRNA fragments, which were termed 'arc-RNAs' due to their migration pattern. TUTase activity was localized predominantly to complex T-IV by an in situ gel assay [20].

Several partial in vitro editing systems have been reported. A gRNA-mediated U-deletion [3,22,23] and U-insertion system [8] using a preedited ATPase 6 (A6) mRNA substrate and a 20–35S fraction [19] of a mitochondrial extract from *T. brucei* was described. The 5′ and 3′ mRNA cleavage products predicted by the enzyme cascade model as well as the gRNA-mRNA chimeric molecules predicted by the transesterification model [24] were directly visualized by gel analysis of end-labeled substrates [3,23]. Evidence was presented that the cleavage fragments represented intermediates of the editing reaction and that the chimeric molecules were aberrant by-products [3], suggesting that the enzyme cascade model is the most likely mechanism for RNA editing.

In *L. tarentolae* two types of in vitro U-insertion editing have been reported, one that is independent of gRNA but dependent on the secondary structure of the mRNA [2,5,7], and another gRNA-dependent activity in which the number of inserted U's is guided by base-pairing with guiding nucleotides in an added cognate gRNA [6]. The gRNA-independent editing activity sedimented at 20S in glycerol gradients and was operationally termed the 'G-complex' since evidence indicated the presence of gRNA in this region of the gradient [20].

In this study, we investigate in more detail the RNP complexes in a mitochondrial lysate from *L. tarentolae* which may be involved in RNA editing. We identify an RNA ligase-containing high molecular weight complex in addition to a heterodisperse class of complexes that contain gRNA, mRNA, TUTase and a 70 kDa protein of unknown function.

2. Materials and methods

2.1. Cell culture of Leishmania tarentolae and mitochondrial isolation

L. tarentolae (UC strain) cells were grown as described previously to late log phase ($1–2 \times 10^8$ cells ml^{-1}) [25]. The kinetoplast–mitochondrial

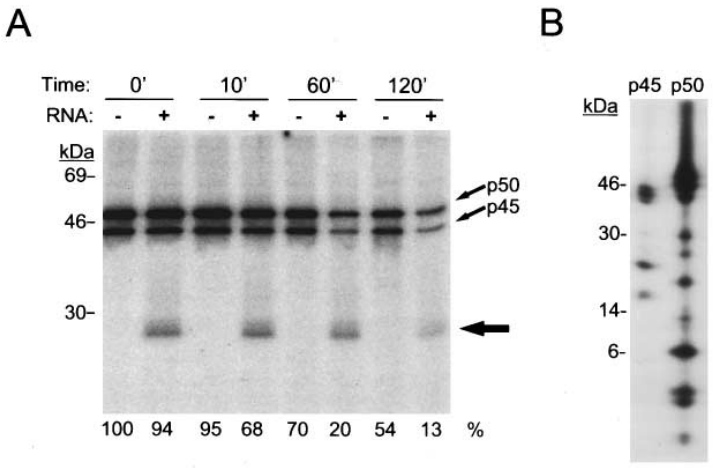


Fig. 3. Analysis of the [α-^{32}P]ATP-labeled p45 and p50 proteins. (A) Deadenylation of p45 and p50 upon incubation with a ligatable RNA. The 20S glycerol gradient fraction (8) was incubated with [α-^{32}P]ATP under standard conditions. The reaction was divided and a ligatable 100 nt substrate RNA (RNA: +) was added to one reaction and incubation continued. The reactions were stopped with denaturing buffer at the indicated times and the products separated on a 12% SDS acrylamide gel. The arrow indicates the 100 nt RNA that was labeled during the course of the chase. The percent of remaining radioactivity in the combined p45 + p50 bands in each lane is indicated at the bottom. (B) In situ partial proteolysis was performed on gel slices of [α-^{32}P]ATP-labeled p45 and p50, and the products fractionated on a 12% SDS acrylamide gel. Protein molecular weight markers in kDa are shown on the left.

fraction was isolated by floatation in Renografin density gradients [26,27] and resuspended either in buffer 1: 25 mM HEPES–KOH (pH 7.9), 50 mM KCl, 1 mM EDTA, 1 mM ATP, 0.5 mM DTT, 5% glycerol [14]; buffer 2: 20 mM HEPES–KOH (pH 7.5), 20 mM KCl, 1 mM EDTA, 10% glycerol [20]; or buffer 3: 20 mM HEPES–KOH (pH 7.5), 100 mM KCl, 0.2 mM EDTA, 10% glycerol [9], and stored in aliquots at − 80°C. Protein concentrations were approximately 5–8 mg ml^{-1}. In general, these different mitochondrial resuspension buffers had no effect on the results of sedimentation or native gradient gel analysis (data not shown).

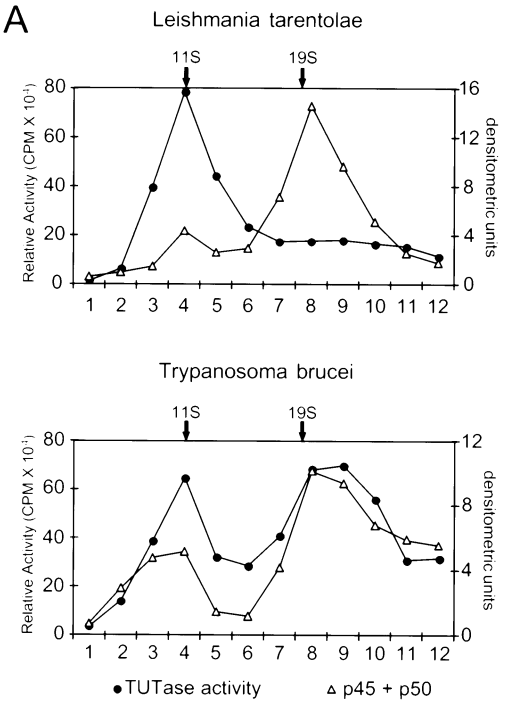
2.2. Cell culture of Trypanosoma brucei and mitochondrial isolation

T. brucei (strain 427) procyclic cells were grown at 27°C in T-flasks in SDM medium [28]. The mitochondrial fraction was isolated and processed as described above for *L. tarentolae*. The isolated mitochondria were stored in buffer 1 at − 80°C.

2.3. Mitochondrial extracts and sedimentation analysis of mitochondrial extracts

Triton X-100 (0.3%) was added to the thawed mitochondrial suspension which was homoge-

Fig. 4. Comparison of sedimentation profiles of TUTase activity and ATP-labeled p45 + p50 proteins in mitochondrial extracts from *L. tarentolae* and *T. brucei*. (A) *L. tarentolae* and procyclic *T. brucei* TS extracts were fractionated on glycerol gradients, and the TUTase activity of each fraction was assayed by the labeling of cytoplasmic rRNA with [α-^{32}P]UTP. Aliquots of each fraction were also incubated with [α-^{32}P]ATP and the labeled proteins detected by SDS-acrylamide gel electrophoresis and autoradiography. Two labeled proteins with identical gel mobility to the p45 and p50 proteins from *L. tarentolae* were detected in the *T. brucei* fractions (data not shown). Quantitation of the labeled p45 + p50 proteins was performed by densitometry of the scanned autoradiogram. (B)ATP-labeling of a high molecular weight complex sedimenting at 20S and migrating as a single band at approximately 1800 kDa in the native gel (arrow). Fractions from glycerol gradients of *L. tarentolae* and *T. brucei* TS extracts were incubated with [α-^{32}P]ATP and electrophoresed in native gradient gels (80-1, acrylamide-bisacrylamide). The locations of the native gel size markers are indicated on the right and S values of cosedimented standards are indicated at the bottom. The ATP-labeled I doublet which peaks in Fraction 6 is indicated.


A
Leishmania tarentolae
11S
19S
Relative Activity (CPM X 10⁻¹)
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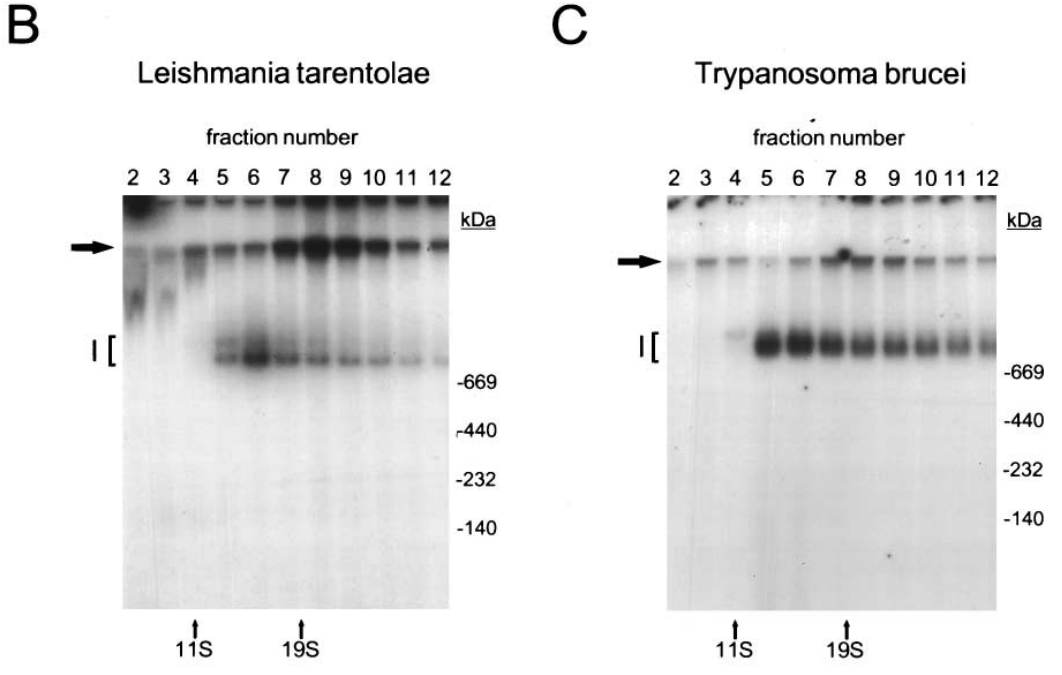
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nized for 15 s with a Kontes disposable pellet homogenizer. The lysate was centrifuged at $14\,000 \times g$ for 30 min at 4°C to yield the TS extract [12]. Gentle inversion in 0.5% Triton X-100 for 10 min at 4°C and clarification at $14\,000 \times g$ for 2 min at 4°C was also used [14], with identical native gradient gel and sedimentation results (data not shown).

TS extract (150 μl) was layered on a 10–30% linear glycerol gradient prepared using a Gradient Master (BioComp Instruments) The gradients contained 25 mM HEPES–KOH (pH 7.5), 60 mM KCl, and 10 mM $MgCl_2$, and were centrifuged either for 14.5 h at $33\,000 \times g$ or 17.5 h at $30\,000 \times g$ at 4°C in an SW41 rotor (Beckman). In the experiments to compare the *L. tarentolae* and *T. brucei* extracts, the gradients contained 20 mM HEPES–KOH (pH 7.9), 50 mM KCl, 10 mM Mg-acetate and 10 mM DTT [14], and were centrifuged at $30\,000 \times g$ for 17.5 h in the SW41 rotor. S values were calculated by cosedimentation with alcohol dehydrogenase (7.5S, Sigma), catalase (11S, Sigma), thyroglobulin, (19S, Sigma) and *E. coli* 30S ribosomal subunits. Gradients were fractionated on an ISCO UA6 apparatus, and 16 0.75 ml fractions were collected.

2.4. Enzymatic assays: TUTase, gRNA-independent U-insertion activity, RNA ligase

Gradient fractions were assayed for TUTase and gRNA-independent U-insertion activity as previously described [2,12,20]. RNA ligase activity was measured by the dimerization of [γ-^{32}P]ATP 5′ end-labeled synthetic RNA substrate (100 nt). The synthetic RNA substrate was derived by T7 transcription of the pBluescript SK$^+$ plasmid linearized with Not I (Stratagene). The RNA was dephosphorylated with calf alkaline phosphatase (Gibco) and 5′ end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (Gibco) using standard conditions. RNA ligase reaction mixtures contained 1.0×10^4 cpm labeled RNA, 1 mM ATP, 100 mM HEPES–KOH (pH 7.5), 25 mM KCl, 20 mM $MgCl_2$, 1 mM EDTA, 2 mM DTT, 20% DMSO, and 25 μl gradient fraction or 70 μg total mitochondrial protein (TS) in a final volume of 50 μl. Incubation was overnight at 4°C. RNA was extracted by phenol/chloroform, ethanol-precipitated with glycogen (100 μg ml^{-1}) as a carrier and run on a 8% acrylamide urea gel. The fixed and dried gel was analyzed using a PhosphoImager (Molecular Dynamics).

2.5. Adenylation/deadenylation of proteins

To visualize the adenylated proteins and complexes, gradient fractions (25 μl) or TS extract (70 μg protein) were incubated with [α-^{32}P]ATP in 20 mM HEPES–KOH (pH 7.5), 25 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, 100 μg ml^{-1} BSA and 10 μCi [α-^{32}P]ATP (800 Ci mmol^{-1}) for 30 min at 27°C in a reaction volume of 50 μl. An equal volume of 2 × SDS gel loading buffer [29] was added to 25 μl of each reaction and heated to 100°C for 3 min before electrophoresis on a 10 or 12% SDS acrylamide gel. Native gel loading buffer (50% glycerol in 50 mM Tris–glycine buffer) was added to the remainder of the reaction to a final concentration of 12.5% glycerol and electrophoresed on a 14 cm × 14 cm × 1 mm, 4–16% linear native polyacrylamide (79:1) gel with a 10–30% glycerol gradient for stabilization. The running conditions were 50 mM Tris–glycine (pH 8.8), at 130 V and 4°C for approximately 18 h [30]. Size markers included thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa) and lactate dehydrogenase (140 kDa) (Pharmacia Marker Calibration Kit).

To assay the deadenylation of p45 and p50, the fraction from a glycerol gradient that exhibited the peak of the adenylated proteins (Fraction 8) was labeled as described above in a reaction volume of 75 μl. The reaction was divided into three equal aliquots. SDS loading buffer (2 ×) was added to one aliquot and it was stored at −20°C. Either water or the ligatable RNA substrate (100 pmol) described above was added to the remaining aliquots to a final volume of 30 μl and the incubation was continued for 0, 10, 60 or 120 min at 27°C. Reactions were stopped with an equal

volume of 2 × SDS buffer and electrophoresed on a 12% SDS acrylamide gel.

To assay for deadenylation of the 20S complex, Fraction 8 was incubated with [α-^{32}P]ATP for 30 min and aliquoted as described above. Native gel loading buffer was added to the first aliquot immediately and it was kept on ice until the gel was loaded. Water or ligatable RNA was added to the remaining reactions and incubation continued for 90 min at 27°C. After addition of native gel loading buffer, the aliquots were electrophoresed on a 4–16% native acrylamide gel.

All SDS and acrylamide-urea gels were dried, fixed and quantified on a PhosphoImager (Molecular Dynamics). The native gels were either autoradiographed wet or fixed and dried and quantified on a PhosphoImager.

2.6. Partial proteolysis of p45 and p50

Gradient Fractions 8–11 were pooled and incubated with [α-^{32}P]ATP. The proteins were concentrated by acetone precipitation and separated in a 10% SDS acrylamide gel. The gel was exposed wet and the gel slices containing the labeled p50 and p45 bands were excised and fixed in 40% methanol/10% acetic acid overnight. The washed gel slices were then incubated in 40% acetic acid/10% urea. Partial proteolysis was performed by incubation of the gel slices in a solution of 15 mM *N*-chlorosuccinimide, 40% acetic acid/10% urea [31]. The gel slices were equilibrated overnight in 10% glycerol/15% 2-mercaptoethanol/3% SDS and layered onto a second dimension 12% SDS acrylamide gel. The gel was fixed, dried and subjected to autoradiography.

2.7. Two dimensional gel electrophoresis of the 20S complex

The 20S fraction from the glycerol gradient of TS extract (Fraction 8) was incubated with [α-^{32}P]ATP and electrophoresed on a 4–16% native polyacrylamide gel as described above. The wet gel was exposed overnight at 4°C. The labeled lane was excised, incubated in 1 × SDS buffer at 65°C for 5 min and then electrophoresed into a 12% SDS acrylamide gel (1.5 mm). Labeled Fraction 8 was run in a separate lane as a marker.

2.8. Northern analysis of native gradient gels

TS extract (15 μl) was incubated under metabolic labeling conditions as previously described [20] with 12.5 μM unlabeled UTP in a reaction volume of 50 μl. Pre-incubated TS, non-incubated TS (15 μl) or glycerol gradient fractions (40 μl fraction) were electrophoresed on 4–16% native gradient gels (79:1) without a stabilizing glycerol gradient for 12 h at 120 V and 4°C. After electrophoresis, the native gels were incubated in 50% urea/25 mM Tris–glycine, 0.5 mM EDTA buffer for 15 min and electroblotted overnight onto Nytran Maximum Strength (0.2 μm; Schleicher and Schuell) in 6 mM trisodium citrate, 8 mM Na_2HPO_4 buffer at 250 mA and 4°C [32]. The RNA was ultra violet (UV) cross-linked to the filters using a Stratalinker 2400 (Stratagene). gRNA- and mRNA-containing complexes were visualized by hybridization of electroblots with [γ-^{32}P]ATP-labeled gRNA-specific oligonucleotides or mRNA-specific PCR probes (see below) and analyzed on a PhosphoImager.

The following 5′end-labeled oligonucleotides were used as hybridization probes for gRNA detection: Cyb-II gRNA, S-530 [20], Cyb-I gRNA, S-2003, 5′-AAATATTATTTAAAAATTTATATTATCTTTTAACTTCAAGTCATATGTGCC-3′; six A6 gRNAs: S-312, S-514, S-515, S-517, S-516 and S-574; eight RPS12 gRNAs: S-509, S-511, S-508, S-252, S-510, S-582, S-513, and S-512 [33]; and two COIII gRNAs S-255 [24], and S-518 [33]. The preedited Cyb mRNA probe was generated by PCR amplification of kinetoplast DNA and labeling with the Prime IT-II random priming kit (Stratagene) as previously described. The Cyb mRNA PCR fragment represents nt 5371–5533 in GenBank entry LEIKPMAX.

2.9. Northern analysis of gGNA in glycerol gradient fractions

To control for recovery of gRNA from gradient fractions, 250 ng of in vitro transcribed heterologous RNA was added to each fraction before RNA isolation. The 746 nt RNA was transcribed from plasmid pGEM7ZF(+) (Promega) DNA linearized with DdeI (BRL). The pGEM RNA

was added to each fraction together with 1 μg of carrier *E. coli* tRNA (Sigma). Total RNA from each fraction was phenol/chloroform-extracted and ethanol-precipitated. Isolated RNA was electrophoresed in a 1.8% agarose formaldehyde gel which was blotted onto nylon filters (MSI, 0.45 μm) as previously described [1]. Kinetoplast RNA was included as a control in a separate lane. The blots were UV cross-linked and hybridized with a 5′-end labeled oligonucleotide antisense to Cyb-II gRNA (S-530). The blots were then stripped in 0.1% SDS at 95°C, and re-hybridized with a mixture of six 5′-end labeled oligonucleotides antisense to all A6 gRNAs (S-312, S-514, S-515, S-517, S-516 and S-574). Blots were also hybridized with pGEM plasmid DNA that was labeled with [α-^{32}P]ATP using the PRIME-IT II kit (Stratagene).

2.10. Generation of polyclonal antiserum against p70 and immunodetection

A 70 kDa polypeptide was found to cofractionate at approximately 200 kDa together with TUTase activity in Superose 6 gel filtration of the TS mitochondrial extract and at 10–13S in subsequent glycerol gradient sedimentation (data not shown). This protein was separated by SDS-acrylamide gel electrophoresis and the gel slices used to generate polyclonal antibodies in a rabbit (Caltag Laboratory). The antiserum at a 1:1000 titer detected a major 70 kDa mitochondrial polypeptide and a minor 69 kDa polypeptide by Western analysis (data not shown).

To localize the p70 protein in the various RNP complexes separable on a native gel, TS extract was fractionated on a 10–30% glycerol gradient and 15 μl of each gradient fraction was electrophoresed on a 4–16% native gel as described above. The gel was electroblotted to nitrocellulose (Protran BA83, Schleicher and Schuell) for 4 h at 500 mA. The filter was blocked for 15 min with TPBC-milk (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 0.05% Tween 20, 5% low fat dry milk) and subsequently incubated with p70 antiserum at 1:2000 or 1:10 000 dilution in TPBS-milk for 1 h. The filter was then washed with TPBS and incubated with goat anti-rabbit IgG (1:2000) conjugated to horseradish peroxidase (BioRad) for an additional hour. After a second washing in TPBS, the signal was developed with Pierce SuperSignal CL-HRP and visualized by Kodak X-OMAT film.

3. Results

3.1. Two [α-^{32}p]ATP-labeled proteins of 45 and 50 kDa cosediment at 20S with RNA ligase activity and gRNA-independent U-insertion activity

When the TS extract was sedimented through a glycerol gradient and each fraction was incubated with [α-^{32}P]ATP and analyzed by SDS acrylamide gel electrophoresis, two major labeled polypeptides of 50 and 45 kDa were detected, comigrating as a minor 10S peak and a major 20S peak (Figs. 1 and 2). The 10S peak cosedimented with the major peak of TUTase activity (Fig. 1B), and the 20S peak with a small shoulder of TUTase activity and also with a gRNA-independent U-insertion activity in which the incorporation of [α-^{32}P]UTP into a preedited cytochrome b mRNA substrate was assayed by an RNase H procedure [2,7] (Fig. 1A,B).

A minor ATP-labeled 33 kDa polypeptide was present in gradient fractions 2 and 3 (Fig. 1A). This labeled band was relatively more prominent than the p45 and p50 bands when unfractionated TS extract was labeled with [α-^{32}P]ATP (data not shown).

RNA ligase activity in each gradient fraction was assayed by dimerization of a 100 nt substrate RNA (Fig. 2A). As shown in Fig. 2AFig. 2B, ligase activity comigrated with the p50 and p45 adenylated proteins at 10 and 20S. RNA ligase activity was also assayed by 3′-end labeling of endogenous and exogenous tRNA with [α-^{32}P]pCp. By this assay, ligase activity was also observed in gradient fractions 1 and 2, suggesting the presence of free RNA ligase which has a preference for tRNA as substrate (data not shown).

Unlabeled 100 nt RNA was incubated with [α-^{32}P]ATP-labeled gradient fraction 8 for differ-

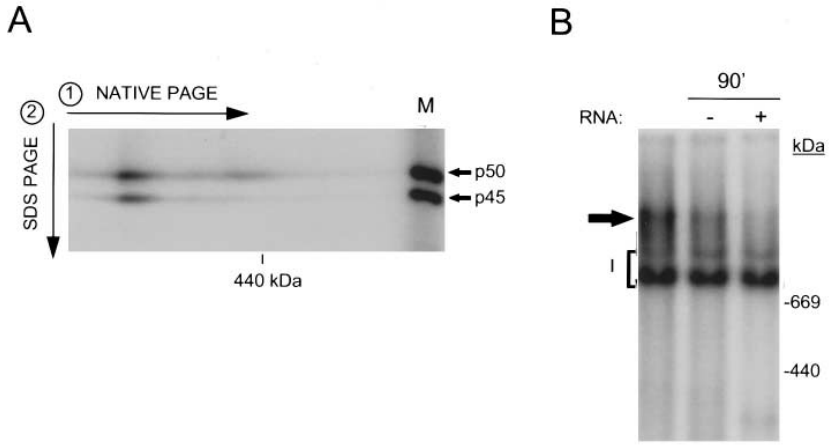

Fig. 5. The p45 and p50 adenylated proteins are components of the 1800 kDa ATP-labeled complex in the native gel. (A) The 20S fraction (8) of a glycerol gradient was labeled with [α-^{32}P]ATP and run on a native gradient gel. The excised lane was incubated in denaturing buffer and then layered on a 12% SDS acrylamide gel for the second dimension. M, ATP-labeled fraction 8 was run in a separate lane as a control for the position of the p45 and p50 bands. (B) Deadenylation of the p45 + p50-containing complex upon incubation with ligatable RNA. Fraction 8 (75 μl) was labeled with [α-^{32}P]ATP and divided in two portions. The first was maintained at 4°C. To the other, either water (− RNA) or a ligatable 100 nt substrate RNA (+ RNA) was added and incubation continued for 90 min. All three reactions were electrophoresed on a native gradient gel. The position of the p45 + p50-contaning complex is indicated by an arrow and the I doublet is indicated.

ent periods of time (Fig. 3A). After 60 min incubation, the label in the p45 + p50 bands decreased 80% in the presence of the ligatable substrate, but only decreased 30% in the absence of this substrate (Fig. 3A). A transfer of label to the RNA substrate (arrow) can also be observed in this experiment. The release of covalently bound AMP was monitored by thin layer chromatography and found to increase with time of incubation (data not shown). These results and the comigration results suggest that the adenylated p45 and p50 proteins represent AMP-charged intermediates [34] of the RNA ligase [12,13].

Pooled glycerol gradient fractions representing the 20S peak (Fractions 8–11) were incubated with [α-^{32}P]ATP and the labeled proteins precipitated with acetone. In situ partial proteolysis of the labeled p45 and p50 proteins was performed, and the products separated in an SDS acrylamide gel. The partial digestion patterns of each protein were unique, suggesting that p45 and p50 represent different proteins (Fig. 3B).

A TS mitochondrial extract from procyclic *T. brucei* was also subjected to glycerol gradient fractionation and the fractions assayed for the presence of adenylated proteins. Two adenylated proteins, which were reported to be 57 and 50 kDa and 54 and 47 kDa in size by different laboratories [13,19], were previously identified in a mitochondrial extract from *T. brucei* and shown to represent AMP-charged intermediates of a mitochondrial RNA ligase [13]. In our hands, the two adenylated proteins from *T. brucei* comigrated at 45 and 50 kDa with the adenylated proteins from *L. tarentolae*, suggesting that the reported size differences reflect gel calibration problems and not a species difference (data not shown).

3.2. The p45 and p50 proteins from L. tarentolae and T. brucei mitochondrial extracts are components of a high molecular weight ATP-labeled complex which can be visualized by native gel electrophoresis

A comparison of the sedimentation profiles of TUTase activity and [α-^{32}P]ATP-labeled p45 and p50 proteins from *L. tarentolae* and procyclic *T. brucei* mitochondrial extracts fractionated on a glycerol gradient is shown in Fig. 4A. The *T.*

brucei extract exhibited two TUTase peaks at 10–13S and 20S but the peaks were of equal size, unlike the situation with the *L. tarentolae* extract, in which most of the TUTase activity was in the 10–13S peak with a shoulder at 20S. Cosedimentation of RNA ligase activity with both the 10S and the 20S TUTase peaks was also observed for the *T. brucei* extract (data not shown).

Gradient fractions of a *L. tarentolae* mitochondrial extract were incubated with [α-^{32}P]ATP and electrophoresed in a native gradient gel (79:1, acrylamide:bisacrylamide). A high molecular weight labeled band migrating at approximately 1800 kDa in the native gel, assuming a linear extrapolation of the calibration curve, peaked at 20S in gradient fractions 7–10 (Fig. 4B) together with gRNA-independent U-insertion activity (Fig. 1B). Similar results were obtained using a mitochondrial extract from *T. brucei* procyclic cells (Fig. 4C). This molecular weight determined by migration in a native gradient gel is larger than the approximately 700 kDa value expected from the 20S sedimentation in the glycerol gradient, but will be used in this paper as an operational size for this complex. Further work is required to determine the accurate size of this complex.

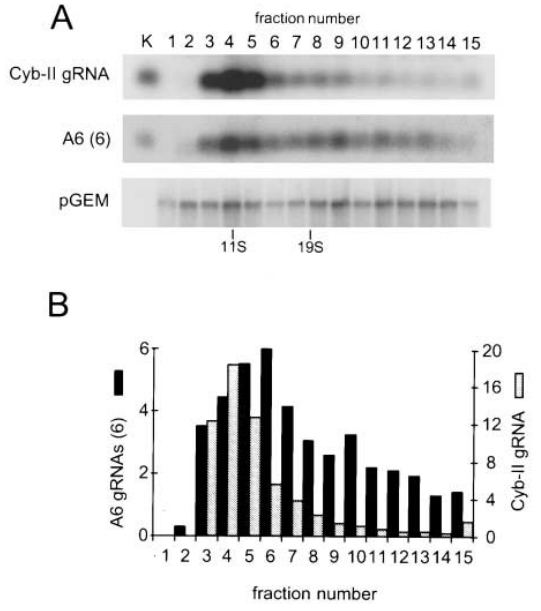

Fig. 6. Distribution of gRNA in glycerol gradient fractions of the *L. tarentolae* TS extract. (A) Total RNA was isolated from each fraction and a pGEM transcript (250 ng) was added to each fraction prior to RNA isolation to control for recovery. The RNAs were fractionated in a formaldehyde agarose gel and the blot was hybridized with a labeled oligonucleotide probe for Cyb-II gRNA and also with random prime-labeled pGEM plasmid DNA. The blot was then stripped and rehybridized with a mixture of labeled oligonucleotide probes for the six A6 gRNAs [33]. Lane K contains a kinetoplast RNA control. (B) Histogram of the normalized recovery of gRNAs from gradient fractions.

An ATP-labeled doublet peaking in gradient fraction 6 was also present in the native gels of both the *L. tarentolae* and the *T. brucei* extracts (bands labeled 'I' in Fig. 4B,C). However, the I-bands were resistant to a 20 min incubation of the extract at 65°C prior to gradient fractionation, whereas the 1800 kDa band was sensitive to such treatment (M. Tayag, J. Alphonso and L. Simpson, unpublished results). The role of the I-doublet is unknown.

Two dimensional gel electrophoresis was used to show that the p45 and p50 proteins represent the labeled components of the ATP-labeled 1800 kDa band in a native gel. The gradient fraction representing the 20S peak (Fraction 8) was incubated with [α-^{32}P]ATP and electrophoresed on a native gel. The gel lane was excised and layered onto a denaturing gel for the second dimension. As shown in Fig. 5A, two labeled proteins which comigrated with the adenylated p45 and p50 proteins were liberated from the native gel band.

Deadenylation of the ATP-labeled 1800 kDa native gel band was observed upon incubation of Gradient Fraction 8 with exogenous RNA (Fig. 5B). The fraction was labeled with [α-^{32}P]ATP for 40 min, and then a ligatable 100 nt substrate RNA was added to the fraction and incubation continued for 90 min, and the sample was electrophoresed on a native gel. As shown in Fig. 5B, the labeling of the native gel band was decreased by incubation in the presence of the ligatable RNA as compared to incubation in the absence of this RNA. Fig. 5B also shows that incubation with a ligatable RNA had no effect on the labeled I bands.

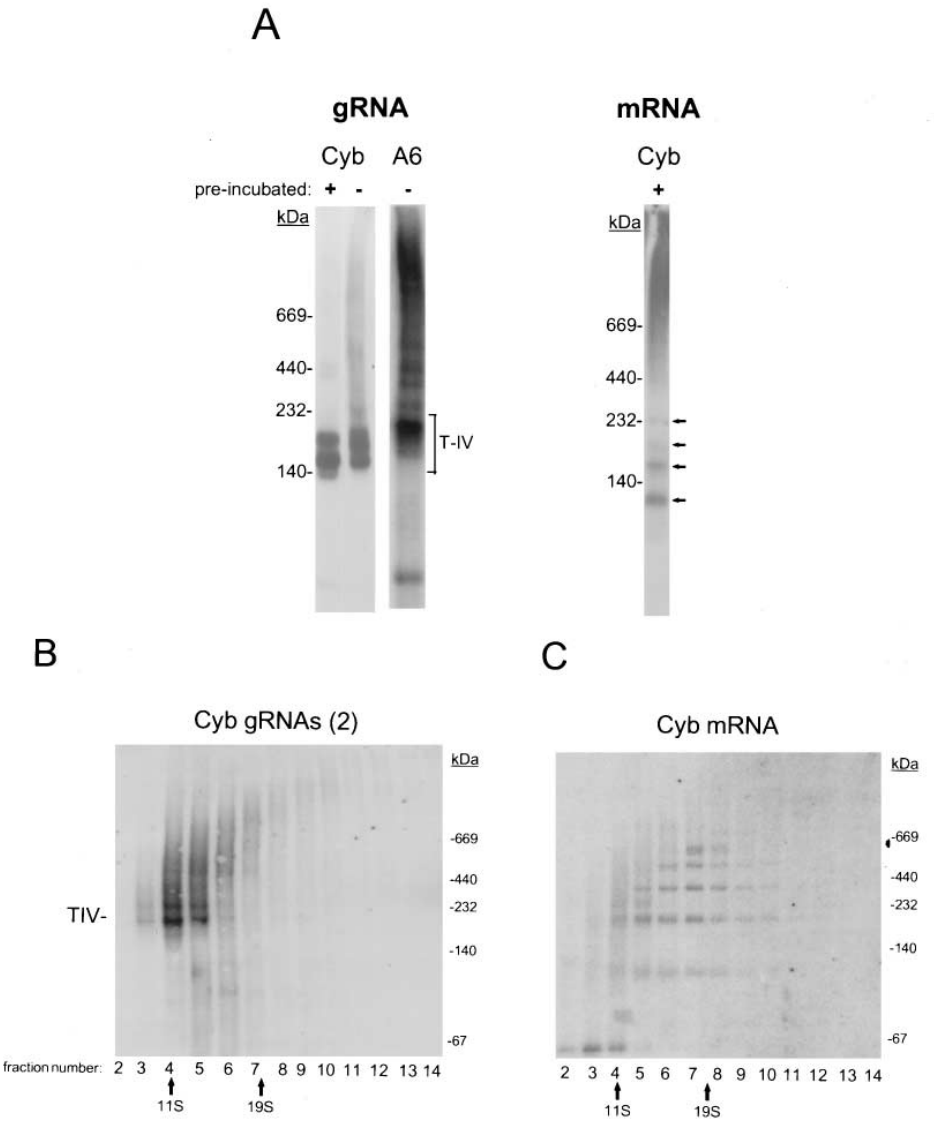

Fig. 7. Identification of gRNA- and mRNA-containing complexes by Northern analysis of native gels of *L. tarentolae* TS extract. (A) TS extract was electrophoresed on a native gel and the gel electroblotted onto Nytran filter. Pre-incubation (+) was at 27°C for 40 min under metabolic labeling conditions without isotope. The electroblots were hybridized with an oligonucleotide probe for gCyb-II gRNA, a mixture of labeled oligonucleotides antisense to the six A6 gRNAs, or a random-primed PCR-amplified DNA probe for Cyb mRNA. The arrows to the right of the mRNA indicate the previously identified 'arc RNA' bands. (B) TS extract was sedimented on a glycerol gradient and the fractions were analyzed by native gel electrophoresis. The gel was electroblotted onto Nytran filter and the filter hybridized with labeled oligonucleotide probes for the two gCyb gRNAs. (C) An identical electroblot was hybridized with a randomised PCR product that covers the 5′ end of preedited Cyb mRNA. The positions of the size markers in the native gels are indicated on the right and the positions of size markers in the glycerol gradient indicated on the bottom.

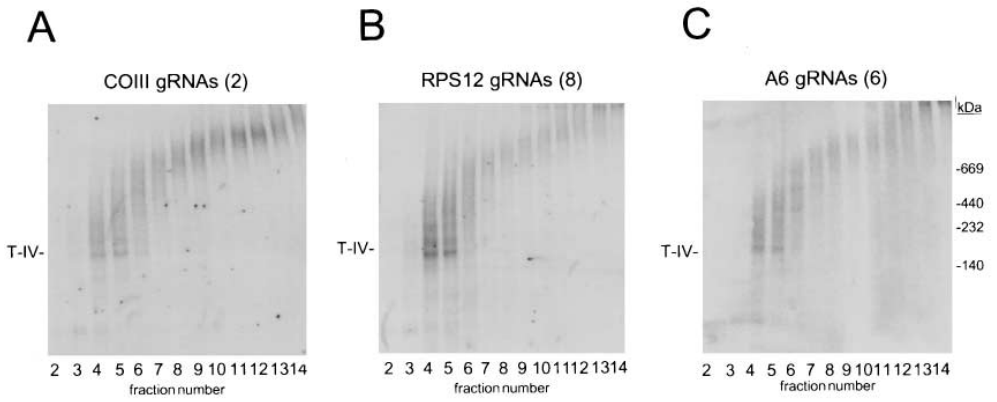

Fig. 8. Sedimentation analysis of gRNA-containing complexes. *L. tarentolae* mitochondrial extract was sedimented on a glycerol gradient and fractions analyzed by native gel electrophoresis. The gels were electroblotted and the blots hybridized with labeled probes for the gRNA mixtures indicated above each panel and exposed on a PhosphoImager (Molecular Dynamics). The localization of the T-IV complex is shown on the left of each panel and the positions of native gel size markers shown on the right. (A) 2 COIII gRNAs. (B) 8 RPS12 gRNAs. (C) 6 A6 gRNAs.

3.3. Localization of gRNAs in glycerol gradient fractions of the mitochondrial extract

The distribution of two different gRNAs in glycerol gradient fractions of the mitochondrial extract was analyzed by Northern analysis of total RNA extracted from each fraction, using oligonucleotide probes for the gCyb-II gRNA and for a mixture of all six gA6 gRNAs. The results were normalized for the recovery of a known amount of a control pGEM transcript added to each gradient fraction. As shown in Fig. 6, the gCyb-II gRNA peaked in the 10–13S region, whereas the gA6 gRNAs showed the presence of a significant proportion of higher molecular weight material. These results differ from those previously reported from our laboratory [20], in which a bimodal distribution pattern was observed with the highest concentration of gRNA being in the 20S region of the gradient. The difference apparently was due to the use of a microfiltration concentration step prior to RNA isolation in the previous work, which resulted in the selective loss and under-representation of low molecular weight gRNA-containing complexes, and to the fact that different gRNAs show widely different distribution patterns, as shown in Fig. 6 (and data not shown).

3.4. Localization of gRNA- and mRNA-containing complexes by Northern analysis of native gels

Hybridization of electroblots of native gels was used to identify the RNA components of specific RNP complexes. As shown in Fig. 7A, hybridization to two bands of approximately 200 kD was observed using oligonucleotide probes for gCyb-II gRNA or for a mixture of all six gA6 gRNAs, as has been reported previously using a two dimensional gel system [20]. In addition, hybridization to a previously unobserved high molecular weight smear was observed for both gRNA probes if the extract was not pre-incubated at 27°C under ATP-labeling conditions prior to native gel analysis (Fig. 7A). As also shown in Fig. 7A, hybridization of a blot of a native gel of non-incubated TS extract with a probe for Cyb mRNA resulted in visualization of the previously described arc-RNA bands (arrows), and in addition, a high molecular weight smear which had not been previously observed.

A more detailed analysis of the high molecular weight gRNA- and mRNA-containing material was accomplished by first fractionating the TS extract in a glycerol gradient prior to Northern native gel analysis. As shown in Fig. 7B and Fig. 8A–C for four different sets of gRNAs, the high

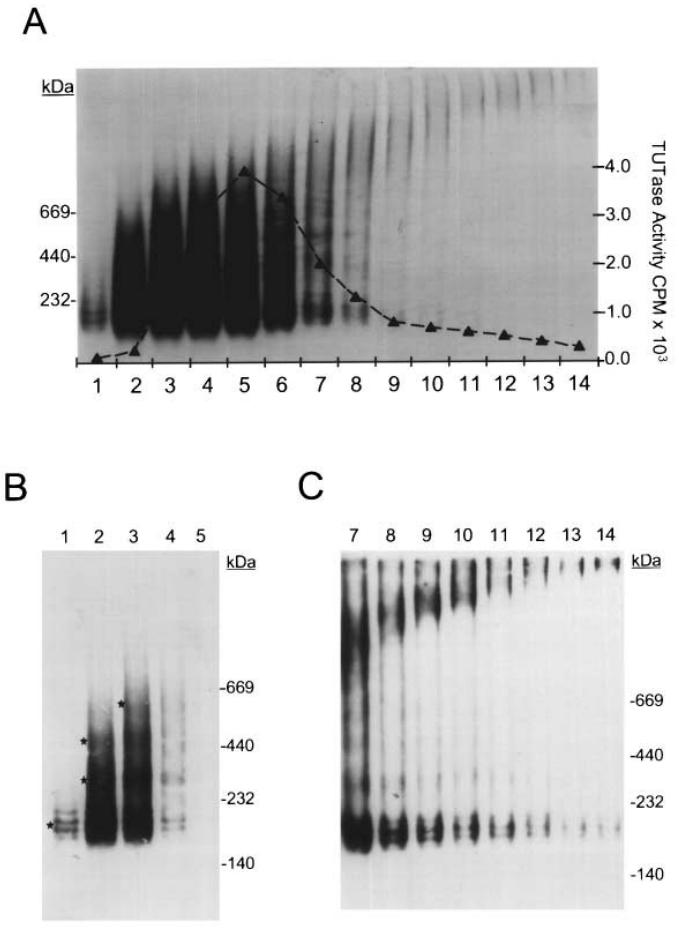

Fig. 9. Immunodetection of the p70 protein. *L. tarentolae* mitochondrial extract was sedimented on a glycerol gradient and each fraction analyzed by native gel electrophoresis. (A) The gel was electroblotted onto nitrocellulose and probed with p70 antiserum (1:10 000). The signal was visualized by exposure to X-ray film after incubation with SuperSignal CL-HRP (Pierce). Exposure time was 6 min. The TUTase activity of each fraction was measured using cytoplasmic rRNA as substrate [20] and is plotted on the autoradiograph. The positions of native gel size markers are indicated on the left. (B) An identical native gel of Gradient Fractions 1–5 with an exposure which shows the p70-positive banding pattern in more detail. The stars indicate the putative oligomeric bands noted in the text. (C) An identical native gel of Gradient Fractions 7–14 with a longer exposure showing the high molecular weight heterodisperse p70-positive material in more detail.

molecular weight gRNA-containing smear resolved into a heterodisperse material sedimenting throughout the gradient. In the lanes of Fractions 4–6 in Fig. 7B and Fig. 8, indications of a multimeric banding pattern can be seen. The relative proportion of the T-IV complexes and apparent multimers and the higher molecular weight heterodisperse complexes varied for different gRNA probes. The 1800 kDa p45 + p50-complex did not appear to contain gRNA, as shown by the complete absence of hybridization of the gRNA probes to this region of the gel. The absence of gRNA in this complex led us to abandon the use of the operational term, 'G-complex', to describe this structure.

The distribution of Cyb mRNA in gradient fractions is shown in the Northern blot of the native gel in Fig. 7C. The presence of a ladder of mRNA bands derived from material sedimenting in the 10–20S region of the gradient is also sug-

gestive of a multimeric composition of the high molecular weight gRNA-containing complexes, in which mRNA fragments are protected from nuclease attack by being bound to proteins. Further work is necessary for a more complete understanding of this phenomenon, but it does appear that mRNA is present as a component of the heterodisperse high molecular weight gRNA-containing complexes. We interpret the disappearance of the high molecular weight heterodisperse gRNA- and mRNA-containing material upon pre-incubation of the lysate, as shown above in Fig. 7, as being due to degradation of mRNA by nuclease in the lysate.

3.5. Immunodetection of a 70 kDA protein component of the T-IV complex and the gRNA-containing heterodisperse complexes

A p70 protein which cofractionated with TUTase activity in glycerol gradients and Superose 6 gel filtration of mitochondrial extract was isolated from a gel and a polyclonal antiserum generated in rabbits. Western analysis of native gels of glycerol gradient fractions of mitochondrial extract in Fig. 9 showed a similar distribution of p70-containing complexes to that of the gRNA-containing complexes in Fig. 7B and FIG. 8. The presence of this protein in T-IV and in several possible T-IV oligomers (✱) can be seen in the lower exposure in Fig. 9B, and the presence of this protein in the heterodisperse higher molecular weight complexes is visible in the over-exposed blot in Fig. 9C. Pre-incubation of the extract under ATP-labeling conditions completely eliminated the high molecular weight heterodisperse p70-containing material but had no effect on the T-IV and putative T-IV oligomers (data not shown).

4. Discussion

We have identified a 20S complex that migrates with a molecular weight of approximately 1800 kDa in a native gradient gel that contains two proteins of 45 and 50 kDa that can be adenylated with ATP and may represent charged intermediates of an RNA ligase. An ATP-labeled band with a similar mobility in a native gel was detected in a *T. brucei* mitochondrial extract. Guide RNAs are not detectable in this complex, but instead are localized in the 200 kDa T-IV region [20], which consists of several bands in a native gel, and also in a higher molecular weight heterodisperse material. Both the T-IV complexes and the heterodisperse gRNA-containing complexes contain a p70 protein. The sensitivity of the high molecular weight p70 + gRNA-complexes to breakdown into 200 kDa T-IV complexes by incubation at 27°C and the presence of mRNA in high molecular weight gradient fractions suggest that the p70 + gRNA-heterodisperse complexes represent multimers of T-IV bound to mRNA, but additional work is required to substantiate this hypothesis.

The ligase-complex comigrates at 20S together with an in vitro gRNA-independent U-insertion activity in *L. tarentolae* and at 20S in *T. brucei*. It has been reported that a 20S fraction from a *T. brucei* mitochondrial extract supports gRNA-dependent U-deletion and U-insertion activities [3,8,23]. We have not yet succeeded in determining the S value of the gRNA-dependent U-insertion activity found in the *L. tarentolae* extract due to the low absolute level of activity [6]. The detection of gRNA-dependent editing in the 20S region of fractionated *T. brucei* mitochondrial extract may be due to the higher relative amount of TUTase activity in this region as compared to that observed with the *L. tarentolae* extract (see data in Fig. 4 above), perhaps due to a greater stability of the editing complexes in *T. brucei*.

It is entirely possible, but not yet proven by the available data, that the ligase-complex alone is responsible for the in vitro gRNA-independent editing and that the in vitro gRNA-dependent editing involves the participation of the p70 + gRNA + mRNA-containing complexes present in those gradient fractions. It is, however, likely that editing in vivo represents a multicomponent activity that involves the interaction of the ligase-containing 1800 kDa complex and the gRNA-containing heterodisperse complexes. Additional work is required to substantiate this hypothesis.

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