

Characterization of two classes of ribonucleoprotein complexes possibly involved in RNA editing from *Leishmania tarentolae* mitochondria

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Communicated by E.M.De Robertis

The molecular mechanism of RNA editing in trypanosomatid mitochondria is an unsolved problem. We show that two classes of ribonucleoprotein complexes exist in a mitochondrial extract from *Leishmania tarentolae* and appear to be involved in RNA editing. The 'G' class of RNP complexes consists of 170–300 Å particles which contain guide RNAs and proteins, show little terminal uridylyl transferase (TUTase) activity and exhibit an *in vitro* RNA editing-like activity. The 'T' class consists of approximately six RNP complexes, the endogenous RNA of which can be self-labeled with [α -³²P]UTP. The most abundant T complex, T-IV, is visualized by electron microscopy as 80–140 Å particles. This complex exhibits TUTase activity in the native gel and contains guide RNAs. Both G and T complexes are possibly involved with RNA editing *in vivo*. These results are a starting point for the analysis of the biochemistry of RNA editing.

Key words: G complexes/gRNA/*Leishmania*/T complexes/TUTase

Introduction

RNA editing of maxicircle transcripts in mitochondria of kinetoplastid protozoa involves the addition and deletion of uridine (U) residues within coding and, in some cases, untranslated regions. The information for the edited sequence is encoded in guide RNAs (gRNAs), but the precise mechanism of editing is still unclear (for recent reviews see Hajduk *et al.*, 1993; Simpson *et al.*, 1993; Sloof and Benne, 1993; Stuart, 1993). An *in vitro* gRNA–mRNA chimera-forming activity has been demonstrated in mitochondrial extracts from *Trypanosoma brucei* (Harris and Hajduk, 1992; Koslowsky *et al.*, 1992) and *Leishmania tarentolae* (Blum and Simpson, 1992), and an *in vitro* internal U-incorporation RNA editing-like activity specific for several pre-edited mRNAs has been described using a mitochondrial extract from *L.tarentolae* (G.Frech, N.Bakalara, L.Simpson and A.Simpson, unpublished results).

Pollard *et al.* (1992) have reported the existence of two multicomponent complexes in a *T.brucei* mitochondrial extract, one (Complex I) sedimenting in glycerol gradients

at 19S and containing gRNA, terminal uridylyl transferase (TUTase), RNA ligase and chimera-forming activity, and the other (Complex II) sedimenting at 35–40S and containing gRNA, loosely-bound TUTase, pre-edited mRNA, RNA ligase and chimera-forming activity. They suggested that Complex I could function as a gRNA 'maturation–selection' complex and Complex II as the 'active editing' complex. The results were interpreted in terms of the cleavage–ligation model of editing (Blum *et al.*, 1990; Harris *et al.*, 1992), as distinguished from the transesterification model of editing (Blum *et al.*, 1991; Cech, 1991).

We show here, by native gel analysis and 2-D native/denaturing gel analysis, that the situation, at least in *L.tarentolae*, is more complex in that there are multiple TUTase-labeled complexes with different RNA and protein components. In addition, we provide functional and morphological evidence for the existence of a large ribonucleoprotein complex involved in an *in vitro* editing-like activity.

Results

Identification of several [α -³²P]UTP-labeled ribonucleoprotein complexes (T complexes) by native gel electrophoresis

When a clarified mitochondrial detergent extract (TS, see Materials and methods) was incubated with [α -³²P]UTP under conditions in which the TUTase is active (Bakalara *et al.*, 1989) and was electrophoresed through a native polyacrylamide gradient gel, several high molecular weight labeled bands could be seen (Figure 1A, left side of panel). The apparent molecular weights of these bands range to >700 kDa, according to protein size standards. These ribonucleoprotein complexes are labeled 'T complexes' (for TUTase-labeled), to distinguish them from the second type of RNP complexes described below which are labeled 'G complexes'. Labeling of the T-I and T-VI regions showed some variation. The labeled material that remains in the well and in the interface with the stacking gel probably corresponds to mitochondrial rRNA which is present in the extract and is known to be labeled with UTP by the mitochondrial TUTase (Bakalara *et al.*, 1989).

Phenol-chloroform extraction of the [α -³²P]UTP-labeled TS resulted in a loss of the labeled complexes in a native gradient gel and the coincident appearance of a low molecular weight smear in the region of T-I and below. This material was sensitive to RNase digestion (data not shown). These results suggest that the high molecular weight labeled bands represent protein-bound RNAs which are presumably 3' end-labeled by the TUTase activity in the extract (Bakalara *et al.*, 1989).

Incubation of [α -³²P]UTP-labeled TS with up to 100 mM KCl had no effect on the relative extent of labeling or on

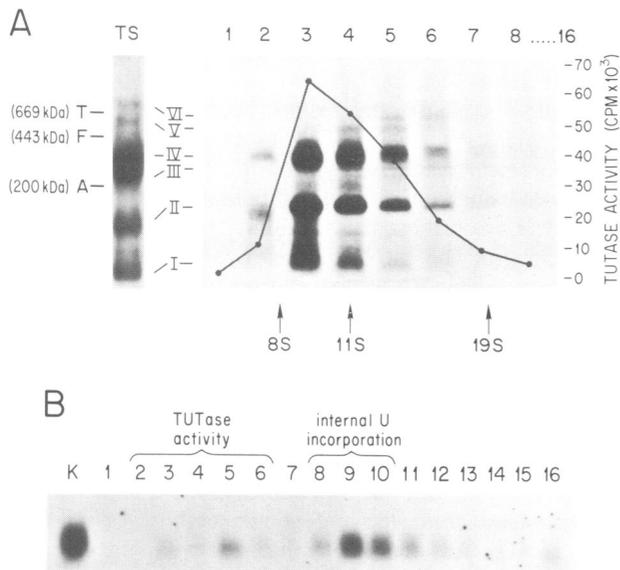


Fig. 1. Sedimentation analysis of TS: TUTase activity, $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ -labeled T complexes and gRNA distribution. (A) On the left of this panel is a native gradient gel fractionation of $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ -labeled TS which shows the six labeled T complexes. The positions of molecular weight standards are indicated on the left: T, thyroglobulin; F, ferritin; A, β -amylase. Also shown in this panel are the fractions from a glycerol gradient. Unlabeled TS was sedimented through a 10–30% glycerol gradient and 16 fractions were collected from the top. Each fraction was concentrated to the same volume and aliquots were used for assay of TUTase activity and labeling of the T complexes by incubation with $[\alpha\text{-}^{32}\text{P}]\text{UTP}$. The T complexes were visualized by native 4–16% gel electrophoresis. The TUTase activity of each fraction, assayed by measuring the labeling of cytosolic rRNA by $[\alpha\text{-}^{32}\text{P}]\text{UTP}$, is plotted on the autoradiograph. Lanes 9–16, corresponding to fractions 9–16, are not shown because they are negative for both labeled bands and TUTase activity. (B) RNA was isolated from equal volumes of each fraction and electrophoresed in formaldehyde–agarose. The gel was blotted and the blot hybridized with a mixture of six 5' ^{32}P -labeled oligonucleotide probes for the MURF4 gRNAs. Lane K, kinetoplast RNA control to show the migration of gRNAs in the gel. The localization of TUTase activity and the internal U-incorporation activity (see Figure 6) is shown in brackets.

the electrophoretic migration profile in the native gradient gel (data not shown), indicating that the complexes are stable in high salt. However, when labeled TS was incubated with increasing amounts of heparin, the labeled bands vanished and most of the label appeared in a low molecular weight smear, and a new high molecular weight band appeared in the vicinity of T-III (E.Byrne and L.Simpson, unpublished results). Addition of *Escherichia coli* rRNA to TS also led to the release of most of the bound labeled endogenous RNA, with the exception of that RNA bound to the lower region of T-IV and T-III (E.Byrne, F.Bringaud and L.Simpson, unpublished results). These results suggest that most of the protein–RNA interactions in the T complexes are not high affinity because the bound RNA can be competed by heparin or exogenous rRNA; however a few interactions appear to be high affinity and specific.

Fractionation of TUTase activity of mitochondrial extract by sedimentation and gel filtration

The mitochondrial TUTase was shown previously to add uridylate residues to the 3' OH-termini of exogenous RNA (Bakalara *et al.*, 1989). When TS was fractionated on a glycerol gradient and each fraction assayed for TUTase

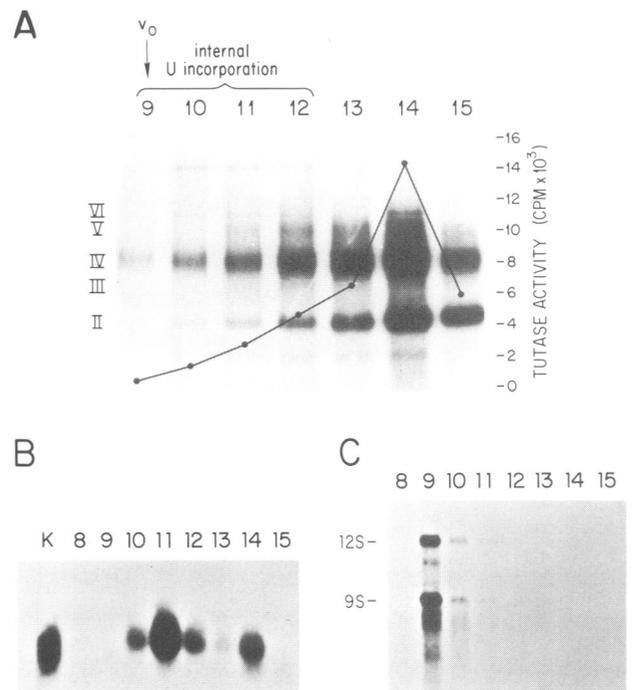


Fig. 2. Gel filtration analysis of TS: TUTase activity, $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ -labeled T complexes and gRNA and rRNA distribution. (A) Unlabeled TS was fractionated on a Superose 6 FPLC column. Aliquots from each fraction were labeled by incubation with $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ and the labeled T complexes visualized by autoradiography of a native 4–16% gel. The void volume is indicated by V_0 and the T complexes are shown on the left. The internal U-incorporation activity is indicated by brackets. The TUTase activity of each fraction, assayed by measuring the labeling of cytosolic rRNA by $[\alpha\text{-}^{32}\text{P}]\text{UTP}$, is plotted on the autoradiograph. (B) RNA was isolated from each Superose 6 column fraction and electrophoresed in formaldehyde–agarose. The gel was blotted and the blot hybridized with a mixture of six 5' ^{32}P -labeled oligonucleotide probes for MURF4 gRNAs. (C) An identical blot as in (B) was hybridized with PCR-amplified 9S and 12S kinetoplast rRNA genes that were random-primed with $[\alpha\text{-}^{32}\text{P}]\text{dATP}$. The two major RNA bands showing hybridization in the void volume fraction 9 represent the 610 nt 9S and the 1172 nt 12S RNAs.

activity using total cell RNA as substrate, the activity sedimented over a broad range with a peak at $\sim 10\text{S}$, as shown in Figure 1A. A correlation of the TUTase labeling of exogenous RNA and the endogenous $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ -labeling of the T complexes is also shown in this experiment. Each gradient fraction was incubated with $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ and then electrophoresed in a native gradient gel. Similar results for the relative mobility of labeled T complexes in the gradient were obtained using TS pre-labeled with $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ (data not shown). This cosedimentation of TUTase activity with labeled T complexes indicates that the labeling of the RNA components of the T complexes is probably due to the same TUTase activity that adds uridylate residues to the 3' OH-termini of exogenous RNA substrates added to the TS.

Fractionation of TS by gel filtration in Superose 6, as shown in Figure 2A, gave similar results to the sedimentation analysis. The TUTase activity eluted in a broad retarded peak. Incubation of each column fraction with $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ and subsequent native gel electrophoresis yielded a distribution of labeled T-II to T-VI bands throughout the retarded TUTase peak.

The presence of TUTase activity in the RNP complexes

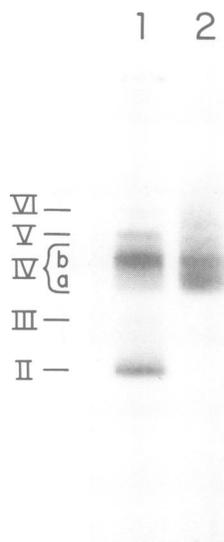


Fig. 3. *In situ* TUTase activity of T complexes in native gel. Lane 1, native gel electrophoresis of labeled TS. T complexes are indicated on the left. Lane 2, unlabeled TS was electrophoresed in a native 4–16% gel and the gel slice incubated with [α - 32 P]UTP for 60 min at 27°C. The gel slice was washed to remove unincorporated label, dried and exposed to X-ray film.

was determined by incubation of a native gel of unlabeled TS in the presence of [α - 32 P]UTP under TUTase conditions. As shown in Figure 3, two discrete bands (labeled a and b) migrating in the T-IV region were labeled by this method, indicating the presence of TUTase activity in these complexes. An unresolved minor smear of incorporation was also present in the regions of T-V and T-VI. In other complexes, TUTase is either absent or unavailable for *in situ* labeling.

[α - 32 P]UTP-labeled RNA components of complexes T-I to T-VI: 2-D gel electrophoresis

[α - 32 P]UTP-labeled TS was electrophoresed in a native gradient gel and the gel lane was excised and layered onto a 10% polyacrylamide denaturing gel, thereby releasing the labeled RNA components from each RNP complex. The patterns obtained were almost identical using either 0.1% SDS (Figure 4A) or 8 M urea (Figure 4B) as the denaturant in the second dimension. RNase A treatment of the native gel slice prior to electrophoresis completely eliminated the labeled bands (data not shown). As shown in Figure 4B, most of the labeled RNA from T-IV migrated in a broad low molecular weight smear. Minor smears of this size range were also released from T-V and T-VI. In addition, there was a second labeled RNA component (arrows in Figure 4B) which showed a size increase and an abundance decrease correlating with the size of the complex. Because of this characteristic migration pattern in the 2-D gel, these latter RNAs are termed ‘arc-RNAs’.

The genomic origins of the various RNA components were determined by hybridization of electroblots of the 2-D native/urea polyacrylamide gels of unlabeled TS with DNA probes. The efficiency of the electroblotting procedure is apparent from Figure 4B, which represents an electroblot of labeled RNA in a 2-D gel. Hybridization of an electroblot of unlabeled TS with a probe for 9S rRNA showed the

presence of rRNA in the well of the native gel and at the interface of the stacking native gel, but not in any of the T complexes (data not shown).

Figure 4C shows the results of a hybridization of an electroblot of unlabeled TS with an oligomeric probe for cytochrome *b* gRNA-II (gCYb-II). Similar results were obtained using a mixture of eight oligomeric probes for the complete set of ribosomal protein S12 (RPS12) gRNAs (data not shown). The gRNA probes hybridized with the low molecular weight RNA released from the T-IV region. These results indicate that gRNAs are present in T-IV. The presence of other low molecular weight RNA species is not ruled out, however, since the region of hybridization is localized to the upper portion of the labeled RNA smear visible in Figure 4A and B.

The blots were also hybridized with an oligonucleotide probe for CYb mRNA, complementary to a sequence 70 nt downstream from the pre-edited region. As shown in Figure 4D, this probe hybridized with the arc-RNA components from the T-III region and above. Similar hybridization results were obtained using a probe for the 5' portion of the NADH dehydrogenase subunit 7 (ND7) mRNA (data not shown). These results suggest that the arc-RNAs contain 5' fragments of maxicircle-derived mRNAs. No hybridization was observed with the RNA released from T-II.

Control experiments (data not shown) suggest that the arc-RNAs represent degradation of endogenous RNA that occurs as a result of experimental manipulation. The lack of hybridization of the CYb mRNA probe to arc-RNA released from T-II in Figure 4D could be due to degradation of that region of the molecule containing the probe sequence.

Fractionation of mitochondrial extract identifies a larger RNP complex (G complex) associated with an *in vitro* editing-like activity

We have demonstrated an *in vitro* editing-like activity, using the *L.tarentolae* TL mitochondrial extract (see Materials and methods), in which U residues are incorporated into the pre-edited region of pNB2 RNA (pNB2 RNA is the 5' portion of pre-edited CYb mRNA) (G.Frech, N.Bakalara, L.Simpson and A.Simpson, unpublished results). The activity is assayed by digesting the labeled pNB2 substrate RNA with RNase H after annealing of a specific complementary oligonucleotide downstream of the pre-edited region and measuring the extent of labeling of the released 5' and 3' fragments by autoradiography of analytical gels.

We show in the experiment in Figure 5 that the internal U-incorporation activity of TL is retained, with some decrease of activity, in TS and S-100 (see Materials and methods) extracts, indicating that this activity is present in a soluble fraction. The percentage of label in the 5' versus the 3' fragment decreased from 39% using TS, to 25% using the S-100 extract. To measure the sedimentation coefficient of the soluble activity, each fraction from a glycerol gradient of TS was assayed for internal U-incorporation activity. As shown in Figure 6A, the internal U-incorporation activity sedimented around 25S, clearly separated from the labeling of the endogenous RNAs in T-I to T-VI and from the peak of the TUTase activity, which was shown above in Figure 1A to sediment around 10S. The addition of U residues to the 3' OH of the substrate pNB2 RNA can also be seen in Figure 6A to peak at ~10S.

RNA was isolated from each fraction of the gradient in Figure 1A and subjected to Northern analysis as shown in Figure 1B, using for hybridization a mixture of six oligonucleotides antisense to the six gRNAs involved in editing of maxicircle unidentified reading frame 4 (MURF4) mRNA. The gRNAs showed a bimodal distribution in the gradient, with >60% of the RNA being associated with the 25S region.

Similar results were obtained by analysis of the Superose 6 gel filtration experiment shown in Figure 2A. Each fraction was assayed for *in vitro* internal U-incorporation activity and, as shown in Figure 6B, the activity eluted in fractions 9–12

with a peak in fraction 11, separate from the main TUTase peak. Northern analysis of RNA isolated from each column fraction also showed a bimodal distribution of MURF4 gRNAs, with 75% of the gRNA being associated with those fractions that also exhibited the internal U-incorporation activity (fractions 10–12 in Figure 2B), and 25% of the gRNA being associated with the peak of TUTase activity (fraction 14 in Figure 2B). Use of a hybridization probe for gCYb-II yielded similar results to MURF4, except that some gRNA was also detected in the void volume in fraction 9 (data not shown). Hybridization of the Northern blots with PCR-amplified DNA probes for the 9S and 12S

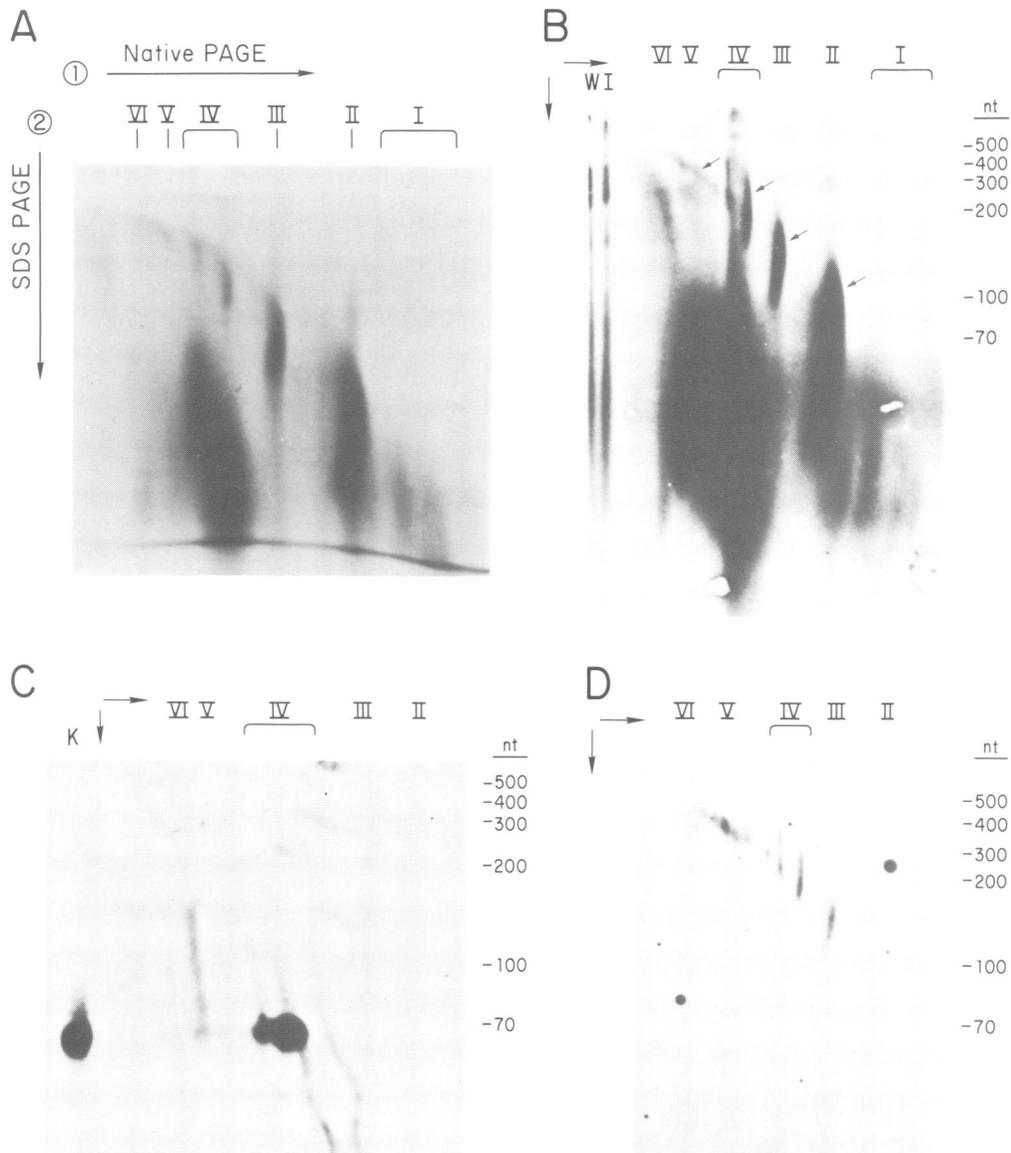


Fig. 4. 2-D gel analysis of RNA components of T complexes. [α - 32 P]UTP-labeled (A and B) or unlabeled (C and D) TS was electrophoresed in a native 4–16% gel and the excised gel lane was layered onto either a 0.1% SDS–10% acrylamide gel (A) or a 8 M urea–10% acrylamide gel (B, C and D). The positions of the six major T complexes in the first dimension are indicated above the gel. (A) Autoradiograph of fixed SDS–polyacrylamide gel. (B) Autoradiograph of electroblot of 8 M urea–polyacrylamide gel. W, well of the first dimension gel; I, interface of the 4% stack in the first dimension. Arrows indicate positions of the arc-RNAs. (C) Hybridization of electroblot of 2-D gel of unlabeled TS probed with oligonucleotide antisense to gRNA for CYb-II (S-530). K, kRNA control. (D) Hybridization of electroblot of 2-D gel of unlabeled TS probed with oligonucleotide antisense to CYb mRNA (S-178). Size standards shown on the right are from an RNA ladder (Ambion) and tRNA.

mitochondrial rRNAs showed that the mitochondrial rRNA eluted mainly in the void volume in fraction 9, as shown in Figure 2C.

The glycerol gradient and gel filtration results together indicate the presence of a class of higher molecular weight RNP complexes which contain the majority of the gRNA

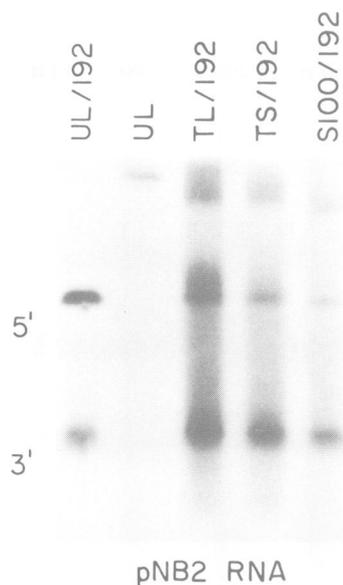


Fig. 5. Internal U-incorporation activity is in a soluble fraction. Unlabeled pNB2 RNA was incubated with [α - 32 P]UTP in the presence of TL, TS or S-100 mitochondrial extracts. The intact labeled RNA was gel-isolated and subjected to RNase H analysis using oligonucleotide S-192, which liberates two fragments indicated as 5' and 3'. The larger fragment is derived from the 5' portion of the molecule, which contains the internal U-incorporation within the pre-edited region and the 3' fragment is labeled due to 3' U addition by the known TUTase activity. UL, pNB2 RNA uniformly labeled with 32 P during T7 transcription.

in TS and exhibit internal U-incorporation activity *in vitro*, but which lack TUTase activity. We term these the 'G' class of RNP complexes (to indicate 'major gRNA-containing').

Identification of two classes of RNP complexes by electron microscopy

Figure 7A shows an electron micrograph of a representative field from a portion of the TUTase peak from a glycerol gradient. The field consists of a fairly monodisperse class of particles (arrow), ~ 100 Å in diameter, which appear to contain a central hollow core. These particles probably correspond to complex T-IV, since this represents the most abundant [α - 32 P]UTP-labeled complex in the TS and because electroelution of T-IV from a preparative native gel also yielded these particles (data not shown).

Each fraction from a Superose 6 gel filtration of TS was prepared for electron microscopy. As shown in Figure 7B, 80–140 Å particles (arrows), similar to those in Figure 7A, were most abundant in column fraction 14, which represented the peak of TUTase activity. A second class of particles 170–300 Å in size was present in column fractions 11 and 12. Representative fields from fraction 11 are shown in Figure 7C and D (putative G particles are indicated by stars). Histograms of the frequencies of particle areas from micrographs of column fractions 14 and 11 are shown in Figure 8A and B. These histograms are consistent with the visual impression of the particles in fraction 11 being larger than those in fraction 14. The data are not sufficient to determine if there are two overlapping size classes in fraction 11 or a continuous gradation of particle size. However, the correlation of the smaller particles with the peak of TUTase activity suggests that these represent the T complexes, and the correlation of the larger particles with the peak of internal U-incorporation activity suggests that these represent the G complexes.

A low abundance (~ 3 –5%) of contaminating cytoplasmic

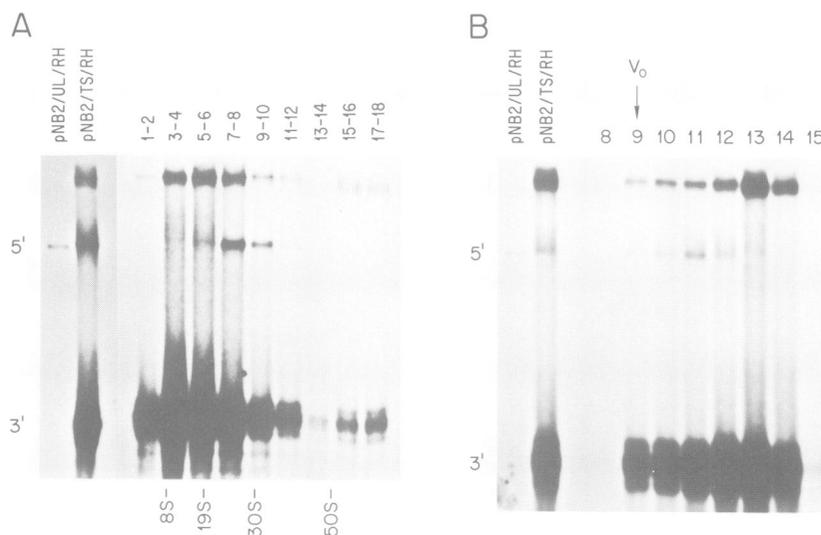


Fig. 6. Distribution of internal U-incorporation activity in glycerol gradient and gel filtration fractionations. (A) Glycerol gradient sedimentation analysis of TS. Gradient fractions were pooled as indicated and assayed for internal U-incorporation activity using pNB2 RNA and the RNase H assay with oligonucleotide S-165. Autoradiograph of dried 8% polyacrylamide–7.5 M urea gel. pNB2/UL/RH, uniformly-labeled pNB2 RNA digested with RNase H. pNB2/TS/RH, TS-treated pNB2 RNA digested with RNase H. Positions of the 5' and 3' fragments are indicated on the left. S values of standards are shown below. Note that 18 fractions were recovered in this experiment. (B) Superose 6 gel filtration analysis of TS. Control lanes on the left are identical to (A). Each column fraction, 8–15, was analyzed for internal U-incorporation activity as in (A). V_0 , void volume. Autoradiograph of dried 8% polyacrylamide–7.5 M urea gel. Positions of the 5' and 3' fragments are indicated on the left.

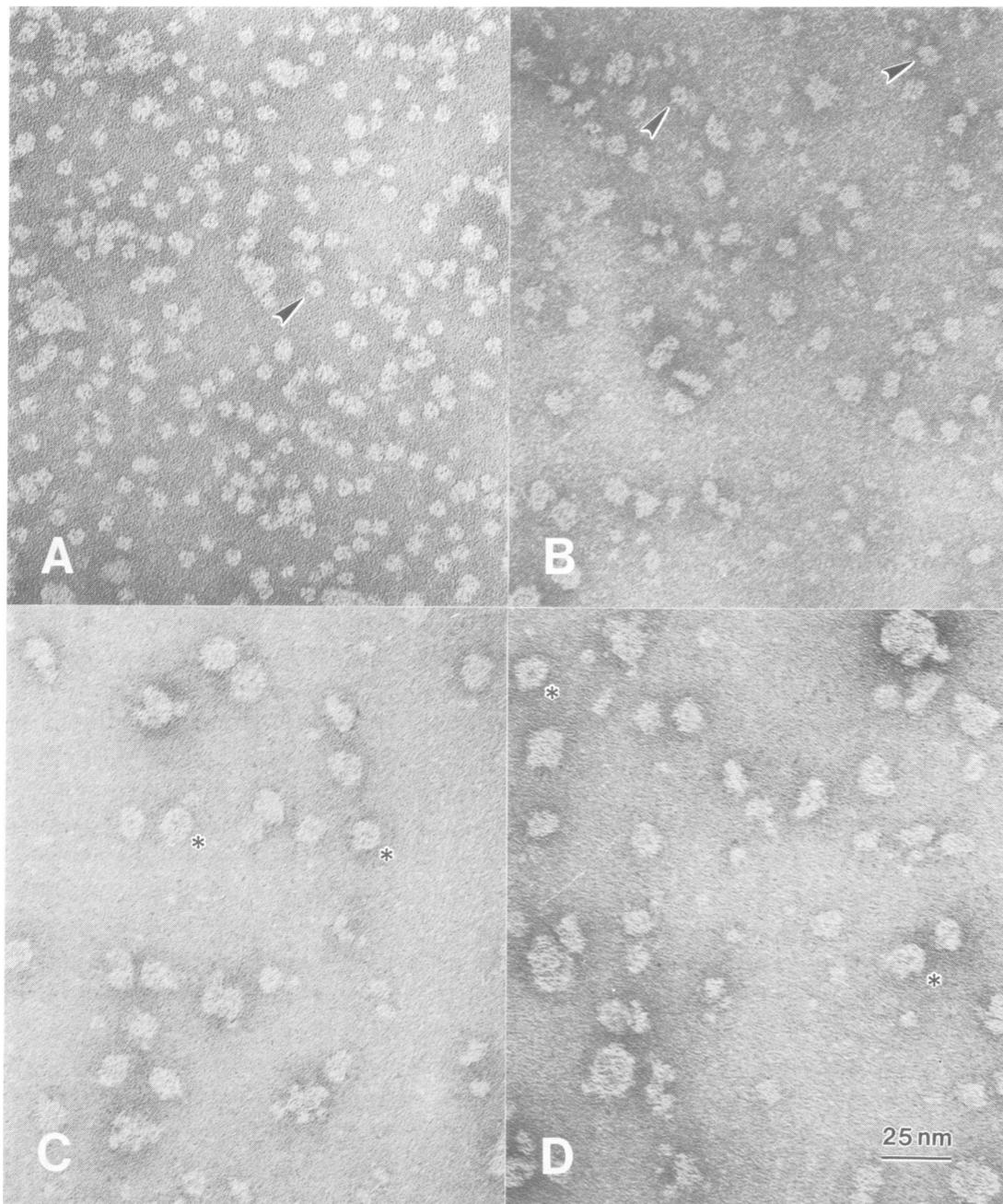


Fig. 7. Electron microscopy of fractions that contain TUTase and internal U-incorporation activity. Samples were negatively stained with 1% uranyl acetate using the double carbon method. (A) TUTase peak from a glycerol gradient. A putative T particle is indicated by an arrowhead. (B) Superose 6, fraction 14, TUTase peak. Two putative T particles are indicated by arrowheads. (C) Superose 6 chromatography, fraction 11, peak of internal U-incorporation activity. Two putative G particles are indicated by asterisks. (D) Another field of fraction 11 from the Superose 6 fractionation. Two putative G particles are indicated by asterisks. All micrographs are at the same magnification.

ribosome subunits was also present in column fraction 11 in some preparations. We assume that the localization of the mitochondrial ribosomes to the void volume, demonstrated in Figure 2C by hybridization of 9S and 12S rRNAs, is caused by an attachment of the ribosomes to mitochondrial membranes which are the main component of the void volume (data not shown).

Discussion

The presence of at least two major classes of RNP complexes possibly involved in RNA editing in kinetoplast-

mitochondrial extracts from *L.tarentolae*, the T class and the G class, is indicated by several lines of evidence. The T class consists of approximately six RNP complexes containing bound RNA which can be labeled by incubation of TS with [α - 32 P]UTP. The most abundant complex is T-IV, which contains most if not all of the gRNA of these complexes and can be visualized in the electron microscope as 80–140 Å particles with a characteristic morphology.

The T complexes probably correspond to the 19S 'Complex I' of Pollard *et al.* (1992) in mitochondrial extracts from *T.brucei*, but there are some significant differences in addition to the sedimentation coefficient. We have shown

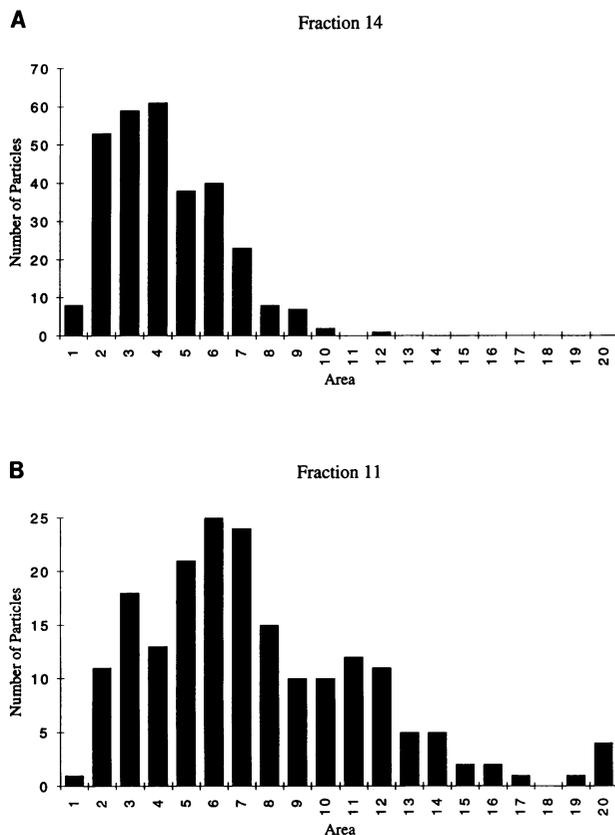


Fig. 8. Histograms of particle size distributions in different fractions. Micrographs at identical magnifications were scanned in a Microtek II scanner and the resulting TIFF files were analyzed using the NIH Image Analysis program. Relative particle areas were measured and a histogram constructed. (A) Superose 6, fraction 14. (B) Superose 6, fraction 11.

by native gradient gel analysis that there are at least six RNP complexes in *L.tarentolae* (and probably also in *T.brucei*) which constitute the main TUTase peak rather than a single complex. The mitochondrial RNA ligase, which Pollard *et al.* (1992) found to be associated with Complexes I and II in *T.brucei*, sediments at the top of the glycerol gradient and is not associated with either T or G complexes in the case of *L.tarentolae* (A.Simpson and L.Simpson, unpublished results). Chimera-forming activity (Blum and Simpson, 1992; Harris and Hajduk, 1992; Koslowsky *et al.*, 1992), which Pollard *et al.* (1992) found to be associated with Complexes I and II in *T.brucei*, sediments at ~15S in a detergent-free *L.tarentolae* extract, but is inactive in the TS extract because of inhibition by Triton X-100 (B.Blum and L.Simpson, unpublished results).

The major RNA component of complex T-IV is gRNA. A minor RNA component, the arc-RNA, is also present in the T complexes. The arc-RNAs show a decrease in relative abundance and a stepwise increase in size, which can be attributed to degradation occurring during experimental manipulation. The fact that the larger complexes yielded larger RNA suggests that there may be protection by proteins in the complexes. It is not known if these RNAs contain unedited, partially edited or edited mRNA sequences. It is also possible that these RNAs represent non-specific sticking of mRNAs or mRNA fragments to protein complexes. Further analysis of the arc-RNAs must be performed to

understand their significance, if any, in the editing process.

TUTase activity using total cell RNA as substrate correlates with the presence of the T complexes in sedimentation and gel filtration fractionations. In addition, an in-gel assay showed the main localization of TUTase activity to be in T-IV, the T complex that contains gRNA. This finding presents an interesting problem for the observed labeling of the endogenous RNAs present in T-I, T-II and T-III obtained by incubation of TS with [α - 32 P]UTP, unless it is assumed that these complexes interact transiently with the active TUTase in T-IV.

The G class of RNP complexes, which contain the majority of the gRNA in the extract but which do not label with [α - 32 P]UTP indicating an absence of TUTase activity, can be correlated with 170–300 Å particles. There is also a correlation between the presence of G complexes and the internal U-incorporation activity, suggesting that these particles are responsible for this editing-like activity. Nothing is known about the protein components of the G complexes or about the relationship between the G and T complexes. The G complexes may correspond to the 35–40S Complex II particles of Pollard *et al.* (1992) in *T.brucei*, but there are several significant differences in addition to sedimentation coefficient. For example, chimera-forming activity does not cosediment with G complexes in *L.tarentolae* (B.Blum and L.Simpson, unpublished results).

We assume that the main function of the T complexes (at least complex T-IV) is the 3' terminal addition of U residues to gRNAs. However, the presence of minor amounts of mRNA sequences (arc-RNAs) is not completely understood and must be further investigated. It is significant that the G complexes appear capable of a concerted series of reactions leading to the internal incorporation of U residues into the pre-edited region of CYb mRNA. The origin of the internalized labeled U residues in the pNB2 RNA presents a problem if one assumes that the Us are transferred from the 3' oligo(U) tail of a gRNA via chimera formation and resolution, as in the transesterification model. The putative 3' end-labeled gRNAs could be derived from the T complexes which contaminate the G complex peak in these separations, or the labeled U residues could cycle rapidly through the G complex gRNA into the pNB2 RNA. Alternatively, if the internal U residues derive directly from [α - 32 P]UTP, as in the enzyme cascade model, then the T complexes, which contain the TUTase activity, must transiently interact with the G complexes to catalyze the reaction. However, this is unlikely because endogenous gRNAs in the G complexes are not 3' end-labeled during the incubation, as might be expected if such an interaction were to occur. It is clear that these results do not enable us to distinguish between the two models for RNA editing.

We feel that it is still premature to term the G complex the 'editosome' because it has not been shown to mediate precise editing *in vitro*. Nevertheless, these results represent a starting point for working out the detailed mechanism of RNA editing in kinetoplast mitochondria.

Materials and methods

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) as described previously (Simpson and Braly, 1970) and the kinetoplast-mitochondrial fraction was isolated by flotation in Renografin

density gradients (Simpson and Simpson, 1978). The mitochondrial fraction was resuspended in 20 mM HEPES-KOH (pH 7.5), 20 mM KCl, 1 mM EDTA and 10% glycerol at a protein concentration of ~5 mg/ml, and was stored in aliquots at -80°C. Triton lysate (TL), Triton supernatant (TS) and S-100 mitochondrial extracts were prepared as described previously (Bakalara *et al.*, 1989).

Labeling of mitochondrial extracts and TUTase assay

TS (25 μ l) was endogenously labeled by incubation for 40 min at 27°C in 1 mM ATP, 1 mM GTP, 20 mM DTT, 5 mM HEPES-KOH (pH 7.5), 60 mM KCl, 6 mM Mg acetate, 3 mM potassium phosphate (pH 7.5) and 20 μ Ci [α -³²P]UTP (800 Ci/mmol), in a 50 μ l total reaction volume. CTP was omitted to prevent transcription (Bakalara *et al.*, 1989). To assay TUTase activity with exogenous substrate RNA, 25 μ l extract or fraction in a final reaction volume of 50 μ l was incubated, as stated, with the addition of 900–1200 ng *L.tarentolae* total cytosolic RNA isolated as described previously (Bakalara *et al.*, 1989). The reaction was stopped with 0.2% SDS, 0.5% NaPP_i, and spotted onto DE-81 filters, washed and counted.

Native polyacrylamide gradient gels

Labeled TS (50 μ l) was mixed with glycerol to a final concentration of 30% and electrophoresed on a native polyacrylamide 4–16% linear gradient gel (stabilized with a 10–30% glycerol gradient and including 0.1% Tween 20) with a 4% stack, as described previously (Smith *et al.*, 1991). The 14 cm \times 1 mm gels were run at 125 V for 24 h at 4°C in 40 mM Tris-acetate (pH 8) and 1 mM EDTA (TAE buffer). Molecular weight 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.

2-D gel analysis of [α -³²P]UTP-labeled RNA from mitochondrial extract

For analysis of the RNA component of the T complexes, [α -³²P]UTP-labeled TS was run in a 4–16% native gel as described above. The lane was excised and incubated in 50% formamide, 0.1% SDS and TBE for 15 min at 65°C. The lane was layered on a 10% polyacrylamide, 8 M urea gel, which was electrophoresed for 12 h at 80 V. Size markers were *L.tarentolae* cytoplasmic rRNA, or ³²P-labeled 5'-kinased RNA ladder (100–500 nt, Ambion).

For electroblotting, unlabeled TS was electrophoresed in a 2-D native/8 M urea polyacrylamide gel as described above. The gel was electroblotted onto a Nytran filter at 500 mA, 4°C, in 1 \times TAE buffer. Electroblots were hybridized using one of the types of probes described as follows: oligonucleotide probes were 5' end-labeled using [γ -³²P]ATP and T4 polynucleotide kinase (BRL) to specific activities of 10⁸ c.p.m./ μ g and were purified using Nucletrap columns (Stratagene). The following oligonucleotides were used for hybridization: S-530, 5'-GTTACTTTTTTATTATTTAG-AAAAG-3' (probe for gCYb-II); S-178, 5'-CATGCTAAGCAAACACCA-CA-3' (probe for CYb mRNA, downstream of pre-edited region); S-515, S-517, S-312, S-574, S-516 and S-514 (oligonucleotide probes for the six MURF4 gRNAs) (Maslov and Simpson, 1992); and S-252, S-508, S-509, S-510, S-511, S-512, S-513 and S-582 (oligonucleotide probes for the eight RPS12 gRNAs) (Maslov and Simpson, 1992).

Gene fragments to be used as probes were amplified by PCR from *L.tarentolae* kDNA, isolated as described previously (Simpson and Berliner, 1974) and used as hybridization probes. The PCR products were labeled using Prime-IT II random priming kit (Stratagene) and were purified using Nucletrap columns. The following gene fragments were used: 9S, nt 1639–1999; 12S, nt 1043–1610; ND7, nt 3240–3451 (localization on GenBank entry LEIKPMAX is given for each sequence).

Native gel in situ TUTase assay

Native 4–16% gel electrophoresis of unlabeled TS was performed as described above. Gel lanes were excised, transferred to 15 ml centrifuge tubes and incubated at room temperature with 3 ml of a TUTase labeling mix, as described above. The gel slice was then washed with H₂O, fixed and stained with Rapid Coomassie Stain, dried under vacuum and autoradiographed.

Internal U-incorporation assay

Construction of the pNB2 plasmid, which contains the pre-edited region and some 5' and 3' flanking sequences of the *L.tarentolae* CYb gene, has been described previously (Simpson *et al.*, 1992). The pNB2 plasmid was transcribed *in vitro* as described previously (Bakalara *et al.*, 1989; Simpson *et al.*, 1992). T7 RNA polymerase-synthesized pNB2 RNA (0.5–1 μ g) was incubated in the presence of TL, TS, S-100 extract, or with glycerol

gradient or gel filtration fractions at 27°C for 40–60 min in a 50 μ l reaction volume. The reaction contained 5 mM HEPES (pH 7.5), 60 mM KCl, 3 mM potassium-PO₄ (pH 7.5), 6 mM Mg-acetate, 20 mM DTT, 2 mM spermidine, 1 mM ATP, 1 mM GTP, 1 mM unlabeled UTP and 25 μ Ci [α -³²P]UTP (800 Ci/mmol). CTP was not included in the reaction mixture to eliminate transcription, which has been shown to require the presence of all four triphosphates (Bakalara *et al.*, 1989). The intact RNA was gel-isolated and digested with RNase H, together with a short oligonucleotide (S-192 or S-165) antisense to a region 3' to the pre-edited region (Donis-Keller, 1979). Each 20 μ l reaction mixture contained 0.5–1.0 μ g RNA, 0.1–0.2 μ g DNA oligomer, 50 mM Tris-HCl (pH 7.5), 0.1 M KCl, 10 mM MgCl₂, 0.1 mM DTT, 10 μ g/ml BSA and 0.16 U RNase H (Pharmacia). RNase H digestion was allowed to proceed for 1 h at 37°C. The 3' and 5' fragments from the RNase H digestion were then separated by analytical gel electrophoresis. Label in the 5' fragment is derived from internal U-incorporation and label in the 3' fragment is derived from 3' terminal U addition by the known TUTase (Bakalara *et al.*, 1989). The oligonucleotides are as follows: S-165, 5'-CCTAACTAAAACCTACACC-3'; and S-192, 5'-ATACTCGTAATAAACAACT-3'.

Sedimentation analysis of mitochondrial extract

TS (200 μ l) was layered on 10–30% glycerol gradients in 20 mM HEPES (pH 7.5), 20 mM KCl and 1 mM EDTA, which were centrifuged in a Beckman SW-41 rotor for 14 h at 33 000 r.p.m. and 4°C. S values were calculated by cosedimentation with catalase (11.5S; Pharmacia), alcohol dehydrogenase (7.6S; Sigma), thyroglobulin (19.3S; Pharmacia) and *E.coli* small (30S) and large (50S) ribosomal subunits. Either 16 or 18 fractions, ~750 or 670 μ l, respectively, were collected from the top of each gradient. Fractions were analyzed for TUTase activity and internal U-incorporation activity as described above. RNA was isolated from the remainder of the fractions and used for Northern analysis.

Superose 6 gel filtration analysis of mitochondrial extract

Gel filtrations were performed using a Superose 6 (10/30) FPLC pre-packed column (Pharmacia). The column was first equilibrated with 20 mM HEPES-KOH (pH 7.5), 20 mM KCl, 1 mM EDTA. TS (200 μ l) was filtered through a 0.22 μ m filter before applying to the column. The flow rate was 0.5 ml/min and 1 ml fractions were collected. Assays for TUTase activity and internal U-incorporation were performed as described above. Proteins were assayed by the BCA protein assay (Pierce). The remainder of the fractions were used for RNA isolation.

Northern analysis

RNAs were electrophoresed in agarose-formaldehyde gels and blotted onto Nytran filters as described previously (Blum and Simpson, 1990). kRNA was included in a separate lane as a control. Blots were probed with oligonucleotides for all six MURF4 gRNAs which were 5' end-labeled with ³²P by polynucleotide kinase.

Electron microscopy

To remove glycerol, gradient fractions were dialyzed against 20 mM KCl, 20 mM HEPES (pH 7.5) and 1 mM EDTA using a Centricon 10 microconcentrator. Both glycerol gradient fractions and Superose 6 gel filtration fractions were negatively stained at a protein concentration of 40 μ g/ml using the double layer method of Lake (1979). The grids (400 mesh) were examined using a Hitachi 7000 electron microscope at 75 kV acceleration and a magnification of 80 000 \times . Histograms of the size distributions of particles in the micrographs were constructed using a Microtek II Scanner and the NIH Image Analysis program.

Acknowledgements

We would like to thank Dr D.Maslov and all other members of the Simpson laboratory for advice and discussion. We also thank Dr F.Eiserling for use of the electron microscope and members of the Eiserling laboratory for technical assistance with electron microscopy. We thank Dr J.Lake for assistance with the interpretation of micrographs and A.Aguinaldo for the gift of *E.coli* 30S ribosome subunits. This research was supported in part by a research grant from the National Institutes of Allergies and Infectious Diseases to L.S. G.C.F. would like to acknowledge support from the Swiss National Science Foundation and from the American Cancer Society (# PF-3824).

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Received on October 25, 1993; revised on January 13, 1994