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RNA-protein interactions in the ribonucleoprotein T-complexes in a mitochondrial extract from *Leishmania tarentolae*

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Abstract

We have investigated protein-RNA interactions and the incorporation of [α-^{32}P]UTP into the guide RNA and mRNA components of the 'T-complexes' in a mitochondrial extract from *Leishmania tarentolae*. The terminal uridylyl transferase-containing complex T-IV is probably involved in the maturation of the 3'-oligo(U) tail of the gRNAs, but the biological function and biochemical nature of the remaining T-complexes is not known. We have found that the relative extent of labeling of the RNA components is dependent on the UTP concentration: at low levels, the main endogenous RNA components labeled are the gRNAs in T-IV; at higher levels, the mRNAs in all of the T-complexes are preferentially labeled. We also show a tentative correlation in the migration pattern of UTP-labeled T-complexes and complexes which bind exogenous labeled RNA. The relative extent of binding to specific complexes is dependent upon the type of RNA. Most of the interactions between the labeled RNAs and proteins can be disrupted by heparin or a large excess of rRNA, but two labeled complexes were resistant to competition. Most of the binding of labeled exogenous gRNA is disrupted by competition with a large excess of rRNA, but predigestion of the extract with micrococcal nuclease and saturation with rRNA uncovered a high affinity complex, which involves at least two proteins interacting with the bound gRNAs. A knowledge of the RNA and protein components may aid in understanding the biological roles of these RNP complexes.

Keywords: *Leishmania tarentolae*; RNA editing; Ribonucleoprotein complex

Abbreviations: CYb, cytochrome b; gRNA, guide RNA; MN, micrococcal nuclease; ND7, NADH dehydrogenase subunit 7; RNP, ribonucleoprotein; TS, triton supernatant; TUTase, terminal uridylyl transferase; U, uridine.

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1. Introduction

Guide RNAs (gRNAs) are small RNAs that mediate the insertion and deletion of uridine residues in pre-edited mitochondrial transcripts of kinetoplastid protozoa by a base-pairing mechanism, the precise details of which are uncertain [1–3]. The gRNAs also possess a non-encoded 3′ oligo(U) tail of variable length which may possibly be involved in donating U bases to the editing sites [4,5]. U residues are added to the 3′ end of gRNAs by a mitochondrial terminal uridylyl transferase (TUTase) [6]. This TUTase activity labels endogenous RNAs with [α-^{32}P]UTP, giving rise to a reproducible pattern of approx. 6 ribonucleoprotein (RNP) complexes visualized in native gels, the so-called 'T-complexes' [7]. TUTase activity has been localized to specific complexes which we have termed T-IVa and T-IVb, which also contain gRNAs [7]. The other T-complexes that are labeled with [α-^{32}P]UTP were shown to contain mRNA [7].

A variety of mitochondrial RNP complexes containing putative components of the editing machinery have been reported previously in the related trypanosomatid *Trypanosoma brucei*. Pollard et al [8] reported that two gRNA-containing RNP complexes in a mitochondrial extract from *T. brucei* can be detected by sedimentation in glycerol gradients: a 19S complex which contains gRNA, TUTase, RNA ligase and chimera-forming activity and a 35S complex which has in addition pre-edited RNA, but lacks tightly bound TUTase. Read et al. [10], Goringer et al. [9] and Koller et al. [11] have identified by gel-retardation analysis several mitochondrial RNP complexes from *T. brucei* which interact with exogenous synthetic gRNAs, but the function of these complexes remains unknown.

An investigation of the various T-complexes in *Leishmania tarentolae* is important to determine their biological function. In addition to the apparent role of T-IV in gRNA 3′ metabolism, the presence of mRNAs in other T-complexes [7] raises the possibility of an involvement of these complexes in the process of RNA editing. In this paper we initiate an investigation of the T-complexes of *L. tarentolae* with an examination of the labeling of the endogenous RNA components and the interaction of endogenous and exogenous RNAs with specific protein components.

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}) as described previously [12], and the kinetoplastid-mitochondrial fraction was isolated by flotation in Renografin density gradients [13,14]. The mitochondrial fraction was resuspended in 20 mM Hepes-KOH (pH 7.5)/20 mM KCl/1 mM EDTA/10% glycerol, at a protein concentration of approx. 5 mg ml^{-1}, and stored in aliquots at −80°C. Triton supernatant (TS) was prepared by homogenization of the mitochondrial fraction in 0.3% Triton X-100 and clarification at 12000 $\times g$ for 30 min, as described previously [6].

2.2. Self-labeling of mitochondrial extracts with [α-^{32}P]UTP

TS (usually 5 μl) was incubated in a volume of 25 μl 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 · acetate/3 mM K · phosphate (pH 7.5), with 2 μCi [α-^{32}P]UTP (800 Ci mmol^{-1}; for a final concentration of 0.1 μM). Note that CTP was omitted in order to prevent transcription [6]. For some experiments (high-UTP conditions), 40 μM unlabeled UTP was included.

2.3. Superose-6 gel filtration of mitochondrial extract

For UV-crosslinking of [α-^{32}P]UTP-labeled complexes, material from a gel-filtration fraction of TS containing the peak of TUTase activity was used. TS was fractionated on a Superose-6 (10/30) FPLC column (Pharmacia) equilibrated with the same buffer as used for the storage of purified kinetoplasts (see above). 200 μl of TS, clarified through a 0.22-μm filter, was passed through the column at a rate of 0.5 ml min^{-1} and 1-ml fractions were collected. The

fractions were assayed for TUTase activity by performing an [α-^{32}P]UTP labeling reaction, as described above for the self-labeling reaction for TS, but using 25 μl of each fraction in a final volume of 50 μl and adding 1 μg of total *L. tarentolae* RNA (isolated as described in Ref. 6) as substrate. The reactions were stopped with 0.2% SDS in 0.5% Na · pyrophosphate and spotted on DE-81 filters, which were washed and counted. The fraction containing the peak of activity was then concentrated five-fold using a Centricon 10 microconcentrator (Amicon). This fraction also showed maximal labeling of T-complexes with [α-^{32}P]UTP under high-UTP conditions, as assayed by native gel electrophoresis.

2.4. RNAs used in binding studies

Synthetic gRNAs and the 69-nt pGEM T7 transcript were transcribed in vitro by a method based on that described [15] for small RNAs. T7 promoter-containing templates for the gRNAs were produced by PCR, using kinetoplast DNA or cloned maxicircle DNA sequences with the following primers: S-270 5′-TAATACGACTCACTATAGGGATTTTTA-CAAGTGTAGAAAA-3′ and S-288 5′-AAAAAA-AAAAAAATAATCATACTCCATAT-3′ for gND7-II (guide RNA for block II of NADH dehydrogenase subunit 7) with a 3′ poly(U) tail of 13 U residues; S-658 5′-TAATACGACTCACTATAGGGCTTT-TCTAAATAATAAAAAAAG-3′ and S-659 AAA-AAAAAAAAAAAAAATATATTTTCTCATGTTA for gCYb-II (guide RNA for block II of cytochrome *b*) with a 3′ poly(U) tail of 17 U residues. The 69-nt pGEM transcript was prepared using pGEM-7Z (Promega) linearised at the *Cla*I site. Transcription reactions contained 40 mM Tris (pH 8.1)/20 mM $MgCl_2$/1 mM spermidine/5 mM DTT/1 mM rNTPs/0.6 u μl^{-1} RNAguard (Pharmacia)/3.5 u μl^{-1} T7 RNA polymerase/5 ng μl^{-1} PCR product or approx. 100 ng ml^{-1} plasmid. For labeled RNA, 0.3 μl [α-^{32}P]UTP (10 mCi ml^{-1}, 800 Ci $mmol^{-1}$) per μl of reaction was included. Reactions were at 37°C for 2 h, followed by digestion of the templates with 10 u μl^{-1} RNase-free DNase for 15 min. Labeled RNAs were purified by electrophoresis in urea-polyacrylamide gels.

Synthetic mRNA sequences were prepared as described in Ref. 6, using the plasmids pNB2 [16], S(I + II) (G. Frech, L. Simpson and A. Simpson, unpublished results) and pND7. pND7 was constructed by cloning a PCR product containing the 5′ region of the ND7 gene into pBluescript SK(-) at *Bam*HI/*Eco*RI; the 5′ primer was S-602 5′-ATC-GAATTCTTAAATTTTATTTAGCCGACTA-3′ and the 3′ primer was S-603 5′-AGTGGATCCCAA-TAATTACATCTATATAC-3′.

Escherichia coli rRNA was from Boehringer-Mannheim and poly(U) from Pharmacia.

2.5. Incubation of TS with ^{32}P-labeled exogenous RNAs

^{32}P-labeled exogenous RNAs were incubated with TS (10 ng RNA with 10 μg TS protein, unless otherwise stated) for 15 min at 27°C in a volume of 20 μl. The concentrations of HEPES and KCl were maintained at 20 mM, and $MgCl_2$ was added to a final concentration of 3 mM.

2.6. Incubation of micrococcal nuclease-treated E. coli rRNA-saturated TS with ^{32}P-labeled exogenous RNAs

TS (10 μg protein) was digested with micrococcal nuclease (MN; 10 u) for 15 min at 37°C in the presence of 5 mM $CaCl_2$; the reaction volume was brought to 20 μl with 20 mM HEPES/20 mM KCl. EGTA was then added to 12.5 mM to terminate the reaction, followed by 40 μg *E. coli* rRNA (120-fold molar excess), and the mixture placed at 27°C for 15 min. ^{32}P-labeled RNA was then incubated with the treated TS for 40 min at 27°C in a final volume of 25–45 μl, with $MgCl_2$ at a final concentration of 3 mM.

2.7. Native polyacrylamide gradient gels and two dimensional native / urea gels

Samples containing TS labeled with either [α-^{32}P]-UTP or [^{32}P]exogenous RNA as described above were mixed with 1/5 volume of glycerol and electrophoresed in native polyacrylamide 4–18% linear gradient gels. The acrylamide gradients were stabilized with a 10–30% glycerol gradient and included 0.1% Tween 20 [17]. Gels (1 mm thick) were

run at 4°C in 40 mM Tris · acetate/1 mM EDTA (pH 8) (TAE buffer) at 90 V for 19 h (10 cm length). Molecular mass markers were thyroglobulin (669 kDa; Pharmacia), ferritin (443 kDa; Pharmacia) and β-amylase (200 kDa; Sigma). Gels were either autoradiographed wet, or fixed and stained with Rapid Coomassie Stain (Diversified Biotech) and dried under vacuum before autoradiography.

Two-dimensional native/urea gels were run by excising the lane from the native gel, denaturing in 50% formamide/0.1% SDS/TBE for 15 min at 65°C, and then layering on an 8 M urea polyacrylamide gel for the second dimension.

2.8. UV-crosslinking of proteins to RNAs in T-complexes

For labeling of T-III* and T-IV*, 30 μl of TS was labeled by incubation with 1.5 μCi of [α-^{32}P]UTP (800 Ci mmol^{-1}) (under low-UTP conditions), in a volume of 150 μl. 50 μl of the reaction was then further incubated with 10 mM EDTA and 50 μg *E. coli* rRNA to generate T-IV* and T-III$_r^*$, or with 50 μg ml^{-1} heparin to produce T-III$_H^*$. T-α was labeled by incubating MN-treated TS (40 μg protein, 40 u nuclease) with a mixture of 100 μg *E. coli* rRNA and approx. 10^6 cpm (300 ng)

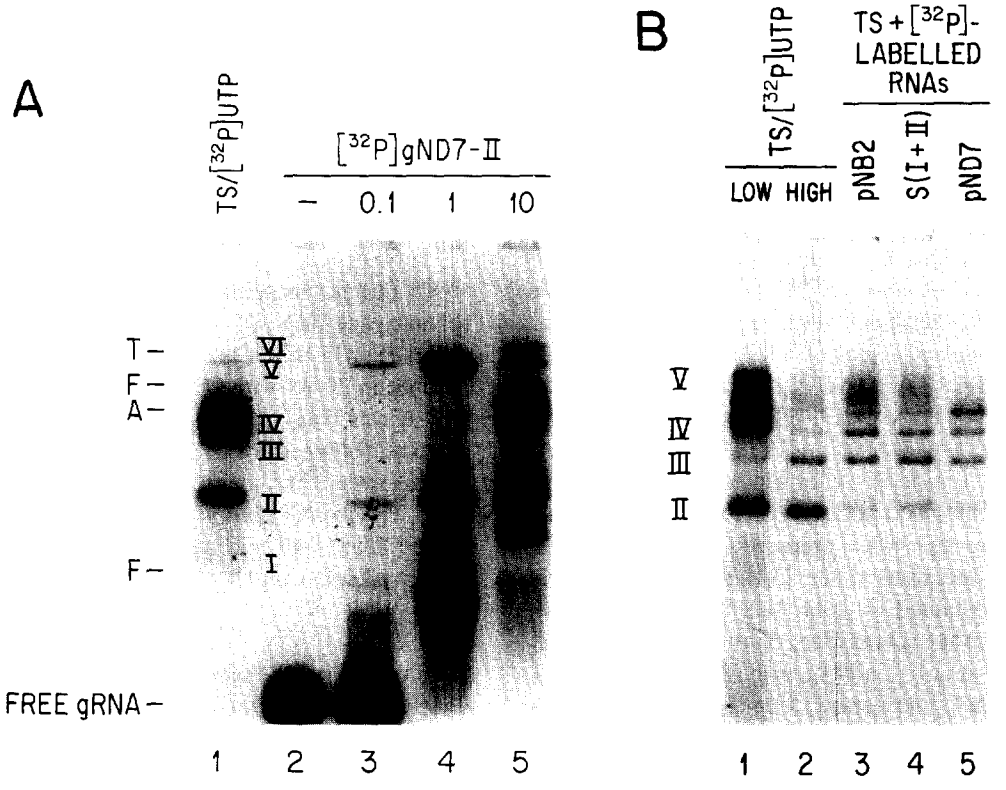


Fig. 1. Native gel showing labeling of complexes in TS by incubation with [α-^{32}P]UTP and ^{32}P-labeled RNAs. (A) Lane 1, TS labeled by incubation with [α-^{32}P]UTP under low-UTP conditions, as described in Materials and methods. Lanes 2–5: 25 ng of [^{32}P]-labeled synthetic gND7-II RNA (70 nt) incubated with the amount (μg protein) of TS extract indicated above each lane. The positions of molecular weight standards and free gRNA are shown on the left: T, thyroglobulin; F, ferritin; A, β-amylase and the positions of the 6 major T-complexes are indicated as I–IV. (B) Lanes 1 and 2: TS labeled by incubation with [α-^{32}P]UTP in low- and high-UTP conditions. Lanes 3–5: TS (10 μg protein) incubated with ^{32}P-labeled T7 transcripts of pNB2 (5′ sequence from the cytochrome *b* gene [16]) and S(I + II) (derivative of pNB2 with non base-pairing nucleotides in anchor sequence) and pND7 (5′ sequence from the ND7 gene), respectively.

[^{32}P]gND7-II RNA for 80 min in a volume of 55 μl. These preparations were electrophoresed in a native gradient gel and the labeled complexes visualized by autoradiography. The bands were excised, irradiated with UV light at 254 nm for 5 min at 25°C (Stratalinker, Stratagene), incubated with RNaseA (10 μg ml^{-1}) for 30 min at 37°C, and then incubated with 50 mM Tris · HCl (pH 6.8)/100 mM DTT/2% SDS/0.1% bromphenol blue for 30 min at 25°C. The gel slice was then layered onto a 0.1% SDS-10% polyacrylamide gel for electrophoresis in the second dimension.

For labeling of endogenous RNAs under high-UTP conditions, 46 μl of a micro-concentrated fraction of a Superose 6 gel filtration of TS was incubated with 40 μCi [α-^{32}P]UTP and 40 μM unlabeled UTP, in a final volume of 100 μl. This material was subjected to two-dimensional native/SDS gel electrophoresis as described above.

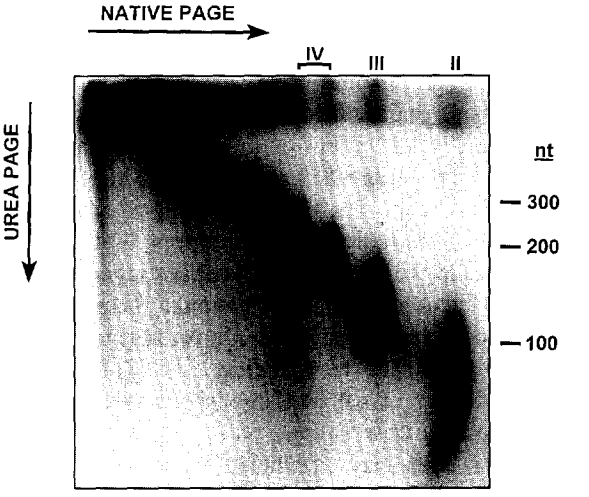


Fig. 2. Two-dimensional native/urea gel of complexes labeled with [α-^{32}P]UTP under high-UTP conditions. Labeled TS (10 μl) was electrophoresed in a native gradient gel, the lane excised, denatured and layered on an 8 M urea-10% polyacrylamide gel for electrophoresis in the second dimension. The positions of the T-complexes in the native gel are indicated, and the positions of RNA size standards (Ambion) run in the second dimension are indicated at the right.

3. Results

3.1. Characterization of two labeling patterns of T-complexes

When a clarified mitochondrial detergent extract (TS, see Materials and methods) of *L. tarentolae* is incubated with submicromolar amounts of [α-^{32}P]UTP under conditions in which the mitochondrial terminal uridylyl transferase (TUTase) is active, the endogenous gRNAs and mRNAs which are components of the RNP T-complexes are labeled, and approx. six bands can be visualized by electrophoresis in a native gel [7] as shown in Fig. 1A,B, lane 1. The major labeled band is the rather broad T-IV, which may represent a number of closely migrating complexes, the major RNA component of which was shown previously to consist of gRNA [7]. A second class of RNA components present in T-II, T-III and the T-IV region, which were termed 'arc RNAs' due to their characteristic migration pattern in a two dimensional native/urea gel, have been shown previously to include mRNA fragments [7].

Increasing the UTP concentration produces an overlapping but distinct labeling pattern (Fig. 1B, lane 2). The major differences between these two patterns are the presence in the high-UTP labeling of two distinct bands in the T-IV region and a more intense labeling of T-III. Two-dimensional native/urea gel analysis of the RNA components of high-UTP-labeled T-complexes yields the 'arc RNA' pattern (Fig. 2), suggesting that the endogenous mRNAs are preferentially labeled at high UTP concentrations, whereas at low UTP concentrations, the endogenous gRNAs in T-IV are preferentially labeled.

Labeled synthetic gND7-II RNA (60 nt; Fig. 1A, lanes 3–5) or gCYb-II RNA (data not shown) added to the TS extract binds to the T-complexes in a pattern resembling that obtained by self-labeling under low-UTP conditions. As shown in Fig. 1A, the relative extent of labeled exogenous gRNA binding to the various T-complexes is dependent on the amount of mitochondrial extract used; binding to the T-IV and T-VI complexes only occurs at higher extract levels. The binding occurs more rapidly than can be measured by a time-course of incubation followed by electrophoresis (data not shown). The evidence that this represents binding to pre-existing complexes, at least for T-I, T-II, T-V and T-VI, and not de novo reconstitution, as suggested by Goringer

et al. [9] for the RNP complexes they observed by gel retardation using mitochondrial extracts from *T. brucei*, is that labeling of these complexes was obtained using high-molecular-mass fractions from a Superose-6 gel filtration of the *L. tarentolae* mitochondrial extract (F. Bringaud and L. Simpson, unpublished results). The lack of labeling of complexes T-III and T-IV under these conditions probably resulted from the low protein concentration in the column fractions, but it still remains possible that these specific complexes undergo reconstitution with exogenous gRNA. Nevertheless, we will use the term, 'binding of gRNA', throughout this paper in discussing our observations.

On the other hand, a labeled synthetic pre-edited cytochrome *b* (CYb) mRNA fragment (pNB2 RNA, 280 nt; [6]) and a labeled synthetic NADH dehydrogenase subunit 7 (ND7) mRNA fragment (pND7, 220 nt) bind to the T-complexes in a pattern resembling that obtained by self-labeling under high-UTP conditions (Fig. 1B, lanes 3 and 5). This binding does not involve hybridization of the anchor sequence, as a CYb mRNA fragment in which the anchor sequence is substituted with non base-pairing nucleotides, S(I + II), binds equally well (Fig. 1B, lane 4). In addition, labeled poly(U) with an average size of 200 nt binds to the T-complexes in a pattern similar to that obtained by labeling TS under high-UTP conditions (data not shown).

3.2. *Effect of heparin and E. coli rRNA on the RNA-protein interactions in T-complexes labeled by incubation with [α-^{32}P]UTP*

In order to examine the stability of the T-complexes containing [α-^{32}P]UTP-labeled endogenous RNA, the resistance of these complexes to heparin and to excess heterologous rRNA was analyzed.

A single band which we have named T-III* retains RNA which was labeled with [α-^{32}P]UTP under low-UTP conditions, after competition with increasing amounts of heparin (Fig. 3A).

Most of the low-UTP-labeled T-complexes are also sensitive to competition with excess *E. coli* rRNA. As shown in Fig. 3B, incubation of labeled TS with rRNA yields a major band which we have

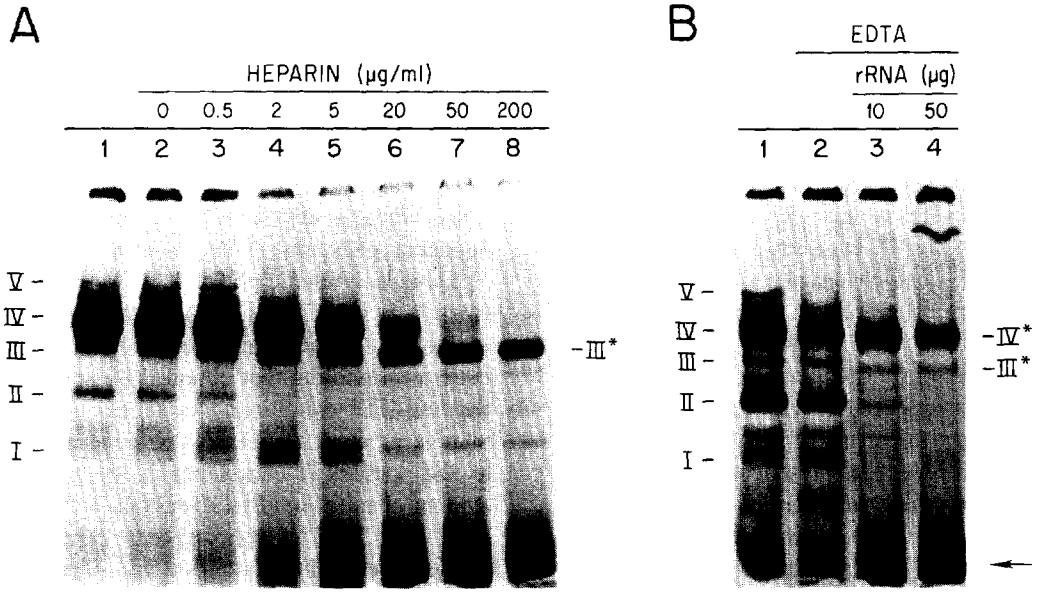
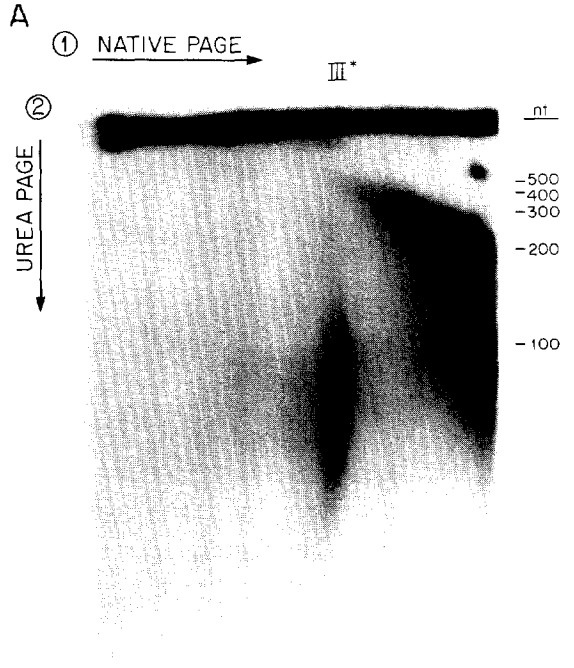
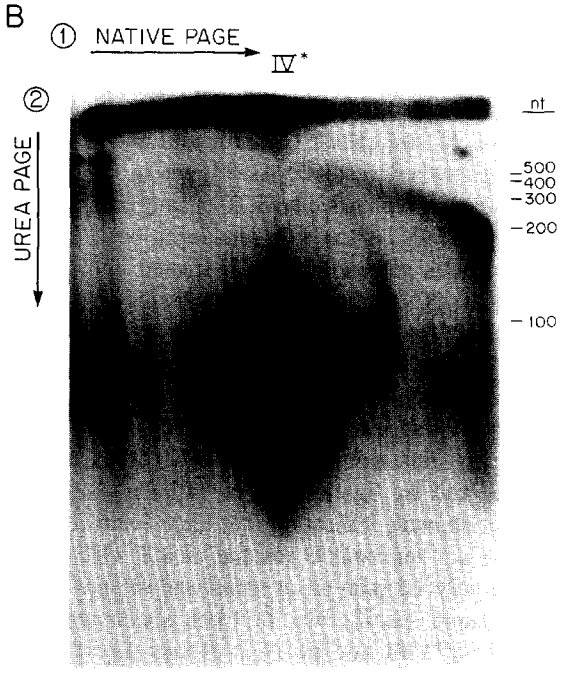


Fig. 3. Effect of heparin and rRNA on complexes labeled by [α-^{32}P]UTP under low-UTP conditions. 20-μl samples of labeled TS were incubated, with the exception of the sample in lane 1 of panels A and B, for 10 min at 27°C in the presence of: (A) lanes 2–8, increasing concentrations of heparin; (B) 10 mM EDTA (lane 2) plus the indicated amount of *E. coli* rRNA (lanes 3 and 4). The samples were electrophoresed on native gradient gels. The T-III* and T-IV* complexes are indicated at the right of the gels, and the released labeled RNA is marked by an arrow in panel B.





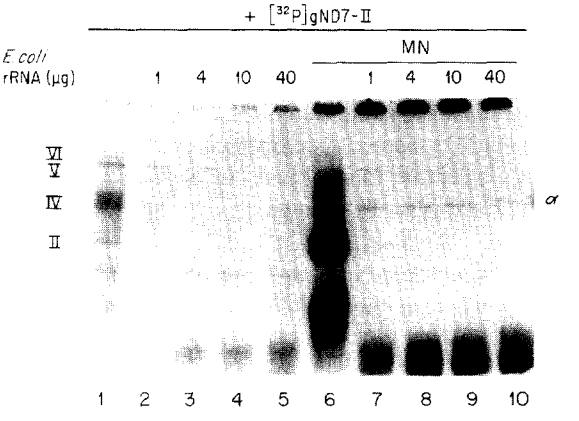


Fig. 5. Binding of [^{32}P]gND7-II RNA to T-complexes in TS extract treated with MN and non-specific RNA. Autoradiograph of native gradient gel. Each lane contains [^{32}P]gND7-II RNA (10 ng) and TS (10 μg protein). The TS extract used in lanes 6–10 was pre-digested with MN which was then inactivated, as described in Materials and methods. The samples for lanes 2–5 and 7–10 were then pre-incubated with the indicated quantities of *E. coli* rRNA for 15 min. All samples were incubated with the labeled gRNA for 40 min. The position of T-α is indicated at the right. Similar results were obtained when the rRNA was added after incubation with the gRNA.

labeled T-IV* and a minor band comigrating with T-III*. EDTA, which was used to inhibit the TU-Tase and prevent labeling of the added rRNA, produces a selective loss of band V (lane 2).

The labeled RNA released from low-UTP-labeled T-complexes by heparin or rRNA saturation migrates as a low-molecular-mass smear (Fig. 4). A two-dimensional native/urea gel analysis of the labeled RNA retained in the heparin-resistant T-III* and in the rRNA-resistant T-IV* shows the RNA to be a low-molecular-mass smear (less than 100 nt) in both cases (Fig. 4A,B), which is consistent with it representing gRNA. The RNA component of complex

Fig. 4. Two-dimensional native/urea gels of labeled RNA in T-III* and T-IV*. TS (60 μl) labeled by [α-^{32}P]UTP under low-UTP conditions was mixed with (A) 50 μg ml^{-1} heparin or (B) 10 mM EDTA plus 75 μg *E. coli* rRNA, in a final volume of 75 μl and then incubated at 27°C for a further 10 min. The samples were electrophoresed in a two-dimensional native/urea gel as in Fig. 2. The positions of the T-III* and T-IV* complexes in the first dimension are indicated above the gels, and the positions of ^{32}P-labeled RNA size standards (Ambion) run in the second dimension are indicated at the right.

III* was confirmed to be gRNA by hybridization of an electroblot of a native gel (M. Peris and L. Simpson, unpublished results).

On the other hand, the mRNA-protein interactions in high-UTP-labeled T-complexes proved to be completely sensitive to the levels of heparin and rRNA used above (data not shown).

3.3. Characteristics of a T-complex which specifically binds exogenous gRNA

The majority of the binding of labeled exogenous gND7-II RNA is relatively low affinity, as the binding can be almost completely competed by a large excess of *E. coli* rRNA (Fig. 5, lanes 1–5). The binding of labeled exogenous mRNA is also competed by a large excess of rRNA (data not shown).

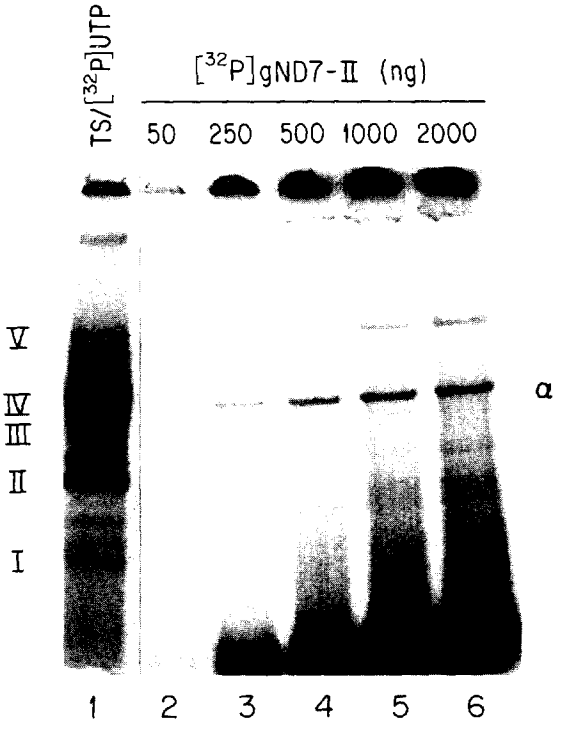


Fig. 6. Binding of [^{32}P]gND7-II RNA to T-α over a range of RNA concentrations. Autoradiograph of native gradient gel. Lane 1, TS labeled by incubation with [α-^{32}P]UTP under low-UTP conditions. The positions of the T-complexes are indicated on the left. Lanes 2–6: MN-digested rRNA-saturated TS prepared as described in Materials and methods was incubated with the indicated quantities (ng) of [^{32}P]gND7-II RNA (lanes 2–6). The position of T-α is indicated.

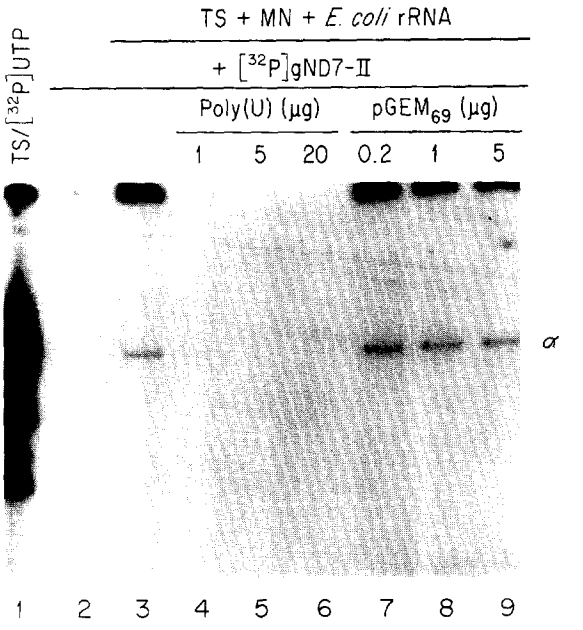
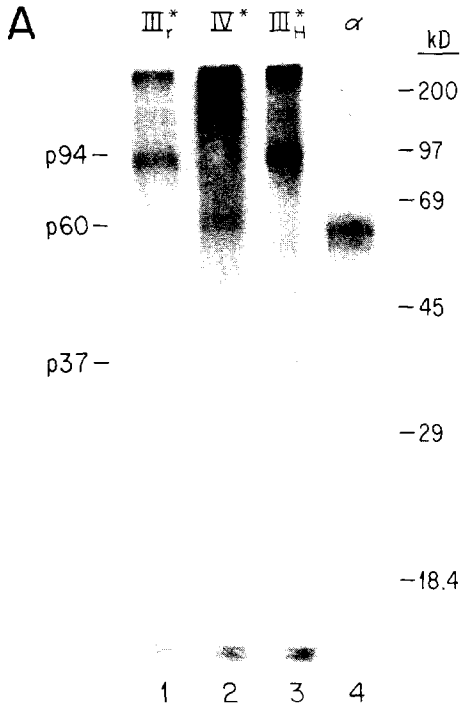
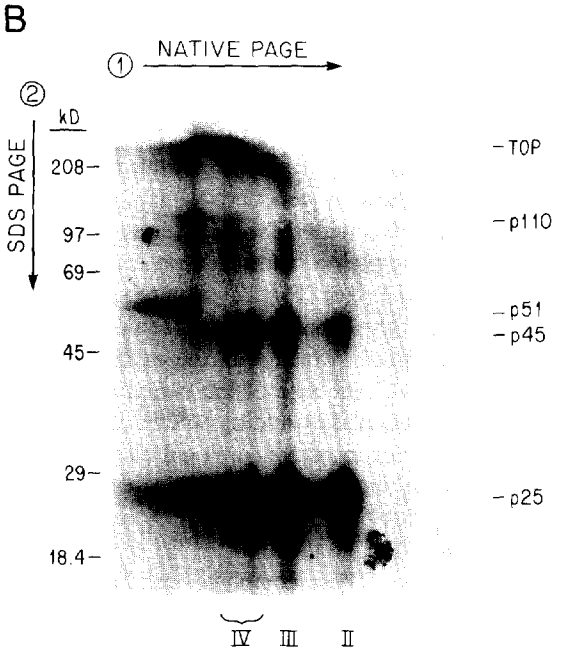


Fig. 7. Effect of heterologous RNAs on binding of [^{32}P]gND7-II RNA to T-α. Autoradiograph of native gel. Lane 1, TS labeled by incubation with [α-^{32}P]UTP under low-UTP conditions. Lanes 2–9: [^{32}P]gND7-II RNA; lanes 3–9 incubated with MN-treated rRNA-saturated TS. The indicated quantities of poly(U) (lanes 4–6) or the 69 nt pGEM T7 transcript (lanes 7–9) were included in the incubations.

However, high-affinity gRNA-specific interactions can be uncovered by use of micrococcal nuclease (MN)-treated TS and competition with a large excess of *E. coli* rRNA (F. Bringaud and L. Simpson, unpublished results). As shown in Fig. 5, an RNP complex which we term T-α, migrating in the vicinity of T-IV, is the sole remaining band labeled by exogenous gRNA when MN-treated TS is saturated with rRNA. Labeled exogenous gCYb-I and gCYb-II RNAs also bind specifically to the α-complex, although less efficiently than gND7-II RNA, and endogenous pooled gRNA represented by [α-^{32}P]GTP-capped kinetoplast RNA also binds specifically (data not shown). gND7-II RNA labeled at the 3′ end by ligation of [α-^{32}P]pCp also binds (data not shown), indicating that there is no requirement for a 3′-terminal U residue or a 3′ OH group. Labeled poly(U) with an average size of approx. 80 nt also shows specific binding (data not shown), and gND7-II, gCYb-I and gCYb-II RNAs modified by removal of the 3′-oligo(U) tails show significantly





less binding than the unmodified gRNAs (data not shown). No specific binding was detected with labeled kinetoplast tRNA or a 69-nt labeled T7 transcript from the pGEM plasmid (data not shown). These data suggest a specific binding of gRNAs, probably mainly involving an interaction with the 3′-oligo(U) tail.

The binding of the labeled exogenous gRNA to the α-complex proceeds for at least 80 min at 27°C, and in the standard 40-min incubation only approx. 1–2% of the input gRNA is incorporated into the complex (Fig. 6). Residual specific binding to a complex migrating in the T-V region was observed in some experiments (Fig. 6), but this was not investigated further. The binding of labeled exogenous gRNA to T-α is relatively slow compared to the rapid non-specific binding of exogenous gRNA to untreated T-complexes. The gRNA-T-α − complex association is resistant to 200 μg ml^{-1} heparin added after binding, but only resistant to 5 μg ml^{-1} heparin added prior to binding (data not shown), suggesting in addition a slow rate of dissociation.

To further test the specificity of gRNA binding to T-α, a variety of RNAs were tested as competitors. Total yeast RNA, yeast tRNA (data not shown) and a 69 nt T7 pGEM transcript (Fig. 7, lanes 7–9) are unable to compete the binding of gND7-II RNA. The only heterologous RNA which competed effectively is poly(U) (Fig. 7, lanes 4–6), again suggesting that the interaction mainly involves the 3′-oligo(U) tail of the gRNA.

Fig. 8. UV-crosslinking analysis of proteins binding to [α-^{32}P]UTP-labeled endogenous RNAs in the T-III*, T-IV* and high-UTP-labeled complexes, and to exogenous labeled gRNA in T-α. (A) Lane 1, complex T-III$_r^*$, which represents the minor band produced in the presence of EDTA and excess *E. coli* rRNA; lane 2, complex T-IV*; lane 3, complex T-III$_H^*$, which represents the band produced in the presence of heparin; lane 4, complex T-α. Protein molecular mass markers are indicated on the right, and the UV-crosslinked proteins, p37, p60 and p94, on the left. (B) T-complexes labeled with [α-^{32}P]UTP under high-UTP conditions. Protein molecular mass markers are indicated on the left, and the positions of the UV cross-linked proteins, p25, p45, p51 and p110, on the right. UV-crosslinking, RNase A digestion and two dimensional native/SDS gel electrophoresis are as described in Materials and methods.

3.4. UV cross-linking analysis of RNA-binding proteins in T-III*, T-IV* and T-α complexes, and also in high-UTP-labeled complexes

An analysis of RNA-binding proteins in the T-III*, T-IV* and T-α complexes is shown in Fig. 8A. Complex T-III_H^* and complex T-III_r^* both have a predominant RNA-binding protein of approx. 94 kDa (p94). Complex T-α contains a major RNA-binding protein (or proteins) of approx. 60 kDa (p60), and a minor one of approx. 37 kDa (p37). T-IV* also contains a 60-kDa protein.

UV cross-linking of complexes labeled either by incubation with [α-^{32}P]UTP under high-UTP conditions (Fig. 8B) or by the binding of labeled pNB2 RNA (data not shown), detects two major proteins of approx. 25 kDa (p25) and 45 kDa (p45), which are components of each of the complexes in the ladder, and which have also been identified in these RNP complexes labeled with [α-^{32}P]UTP under low-UTP conditions (F. Bringaud and L. Simpson, unpublished results). A 110-kDa protein (p110) is detected in the T-V region of the high-UTP pattern, and a 51-kDa protein is distributed among the higher-molecular-mass complexes migrating in the T-VI region. 110- and 51-kDa proteins co-migrating with the T-V and the T-VI complexes, respectively, have also been found to UV-crosslink to endogenous RNAs labeled with [α-^{32}P]UTP under low-UTP conditions (F. Bringaud and L. Simpson, unpublished results).

These UV cross-linking results indicate that those proteins that interact with a high affinity with gRNA differ from those proteins which interact with a lower affinity with endogenous or exogenous gRNA or mRNA.

4. Discussion

The relationship and biological function of the various T-complexes is not known. The T-IV complex is probably involved with the maturation of gRNAs by the 3′ addition of U residues, but the nature and role of the remaining complexes is an open question. The presence of bound mRNAs may represent a functionally significant phenomenon indicating an involvement in the process of RNA editing of pre-edited mitochondrial transcripts, or, on the other hand, may represent a non-specific binding of mitochondrial mRNA molecules to RNP complexes involved in other metabolic processes. In order to eventually distinguish between these possibilities, we have investigated several aspects of the labeling of the endogenous RNA components of the various T-complexes by the endogenous TUTase activity. We have also shown that these complexes bind exogenous gRNAs and mRNAs, and that there are several high-affinity protein-RNA interactions.

The native gel profile of labeled T-complexes was found to be highly dependent on the concentration of UTP during the incubation. The distinction between labeling of the various T-complexes under low-UTP or high-UTP conditions is that the endogenous gRNAs in T-IV are preferentially labeled with sub-micromolar concentrations of UTP and the endogenous mRNAs in T-II, T-III and the T-IV region are preferentially labeled with higher concentrations of UTP. The reason for this differential labeling is uncertain, but could be due to an absolute preference of the endogenous TUTase for a presumed limiting amount of gRNA substrate. At high UTP levels, in this model, the endogenous gRNA substrates would be rapidly saturated with low specific activity U residues, and then the endogenous mRNAs, which may constitute a larger substrate pool [18], would take up the majority of the label.

The presence of mRNAs in T-complexes could be due either to non-specific binding of endogenous mRNAs to proteins in these complexes or to a specific and perhaps functional interaction of endogenous mRNAs with these complexes. The characteristic 'arc' migration pattern [7] observed in a two dimensional gel (see Fig. 2) is probably due to protection of the bound RNAs against nuclease degradation in the extract, with the extent of protection varying with the size of the complex. Evidence for this was obtained by adding labeled 280-nt pNB2 RNA to TS prior to native/urea gel electrophoresis, and observing the characteristic arc pattern in the autoradiograph of the gel (data not shown).

Exogenous RNAs added to the TS extract bind to RNP complexes. These complexes can be tentatively identified as the T-complexes which are labeled under low-UTP conditions, due to a comigration in native gels. In addition, there is a common set of

proteins which can be UV-crosslinked to labeled exogenous or endogenous gRNA (F. Bringaud and L. Simpson, unpublished results). The pattern of binding of synthetic gRNAs to these RNP complexes differs from the pattern of binding of mRNAs and several transcripts unrelated to editing.

Most of the endogenous T-complex RNAs which are labeled by [α-^{32}P]UTP by the endogenous TUTase either under low- or high-UTP conditions can be competed by addition of a large excess of nonspecific RNA (or heparin), implying that most endogenous RNA-protein interactions are relatively low affinity and possibly non-specific. Similarly, the binding of labeled synthetic gRNA and labeled synthetic mRNA are also both susceptible to rRNA (120-fold molar excess) and heparin competition, again suggesting low affinity RNA-protein interactions. However, low and intermediate levels of competitor tRNA (10–40-fold molar excess) or rRNA (3–6-fold molar excess) had only a moderate effect on these RNA-protein interactions (Fig. 5, lane 1; R. Stripecke and L. Simpson, unpublished results), indicating that the level of specificity is similar to that of the reported 8 polypeptides which interact with exogenous gRNAs in mitochondrial extracts from *T. brucei* [9,11]. The *T. brucei* gRNA-protein interactions were similarly sensitive to high levels of heparin or competitor RNA. In addition, it is entirely possible that, as is the case in pre-mRNA splicing [19] or hammerhead ribozyme activity [20], proteins which have a low affinity for RNA in general could nevertheless serve some biological function in these complexes. It remains to be demonstrated, however, that any of the various T-complexes other than T-IV play a role in the process of RNA editing.

Evidence for the presence of several high-affinity RNA-protein interactions was obtained by treating TS, which was labeled with [α-^{32}P]UTP under low-UTP conditions, with heparin or excess rRNA, yielding the T-III* and T-IV* complexes, which may both represent derivatives of the T-IV RNP complex, since they both appear to contain gRNA. Evidence was also obtained for high affinity gRNA-protein interactions involving the binding of exogenous gRNAs to the T-α complex, which may also represent a derivative of T-IV. To detect the T-α complex, the endogenous RNA had to be first depleted by nuclease digestion and non-specific binding sites had to be saturated with excess rRNA. The kinetics with which exogenous gRNAs bind to and dissociate from the T-α complex are slow compared to the kinetics with which the non-specific binding of gRNA occurs. Poly(U) also binds to T-α and can compete with the binding of the gRNAs, suggesting that the 3′-oligo(U) tail of the gRNA is a major determinant of binding specificity.

UV-crosslinking experiments showed that 37- and 60-kDa proteins in the T-α complex represent the major exogenous gRNA-binding components. These proteins are similar in size to two of the gRNA-interacting polypeptides in *T. brucei* [11]. In *T. brucei*, however, polypeptides with molecular masses of 90 and 21 kDa showed the strongest interactions with gRNAs [11]. Interestingly, we found that a 94-kDa protein binds to endogenous [α-^{32}P]UTP-labeled RNA in *L. tarentolae* extracts incubated with heparin or excess rRNA.

Further analysis is required to characterize the biochemical relationship of the various T-complexes and to understand their biological role.

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