The EMBO Journal vol.11 no.12 pp.4429-4438, 1992

Native mRNA editing complexes from *Trypanosoma brucei* mitochondria

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Communicated by P.Borst

The aim of this study was to identify multicomponent complexes involved in kinetoplastid mitochondrial mRNA editing. Mitochondrial extracts from *Trypanosoma brucei* were fractionated on 10-30% glycerol gradients and assayed for RNAs and activities potentially involved in editing, including pre-edited mRNA, guide RNA (gRNA), endonuclease, terminal uridylyltransferase (TUTase), RNA ligase and gRNA-mRNA chimera-forming activities. These experiments suggest that two distinct editing complexes exist. Complex I (19S) consists of gRNA, TUTase, RNA ligase and chimera-forming activity. Complex II (35-40S) is composed of gRNA, pre-edited mRNA, RNA ligase and chimera-forming activity. These studies provide the first evidence that editing occurs in a multicomponent complex. The possible roles of complex I, complex II and RNA ligase in editing are discussed.

Key words: gradient sedimentation/kinetoplastid/mitochondria/ribonucleoprotein complex/RNA editing

Introduction

RNA editing in kinetoplastid protozoa is an unusual form of RNA processing that is required for the expression of a number of mitochondrial genes. RNAs transcribed from these genes (pre-edited mRNAs) require the addition and deletion of uridine residues at specific sites within their coding regions to become functional. Editing corrects frame shifts, creates start codons and in some cases produces essentially the entire functional message (reviewed in Simpson and Shaw, 1989; Benne, 1990; Feagin, 1990). The coding information incorporated into these mRNAs is thought to be stored in small, 55-70 nucleotide (nt) primary transcripts called guide RNAs (gRNAs). gRNAs are complementary to the edited mRNAs and possess non-encoded polyuridine tails (Blum *et al.*, 1990; Blum and Simpson, 1990; Pollard *et al.*, 1990; Sturm and Simpson, 1990; Pollard and Hajduk, 1991). The process by which gRNAs specify the edited sequence is unknown, but it is thought to involve base pairing or comparison between the gRNA and the mRNA being edited. The guide RNA contains a short ~10 nt anchor region at its 5′ end which is complementary to the pre-edited message immediately 3′ to the editing site. The gRNA is thought first to base-pair with the pre-edited message in this region and then direct the editing process, until the mRNA is fully complementary to the gRNA.

In the current model of the editing pathway, the gRNA also serves as the uridine donor for the editing process via the gRNA poly(U) tail. This model, which draws analogies from RNA splicing, proposes that editing occurs through multiple rounds of *trans*-esterification (Blum *et al.*, 1991; Cech, 1991). In the first round, the 3′-OH of the poly(U) tail of the gRNA makes a nucleophilic attack on the phosphodiester bond at the editing site, producing a free 5′ mRNA segment and a chimeric molecule composed of the gRNA covalently linked (5′ to 3′) to the 3′ segment of the mRNA. In the second round, the chimeric molecule is attacked by the 3′-OH of the free 5′ mRNA segment to produce a pre-edited mRNA that has one or more Us inserted or deleted and a gRNA that has the corresponding changes in the length of its poly(U) tail. This process continues until the edited RNA is fully base-paired to the gRNA. In support of this model, gRNA-mRNA chimeric molecules have been shown to exist *in vivo* (Blum *et al.*, 1991) and a chimera-forming activity from mitochondria has been demonstrated *in vitro* (Harris and Hajduk, 1992).

Such chimeric RNAs could also be formed by a mechanism that utilizes endonuclease and RNA ligase activities (Harris and Hajduk, 1992). In this scheme, the pre-edited mRNA is cleaved by an endonuclease at the editing site. The RNA ligase then joins the gRNA to the 3′ fragment of the mRNA creating the chimeric molecule. This chimera could be resolved by a second round of cleavage and ligation. In support of this model, endonuclease activities specific for pre-edited sites (Harris *et al.*, 1992), and RNA ligase activities (Bakalara *et al.*, 1989) have been detected in kinetoplastid mitochondrial extracts. Terminal uridylyltransferase activity (TUTase) has also been reported in kinetoplastid mitochondrial extracts (Bakalara *et al.*, 1989). Both chimeric models propose that the role of TUTase activity is to add the non-encoded poly(U) tail to the gRNA.

The process of editing is complicated. In cases of extensive editing, such as that observed for the cytochrome oxidase III (COIII) message, the entire coding region of the mRNA is created by editing. Over 55% of the ~900 coding nucleotides of the COIII mRNA are added during the process (Feagin *et al.*, 1988; Feagin, 1990). Guide RNAs are typically complementary to only ~40 nt of edited sequence and the regions specified by these gRNAs overlap significantly. Thus, many gRNAs are required for processing these extensively edited transcripts. These gRNAs are proposed to be used sequentially with the 3′-most gRNA creating the anchor sequence for the next gRNA. This would allow editing to occur in blocks specified by gRNAs and move in an overall 3′ to 5′ direction along the mRNA sequence. The editing machinery must have some mechanism for selecting the correct gRNA, which in *Trypanosoma brucei* is no small feat as the gRNA pool numbers in the

hundreds. The anchor sequence, being in some cases only 5 bp (Blum *et al.*, 1990), seems ill-equipped to perform this discrimination without the help of other factors. In addition, the 5′ and 3′ segments of the mRNAs generated during the editing process must be kept in close proximity in order to insure that the final product is not a hybrid of different RNAs being edited. The nature of these problems suggests that RNA editing must occur within a multicomponent complex capable of orchestrating these events.

The purpose of this study was to identify and characterize native complexes involved in editing. Sedimentation analysis through glycerol gradients has proven useful in the initial characterization of many ribonucleoprotein complexes, most notably spliceosomes and polyadenylation complexes (Brody and Abelson, 1985; Frendewey and Keller, 1985; Grabowski *et al.*, 1985; Moore *et al.*, 1988). In this study, *T.brucei* mitochondrial extracts were fractionated over 10–30% glycerol gradients. Gradient fractions were assayed for RNAs, proteins and specific enzymatic activities. From these studies, two editing complexes are proposed to exist, a 19S and a 35–40S particle. The 19S particle, complex I, contains gRNA complexed to TUTase, RNA ligase and chimera-forming activities. The 35–40S particle, complex II, contains pre-edited mRNA, gRNA, RNA ligase and chimera-forming activities.

Results

Pre-edited mRNAs and gRNAs co-sediment in native complexes

Although the exact mechanism of editing is unknown, the process presumably involves an interaction between pre-edited mRNAs and gRNAs. Thus, determining whether these two RNA species are components of a stable complex was our first aim. The primary COIII transcript is an ~450 nt pre-edited, polyadenylated mRNA (Figure 1). This transcript is extensively edited and the majority of the COIII mRNAs exist in the partially edited state ranging in size from ~500 to 1100 nt (Feagin *et al.*, 1988). Examination of the COIII transcript therefore provides an excellent means to observe editing complexes. Glycerol gradient centrifugation was used to separate these proposed complexes from other mitochondrial components. Triton X-100-solubilized *T.brucei* mitochondrial extracts were fractionated on 10–30% glycerol gradients under standard 50 mM KCl conditions. Separate gradients containing sedimentation markers were run concurrently. Gradients were fractionated from the bottom and RNAs were extracted from each fraction of the mitochondrial lysate gradient. RNAs were electrophoresed on an agarose gel under denaturing conditions, transferred to a nylon membrane and probed for different mitochondrial RNAs (Figure 2).

Pre-edited COIII mRNAs (Figure 2D) peak in the center of the gradient in fractions 8–10. The pre-edited mRNA is absent in the top fractions, but trails toward the bottom of the gradient. The COIII gRNAs (Figure 2E) are spread throughout the gradient, with the major peak in fractions 8–10 and a smaller, but reproducible peak in fraction 13. Thus, the pre-edited mRNA and the gRNA peaks overlap in fractions 8–10, suggesting that they are associated. This position in the gradient corresponds to ~35–40S, as determined by the sedimentation markers (Figure 2A). The smaller peak of gRNAs (fraction 13) corresponds to ~19S. The membrane was also probed for edited COIII sequences from two different regions of the mRNA (Figure 1). These probes, being complementary to the center of the COIII mRNAs, recognize RNAs that have been edited (3′ to 5′) up to the probe position, but can be unedited 5′ to the probe (Abraham *et al.*, 1988; Figure 1). The main difference between the RNAs recognized by these edited probes and those recognized by the pre-edited probes is greater length due to editing. The peaks of these RNAs shift, relative to the pre-edited population, further into the gradient. Thus, it appears that the longer or more edited an RNA becomes,

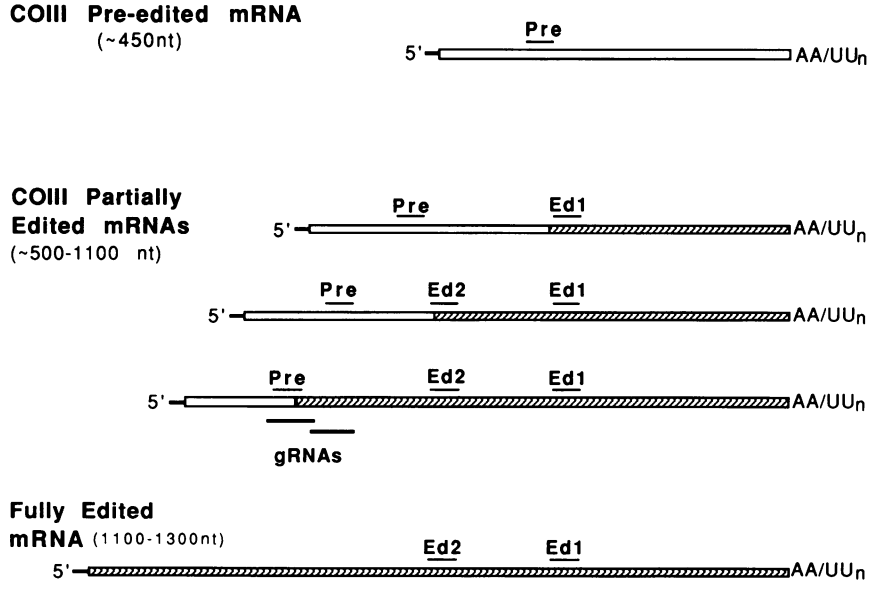

Fig. 1. Progression of COIII pre-edited mRNA to edited mRNA and the probes used to identify these RNAs. COIII is an extensively edited transcript, with the entire coding region being modified by editing. Editing presumably progresses in the 3′ to 5′ direction along the mRNA. Pre-edited RNA is indicated by the open boxed lines. Edited sequence is designated by the hatched boxed lines. Relative positions of the probes for pre-edited mRNA (Pre) and the edited mRNAs (Ed1 and Ed2) are shown. Editing sites specified by the two COIII gRNAs Tb1 and Tb2 are indicated.

the larger its apparent S value. From these data, it also appears that very little fully edited COIII mRNA sediments with the ribosomes relative to the amount of pre- and partially edited RNAs in the gradient. This is due to the fact that >90% of the COIII RNA exists in the partially edited state (Feagin *et al.*, 1988) and to incomplete solubilization of the ribosomes under the conditions used here (data not shown).

In order to determine whether a non-edited mRNA would sediment to the same position of the gradient, the membrane was probed for COI mRNA, which is not modified by editing (Figure 2F). The oligonucleotide probe used recognizes two distinct RNA species transcribed from the same region of the maxicircle (Feagin and Stuart, 1985; Jasmer *et al.*, 1985). The larger, 1700–1900 nt RNAs are the polyadenylated COI mRNAs. These RNAs cosediment with the 9 and 12S ribosomal RNAs (Figure 2A), as expected for a mature mRNA in complex with ribosomes. The smaller 1350 nt RNA species is a non-polyadenylated transcript. This RNA is spread throughout the gradient, showing only a slight peak in fractions 9–11. The function of this transcript and whether it is modified by editing is unknown. The ratio of 1700–1900 nt transcripts to 1350 nt transcripts is skewed in these gradients. Total RNA preparations show that the larger COI mRNA transcripts are much more abundant (Figure 2F, lane T; Figure 3F, lane T). This apparent loss of the COI mRNA is probably due to incomplete ribosome solubilization under the conditions used here (data not shown) and to pelleting of those solubilized ribosomes containing these large mRNAs to the bottom of the tube. It is also possible that an editing complex on the large COI mRNA would have sufficient mass to cause this pelleting, though it is thought unlikely.

The co-sedimentation of the COIII gRNAs and pre-edited mRNAs strongly suggests the association of these RNAs in a stable complex. The high sedimentation coefficients further suggest that these complexes contain other RNAs or proteins.

Pre-edited RNAs and gRNAs are complexed to proteins

In order to determine whether the RNAs observed in Figure 2 were complexed with proteins, mitochondria were solubilized with SDS and the lysates were treated with proteinase K. This lysate was then fractionated on 10–30% glycerol gradients identical to those above. The RNA analysis is shown in Figure 3. All of the RNAs probed shift to the top of the gradient, as expected for free RNA. 9S rRNA peaks in fraction 14, as predicted by the sedimentation markers (Figures 2A and 3B). Pre-edited and edited RNAs

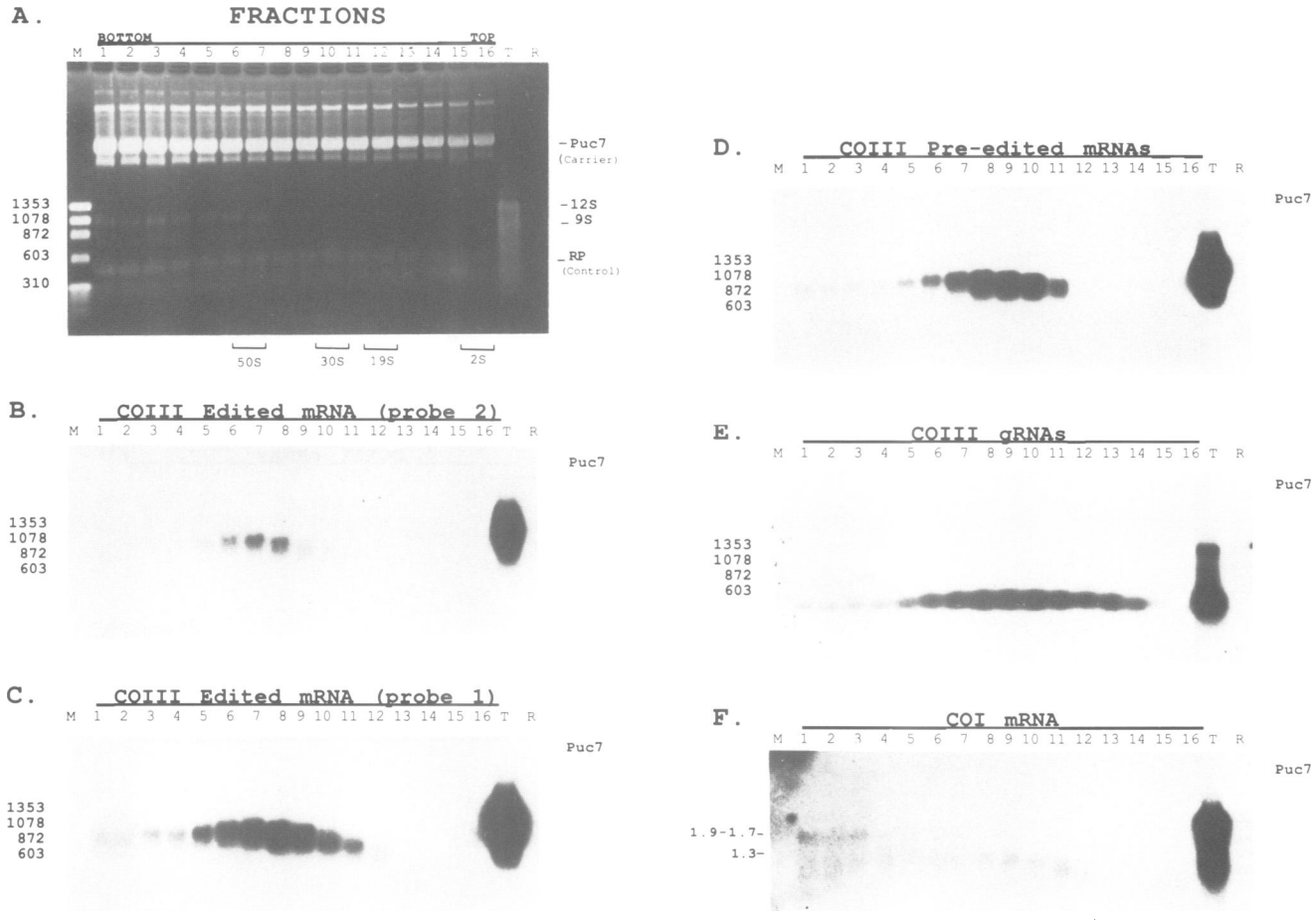

Fig. 2. Northern blot analysis of the RNAs extracted from native gradient fractions. (**A**) Ethidium stained methyl mercury–agarose gel used to separate RNAs extracted from gradient fractions. Lane M contains *Hae*III-digested ΦX174 DNA as markers. Lanes containing RNAs from fractions 1–16 are indicated. Lane T contains total *T.brucei* mitochondrial RNA. Lane R contains Bluescript riboprobe added to all fractions as a degradation and recovery control. This band is indicated by RP. Puc 7 DNA, added as a carrier for precipitation, is also marked. Positions of the 9 and 12S mitochondrial rRNAs are indicated. Peak fractions of the sedimentation markers are shown beneath the gel. (**B**–**F**) Northern blot of the methyl mercury–agarose gel in (A) probed for the indicated RNAs.

also peak in fraction 14, as expected for free RNAs. gRNAs peak in fraction 16, the majority clearly no longer co-sedimenting with the pre-edited RNAs. COI mRNA no longer sediments to the bottom of the gradient, and the majority of the 1700−1900 nt polyadenylated COI mRNA is recovered in the gradient. These results indicate that the mitochondrial mRNAs, rRNAs and gRNAs are complexed with protein in their native state.

From these studies, it is difficult to determine if these RNA−protein complexes are specific. If these complexes are non-specific aggregates, then any RNA added to the mitochondrial lysates should co-sediment with them. In order to test this hypothesis, a 270 nt ^{32}P-labelled non-specific RNA was added to mitochondrial lysates, loaded on glycerol gradients and fractionated as above. The experiment was performed under four different conditions: SDS−proteinase K treatmeant of the lysate as the free RNA control, the standard 50 mM KCl conditions and then 150 and 300 mM KCl higher ionic strength conditions, which should block non-specific binding. Fractions from each of the gradients were analyzed by Cerenkov counting. The results are shown in Figure 4. The RNA peaks in fraction 12 of the SDS−proteinase K gradient. This sedimentation value is unusually large for such a small RNA, but is reproducible and is presumably due to RNA structure. The RNA peaks in fraction 12 in the 50 mM KCl gradient also, but ~70% of the RNA is missing from the gradient. This RNA is found in the insoluble pellet at the bottom of the gradient tube (data not shown) indicating non-specific aggregation. In the 150 mM KCl gradient, the peak of RNA shifts from fraction 12 to 13, presumably due to conformation changes induced by the higher salt and only ~40% of the RNA is trapped in the insoluble pellet. In the 300 mM KCl gradient, essentially all of the transcript is again soluble and peaks in fraction 13. These data indicate that non-specific aggregation does indeed occur in the 50 mM KCl gradients, but it associates with the insoluble pellet at the bottom of the gradient and not the soluble materials in fractions 8−10 where the bulk of the COIII RNAs sediment; 300 mM KCl was sufficient to block this non-specific binding to the pellet, but did not disrupt the co-sedimentation of the COIII gRNAs with the pre-edited mRNAs (see below).

The distribution of proteins across the 50 mM KCl gradient is shown in Figure 5. Protein assays show that the bulk of the protein in these gradients is free in the top fractions. The major peak is in fraction 15 (Figure 5A) and only a small amount of the mitochondrial proteins sediment into the gradients with the 35−40 and 19S RNA complexes. The proteins were analyzed by SDS−PAGE followed by Coomassie blue staining (Figure 5B). Equal volumes of fractions 1−12 were run, while fractions 13−16 were all run at equal protein concentration (45 μg) to avoid

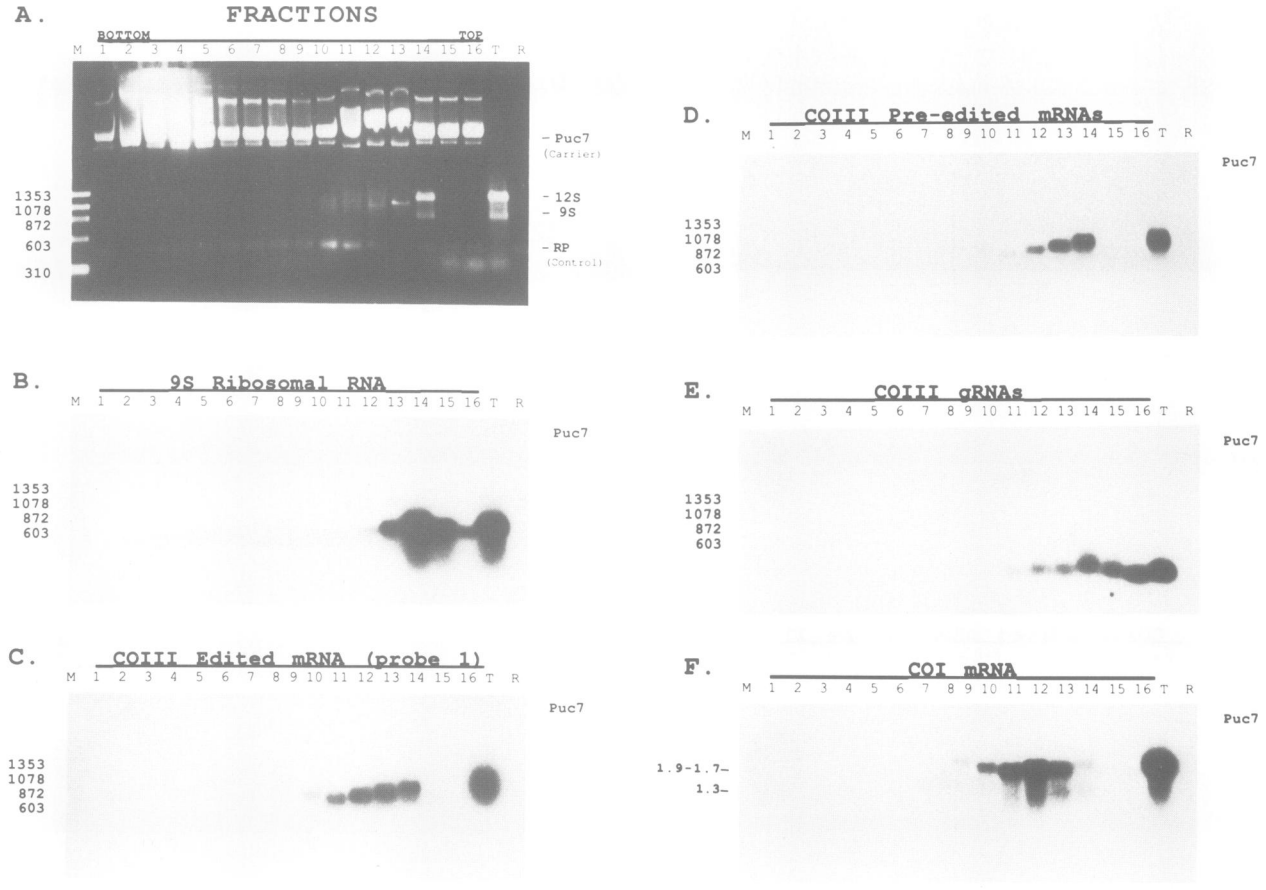

Fig. 3. Northern blot analysis of the RNAs extracted from SDS/proteinase K gradient fractions. (**A**) Ethidium stained methyl mercury−agarose gel used to separate RNAs extracted from the SDS/proteinase K gradient. Lanes are as follows: φX 174 DNA digested with *Hae*III as markers, lane M, RNAs extracted from fractions 1−16, lanes 1−16, total *T.brucei* mitochondrial RNA, lane T, and Bluescript RNA added as a recovery and degradation control, lane R. The mitochondrial 9 and 12S rRNAs, as well as the Puc 7 DNA used as a carrier, are indicated. (**B−F**) Northern blots of the gel shown in (A) probed for the indicated RNAs.

overloading the gel. The SDS–polyacrylamide gel shows a distribution of proteins in the gradient. The three major proteins that form the peak in fraction 8 are probably not related to editing, as they do not exhibit a distribution consistent with the enzymatic activities described below.

TUTase, RNA ligase and gRNA–mRNA chimera-forming activities co-sediment with the pre-edited mRNAs and gRNAs

While the protein analysis in Figure 5 does not implicate any specific proteins in the 35–40 and 19S complexes, the chimera models of editing make specific predictions about the activities associated with editing complexes. These models predict the association of chimera-forming activities with the active editing complex and TUTase with gRNAs. The enzymatic model also predicts the presence of an endonuclease and an RNA ligase. These activities were assayed across the standard 50 mM KCl gradient as described in Materials and methods (Figure 6). Pre-edited RNA specific endonuclease was assayed essentially as described (Harris *et al.*, 1992). Briefly, 5′ end-labelled synthetic pre-edited mRNA was incubated with the gradient fractions. The

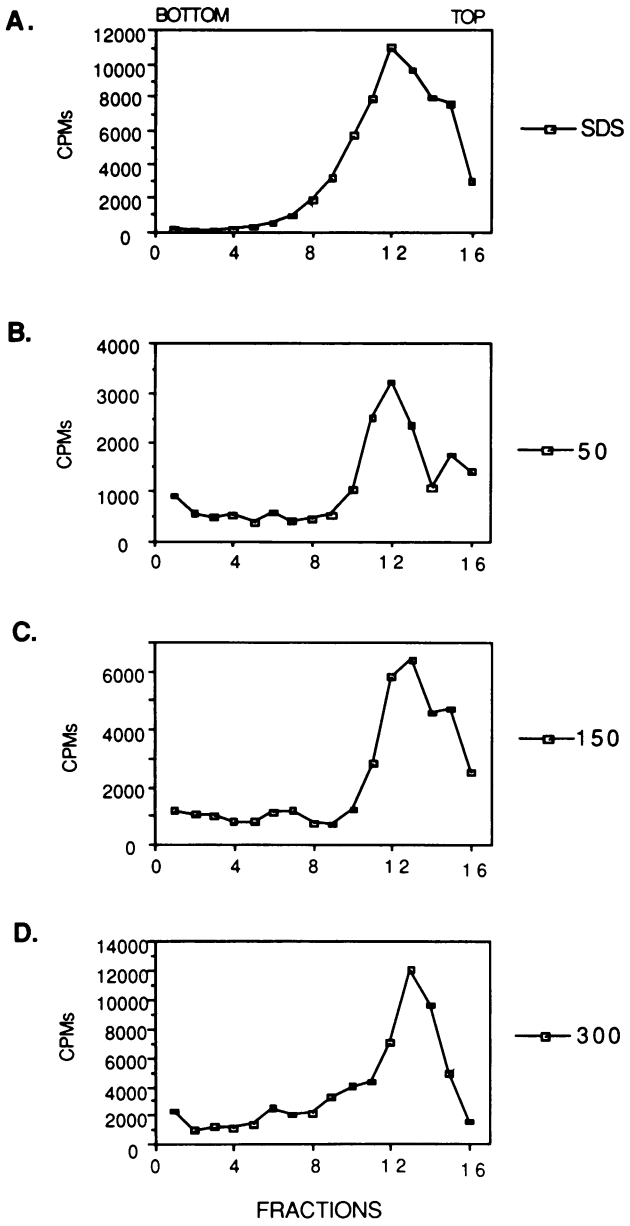

Fig. 4. Sedimentation of a ^{32}P-labelled non-specific RNA. The RNA used in this study is a 270 nt transcript, uniformly labelled with [^{32}P]UTP, transcribed from the T3 promoter of the BlueScript SK+ plasmid and terminating at a *PvuI* site. This region of the plasmid contains the polylinker. (**A**) Sedimentation of the non-specific RNA added to a mitochondrial extract treated with SDS–proteinase K. The *y* axis represents total c.p.m. in each fraction. Note the change in scale between the gradients. (**B**) Sedimentation of the non-specific RNA in the 50 mM KCl gradient. (**C**) Sedimentation of the non-specific RNA in the 150 mM KCl gradient. (**D**) Sedimentation of the non-specific RNA in the 300 mM KCl gradient.

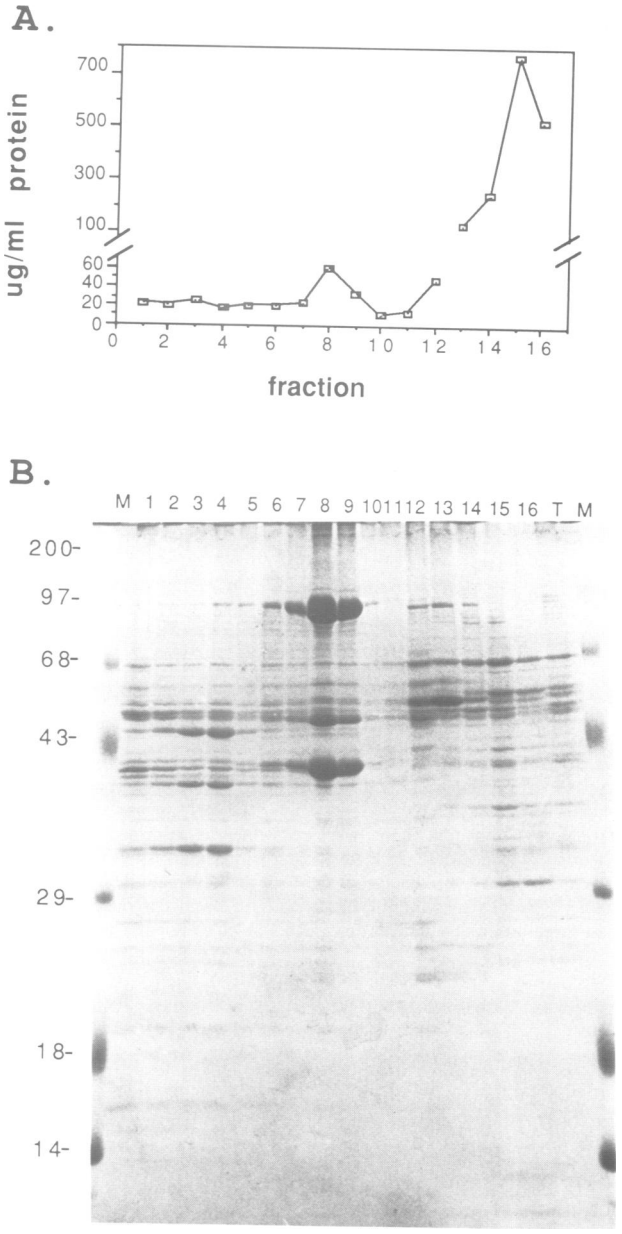

Fig. 5. Distibution of proteins across the native gradient. (**A**) Quantification of proteins in each fraction. Proteins were assayed as described in Materials and methods. (**B**) Proteins from the gradient separated on a 12% SDS–polyacrylamide gel and stained with Coomassie blue. Lane M contains pre-stained protein molecular weight standards, high range (BRL). Lanes 1–16 contain proteins from those respective fractions. Equal volumes of fractions 1–12 were run. Samples from fractions 13–16 and total proteins, lane T, were diluted and equal protein (45 μg) was added to avoid overloading the gel.

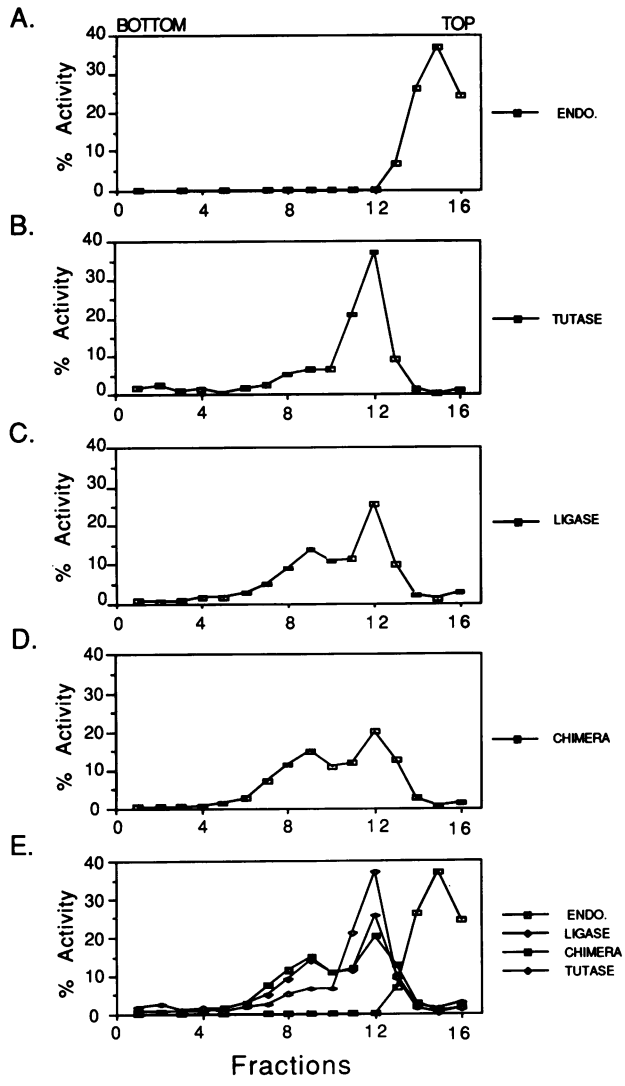

Fig. 6. Analysis of the gradient fractions for enzymatic activities proposed to participate in editing. (**A**) Pre-edited mRNA specific endonuclease assays, (**B**) TUTase assays, (**C**) RNA ligase assays and (**D**) chimera formation assays were performed as described in Materials and methods. (**E**) Comparison of the four assays. All results are expressed as a percentage of the total activity recovered in the gradient. Results shown are typical and have been reproduced in at least three different gradients from different mitochondria preparations.

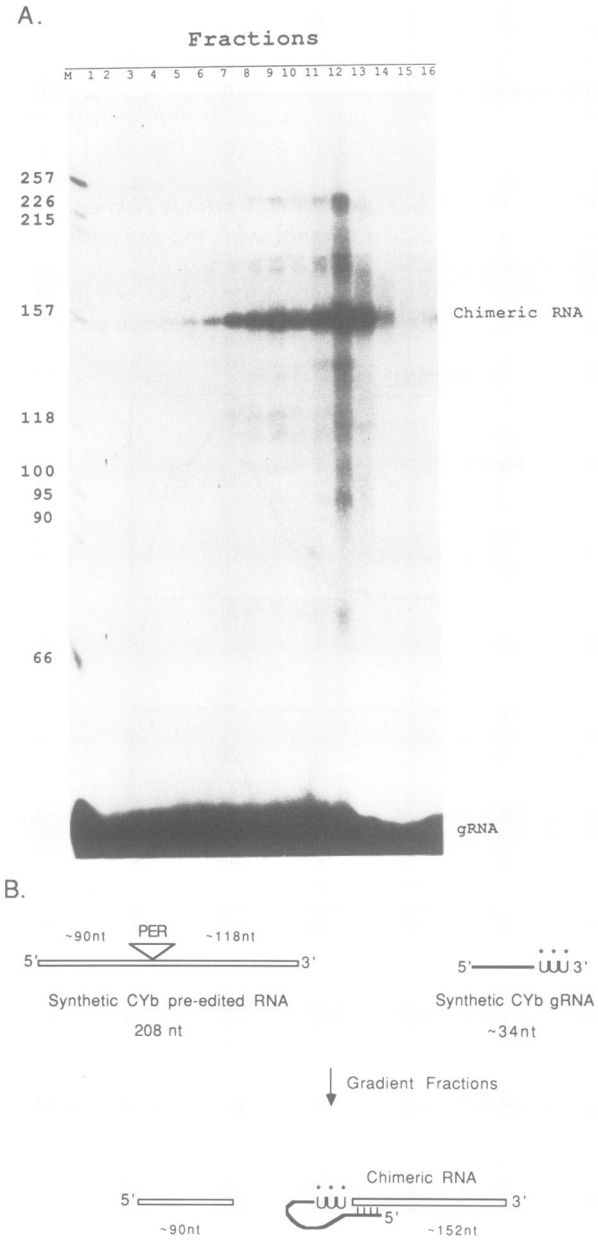

Fig. 7. The chimera formation assay. (**A**) Autoradiograph of the labelled RNAs produced in the reaction. Lane M contains kinase labelled Bluescript SK+ DNA digested with *Alu*I as a marker. Fractions 1–16 are indicated. The uniformly labelled gRNA substrate is marked. All larger labelled bands are reaction products. The RNA species identified as the chimeric RNA product (Harris and Hajduk, 1992) is indicated. The identities of the other products are unknown, but this is being investigated. (**B**) Diagram of the assay showing sizes of the substrates and the chimera product. The pre-edited region of the mRNA (PER) is indicated. Labelled uridine residues are denoted by an asterisk.

products were analyzed by electrophoresis followed by autoradiography. Specific cleavage products were quantified by densitometry. The editing site specific endonuclease activity peaks in fraction 15 (Figure 6A), sedimenting with the bulk of the mitochondrial protein (Figure 5).

TUTase has been shown to add uridine residues non-specifically to any 3′-OH (Bakalara *et al.*, 1989). TUTase activity was measured by incubating yeast tRNA, as well as mitochondrial RNA (data not shown), in the extracts with [α-^{32}P]UTP. Addition of uridines to the RNAs was assayed by TCA precipitation. TUTase activity peaks in fraction 12 and also forms a small but reproducible shoulder to fraction 8 (Figure 6B). RNA ligase activity was also assayed with yeast tRNA and mitochondrial RNA as substrates along with [α-^{32}P]cytidine 3′,5′ bisphosphate (pCp) (Bakalara *et al.*, 1989). Products were analyzed by electrophoresis followed by autoradiography and quantified using densitometry (Figure 6C). The RNA ligase peaks in fraction 12 and shows a smaller peak in fraction 9. A gRNA–mRNA chimera-forming assay described in detail in Harris and Hajduk (1992) is shown in Figure 7 and the results are summarized in Figure 6D. To assay chimera formation, an [α-^{32}P]UTP uniformly labelled, ~34 nt synthetic cytochrome *b* (CYb) gRNA was incubated with an unlabelled 208 nt synthetic CYb pre-edited mRNA in the presence of the extracts (Figure 7B). The pre-edited region of the mRNA is ~90 nt from the 5′ end. Chimera formation results in the labelled

~34 nt gRNA attaching to the ~118 nt 3′ segment of the mRNA. The ~152 nt RNA, identified as the chimeric RNA, peaks in fraction 12 and shows a smaller peak in fraction 9.

Comparison of all the activities assayed (Figure 6E) shows striking co-sedimentation of the TUTase, RNA ligase and chimera-forming activities. These three activities peak together in fraction 12, a position which corresponds well, though not perfectly, to the 19S gRNA peak (fraction 13). The three activities also co-sediment further into the gradient creating a second peak (fraction 9), which is in perfect correspondence with the 35–40S complex of pre-edited mRNA and gRNA (fractions 8–10). This suggests that these activities are present in ribonucleoprotein complexes with the gRNAs and pre-edited mRNAs.

Stability of complexes at higher ionic strengths

In order to determine if these ribonucleoprotein complexes are stable at higher ionic strengths, mitochondrial lysates were prepared and fractionated as above except that the KCl concentration was raised to 150 and 300 mM. RNAs and activities were analyzed as above. In both of these gradients (Figure 8), the COIII pre-edited mRNAs and gRNAs peak in fractions 10–11. This represents a shift of the 35–40S complex in the 50 mM KCl gradient to 25–30S in the 150 and 300 mM KCl gradients. The 19S gRNA peak has also shifted slightly and now contains more of the gRNAs, with the gRNAs being distributed almost evenly between the two peaks. Thus, the gRNAs and pre-edited mRNAs co-sediment in a large 25–30S ribonucleoprotein complex even at higher ionic strengths, while the smaller gRNA complex is more pronounced.

TUTase, RNA ligase and chimera-forming activities were also assayed in these gradients. All of these enzymes lose some activity in the higher ionic strength (data not shown). Each of the enzymes retains only ~50% activity in the 150 mM KCl extracts relative to the 50 mM KCl extracts. In the 300 mM KCl extracts, RNA ligase still retains ~50% activity, while TUTase is down to ~15% activity and chimera-forming activity is apparently absent. In the 150 mM KCl gradient, the TUTase and chimera-forming activities co-sediment, peaking in fraction 13. The RNA ligase activity breaks its strict co-sedimentation with the other two activities and peaks in fraction 12, but still overlaps fraction 13 considerably. All of the activities have lost the second peak, which corresponded to the larger 35–40S RNA complex. The activities in the 300 mM KCl gradient exhibit a pattern similar to the 150 mM pattern except for the absence of the chimera-forming activity. These data suggest that these activities are still sedimenting with the smaller gRNA complex, now 15–19S, but are not stable in the larger 35–40S complex at higher ionic strength. Loss of these activities from the 35–40S complex is consistent with the shift of the RNAs to 25–30S.

Discussion

In this study, glycerol gradient sedimentation was used to identify two putative complexes potentially involved in editing. Co-sedimentation was the criterion used to identify components of these complexes. The sedimentation values exhibited by the RNAs in their native state attest to the fact that they are in complexes (Figure 2). These RNAs co-sedimented with the TUTase, RNA ligase and chimera-

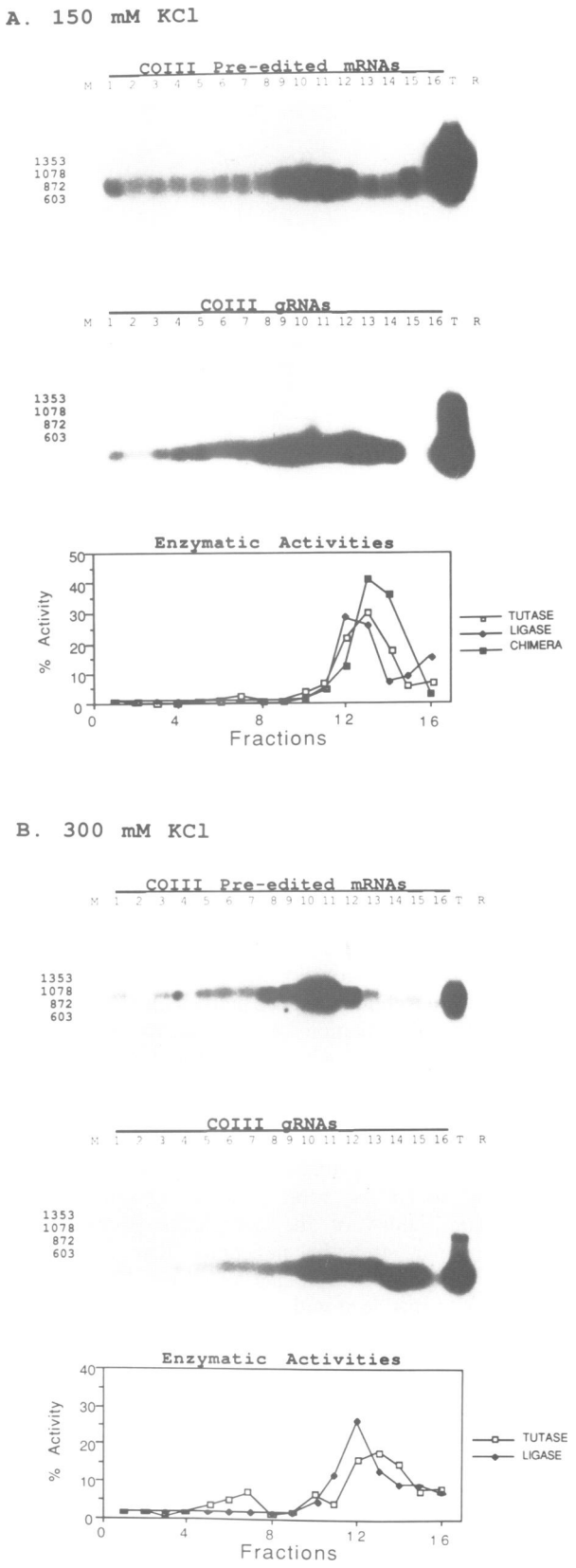

Fig. 8. Sedimentation of RNAs and activities at higher ionic strengths. (**A**) Analysis of RNAs and activities from 150 mM KCl gradients. These experiments were performed exactly as described above for the 50 mM KCl gradients, except that two different hybridization filters from identical gradients are shown due to difficulties in stripping these filters. (**B**) Analysis of RNAs and activities from 300 mM KCl gradients. These experiments were performed exactly as described above for the 50 mM KCl gradients.

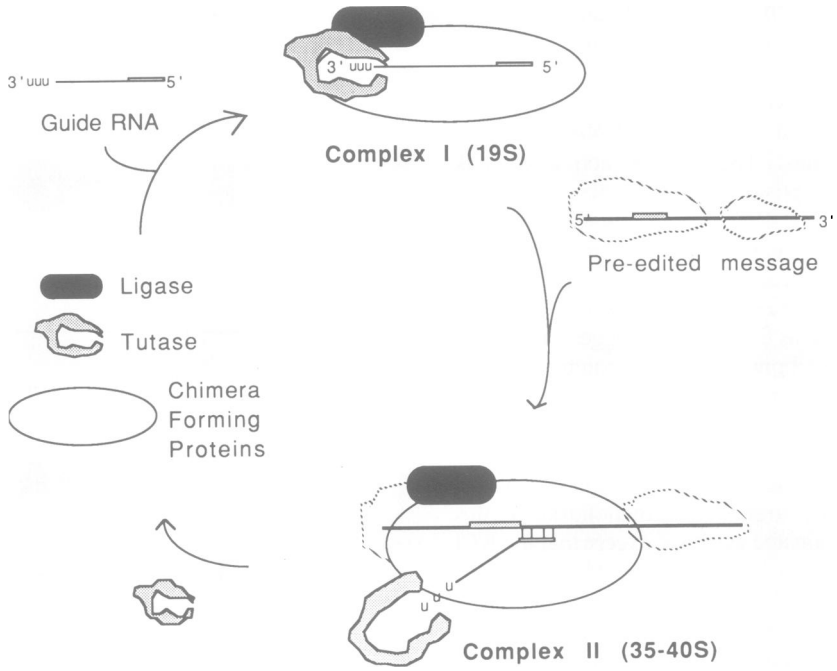

Fig. 9. Proposed editing complexes. RNA ligase, TUTase and chimera-forming proteins are thought to assemble with the gRNA to form complex I. Complex I may then present the gRNA and the associated activities to the pre-edited mRNA and form complex II. The anchor region of the gRNA is shown as an open box. The pre-edited region of the mRNA is represented as a stippled box.

forming activities under standard conditions (Figure 6). These enzymatic activities and RNAs could peak together in the gradient by chance, even though this region of the gradient is well resolved from the majority of the mitochondrial proteins (Figure 5). Co-sedimentation alone does not necessarily prove that disparate activities and RNAs are physically associated. The fact that the enzymatic activities and gRNA show identical biphasic distributions makes co-sedimentation by chance unlikely. It is also unlikely that this co-sedimentation is caused by non-specific aggregation, as exogenously added RNA does not become incorporated into these complexes (Figure 4). We propose therefore that these components are actually associated in specific complexes.

The sedimentation properties of these complexes are affected by increasing ionic strength (Figure 8). The sedimentation values of the complexes decrease with increasing ionic strength, indicating that less tightly associated components are being salted off. This effect is very similar to that seen with increasing ionic strength on the sedimentation values of splicing complexes (Bindereif and Green, 1986). The large 35–40S complex is apparently most sensitive to this affect, moving to a position of 25–30S complex. Concurrent with this shift is the loss of the RNA ligase, chimera-forming activities and TUTase, although the pre-edited mRNAs and ~50% of the gRNAs are still associated. The loss of weakly associated gRNA complexes 'searching' for the correct partially edited mRNA (see below) could also contribute to the lower sedimentation value. Consistent with this possibility is the shift in gRNA distribution from the 25–30S complex into the 15–19S complex. The small 19S complex is also sensitive to the higher ionic strength, but to a lesser degree.

The strongest evidence that these complexes are involved in editing is that they contain the RNAs and enzymatic activities proposed to participate in editing (Figure 9). Complex I, a 19S particle, contains gRNA, TUTase, RNA ligase and chimera-forming activities. The function of TUTase in this complex is probably the addition of the non-encoded poly(U) tail to the gRNA. Thus complex I may play a role in gRNA maturation. The purpose of RNA ligase and chimera-forming activities in complex I is less clear. Complex I may be a precursor to the active editing complex and present the gRNA and associated activities to the pre-edited mRNA for assembly into an active editing complex.

In this scheme, complex II, a 35–40S particle containing pre-edited mRNA, gRNA, RNA ligase and chimera-forming proteins, may be the active editing complex. TUTase activity is also present in complex II, but not with a relative abundance consistent with that of the RNA ligase and chimera activities (Figure 6). This suggests that TUTase may be only transiently associated with complex II. The reduced amount of TUTase activity in complex II suggests that once a gRNA has donated the majority of the uridine residues from its poly(U) tail to the mRNA, it dissociates from complex II. This gRNA is either degraded or recycled back to complex I where the poly(U) tail is regenerated. The abundance of the RNA ligase and chimera-forming activities in complex I and complex II relative to gRNA levels suggests that an excess of pre-formed protein complex exists which co-sediments with complex I. This idea is attractive, as an excess of pre-formed protein complex ready to accept gRNA would facilitate ribonucleoprotein complex formation. Alternatively, these relative levels may simply reflect the fact that we only assayed for two specific gRNAs and not the entire gRNA population. Other gRNAs in the complex I pool may be present in greater excess and account for the relatively high levels of enzyme activities compared with the COIII gRNAs.

Sedimentation of complex II appears to be influenced by the extent of editing exhibited by the mRNA in the complex. The more edited the mRNA, the greater its sedimentation

value (compare Figure 2B, C and D). This may be caused solely by the increased length of the mRNA. The sedimentation of the complex may also increase due to bound gRNAs, which have been proposed to remain hybridized to the edited sequences to protect them from further processing (Decker and Sollner-Webb, 1990). Another possibility is that multiple editing complexes become associated with the mRNA as editing progresses.

The greater sedimentation value exhibited by more fully edited RNAs is an interesting result in light of the more diffuse pattern of sedimentation exhibited by the gRNAs. The gRNAs used in this study specify sequences at sites in the 5′ end of the mRNA (Figure 1). mRNAs in the process of being edited at these sites would exhibit large sedimentation values. These gRNAs co-sediment with the entire range of pre-edited RNA complexes. This brings up the interesting possibility that the majority of these gRNAs co-sediment with the pre-edited mRNAs, not because they are actively editing, but because they are in the process of 'searching' for the correct match between gRNA and mRNA. This selection process is probably carried out by the simple mechanism of trial and error and is potentially directed by complex I. This selection process may be the rate limiting step in the editing pathway.

Several features of the complexes described here have important implications for the mechanism of RNA editing. An intriguing feature is that RNA ligase and chimera-forming activities co-sediment. This suggests that the mechanism of chimera formation is not RNA catalyzed *trans*-esterification (Blum *et al.*, 1991; Cech, 1991), but cleavage followed by ligation (Harris and Hajduk, 1992). Inconsistent with this model is that the editing site specific endonuclease activity assayed in this study does not sediment with the chimera-forming activity. The cleavage during chimera formation, however, may occur by a different activity. Further purification of these editing activities is underway and these possibilities will be addressed.

In this study, we have identified two complexes potentially involved in editing. This is the first evidence that editing actually occurs within a ribonucleoprotein complex. Complex I potentially functions as a gRNA maturation complex, a mediator in selecting the correct gRNA for a particular sequence and as a precursor to the active editing complex. Complex II is presumably the active editing complex. Thus, in keeping with the nomenclature of Abraham *et al.* (1988) and van der Spek *et al.* (1988), complex II could be called an editosome.

Materials and methods

Trypanosomes

Procyclic *T.brucei* TREU 667 were grown in semidefined medium (Cunningham, 1977) supplemented with 10% heat-inactivated fetal bovine serum at 26°C. Cells were collected at or before they reached a density of 1×10^7 cells/ml.

Mitochondrial extract preparation

Mitochondria were isolated as described by Braley *et al.* (1974) and Rohrer *et al.* (1987). Mitochondrial vesicles were washed once with lysis buffer (10 mM magnesium acetate, 50 mM potassium chloride, 25 mM HEPES pH 7.9, 1 mM EDTA, 0.5 mM DTT, 1 mM ATP and 5% glycerol, increased to 150 mM KCl and 300 mM KCl for those specific gradients) and then resuspended at a concentration of 1.5×10^{10} cell equivalents of mitochondria/ml in lysis buffer for Triton solubilization or NET-10−SDS (100 mM NaCl, 10 mM Tris−HCl pH 8.0, 10 mM EDTA and 0.5% SDS) for SDS solubilization. For Triton solubilization, Triton X-100 was added to a final concentration of 0.5% and the extract incubated at 4°C for 10 min with occasional mixing by inversion. SDS-solubilized extracts were treated with proteinase K at a final concentration of 100 μg/ml for 10 min at 37°C. The extracts were then clarified by centrifugation for 2 min at 10 000 *g* to remove the bulk of the insoluble material.

Glycerol gradient sedimentation

500 μl of the lysates were loaded on to 11.5 ml 10−30% linear glycerol gradients containing 20 mM HEPES (pH 7.9), 50 mM KCl (or 150 mM KCl and 300 mM KCl for the higher ionic strength gradients) and 10 mM magnesium acetate. Gradients were centrifuged in a Beckman SW41 rotor at 38 000 r.p.m. for 5 h at 4°C. Sixteen fractions each having a volume of 750 μl were collected from the bottom of each tube. Marker gradients were run concurrently with the experimental gradients. Markers used were cytochrome *c*, thyroglobulin and *Escherichia coli* 30 and 50S ribosomal subunits. Ribosomal subunits were prepared as described by Brimacombe *et al.* (1988). Markers were resuspended at a concentration of ~2−4 mg/ml and 500 μl of each was loaded onto individual gradients. Absorbance at 280 nm was used to quantify protein in each marker gradient fraction.

RNA extraction

Approximately 250 ng of a 270 nt control riboprobe containing the polylinker region (T3 to *Pvu*I transcript) of the plasmid Bluescript SK+ (Stratagene) was added to 500 μl of each fraction. SDS was added to a concentration of 0.5%. Proteinase K was added to a concentration of 100 μg/ml and the extracts were incubated at room temperature for 10 min. After extraction with phenol−chloroform and chloroform, RNase free DNase I was added (100 units/ml) along with $CaCl_2$ (to 2 mM) and the mixture was incubated for 10 min at room temperature. After extraction with phenol−chloroform and chloroform, Puc 7 DNA (1−2 μg) was added as a carrier and the RNAs were precipitated with isopropanol. For the higher ionic strength gradients, Puc 7 DNA was added after the first set of extractions and the RNAs were precipitated before DNase I treatment.

Northern blot analysis

RNAs were run on 1% methyl mercury−agarose gels and stained with ethidium bromide. RNAs were transferred by capillary blot to GeneScreen Plus (Du Pont) nylon membranes according to manufacturer's instructions and UV cross-linked using a Stratagene Stratalinker on standard cycle. Membranes were pre-hybridized in a solution of 1 M $NaCl_2$, 10% dextran sulfate and 1% SDS for 15 min. Salmon sperm DNA (100 μg/ml) and [α-^{32}P]ATP/kinase labelled probe (2×10^6 c.p.m./ml) were added and hybridization was allowed to proceed for 12 h. Filters were washed in 0.15 M sodium chloride, 0.15 M sodium citrate and 1% SDS, according to the manufacturer's instructions. Different probes were hybridized and washed at different temperatures. Probes for the indicated regions and temperatures used were: COIII gRNAs (Pollard *et al.*, 1990) Tb1 5′-ACT TTT TAC AGT TCT TAC ATA GCG AGT TTG-3′ and Tb2 5′-GAC ACT CTT CAC TGT ATT ATC TGA TTT ATG-3′, 42°C; COIII pre-edited mRNA (Feagin *et al.*, 1988) 5′-TCC TCA CTC TCC TCT CTG G-3′, 42°C; COIII edited mRNA (Feagin *et al.*, 1988) #1 5′-GAA CCA AAA ACA CCA CAA C-3′, 37°C; COIII edited mRNA #2 5′-CAA ATA AAC CAC TAA CAA AC-3′ (Feagin *et al.*, 1988), 28°C; COI mRNA (Hensgens *et al.*, 1984) 5′-CCA TAA TCA ACC CAT GTG AAG-3′, 28°C; 9S rRNA (Sloof *et al.*, 1985) 5′-GGG AAT ACT AAC AAT TGA CC-3′, 32°C.

Protein analysis

Proteins in each fraction were quantified using the Bradford protein assay (Bio-Rad) according to manufacturer's instructions. Samples for SDS−PAGE analysis were prepared by precipitating 500 μl of each fraction with 2 vol of acetone, washing with 70% acetone and then drying the pellet. Pellets were resuspended in SDS sample buffer and loaded onto a 12% SDS−polyacrylamide gel. For fractions 1−12, the entire sample was loaded. The other samples were diluted and the appropriate volume necessary to contain 45 μg was loaded. The gel was stained with Coomassie blue.

Enzyme assays

TUTase was assayed essentially as described by Bakalara *et al.* (1989). The TUTase reactions contained the following in a volume of 20 μl: 10 μl of the appropriate fraction, 10 μCi of [α-^{32}P]UTP (3000 Ci/mM), 1 μg of yeast tRNA or 3 μg of mitochondrial RNA, 25 mM HEPES (pH 7.9), 10 mM magnesium acetate, 0.5 mM DTT, 50 mM KCl, 1 mM ATP and 15% glycerol (glycerol was added to bring each fraction to a consistent percentage, but this correction did not influence the results of any of the enzymatic assays). The reaction was allowed to proceed at 26°C for 1 h and then terminated by precipitation with 10% TCA and 100 mM sodium pyrophosphate at 4°C. Labelled products were collected on glass filters (Whatman GF/C) and c.p.m. were quantified by liquid scintillation counting. Endonuclease assays were performed essentially as described by Harris *et al.*

(1992). 5′ end-labelled pre-edited cytochrome *b* synthetic riboprobe was incubated with 10 μl of extract in buffer to give a final concentration of 25 mM HEPES (pH 7.9), 10 mM magnesium acetate, 0.5 mM DTT, 50 mM KCl, 1 mM ATP, 15% glycerol and 50 mM heparin (final volume of 20 μl) at 26°C for 1 h. Reactions were terminated by the addition of 200 μl of stop buffer (0.5% SDS, 100 μg/ml proteinase K and 10 mM HEPES pH 7.9) and incubated at 26°C for 10 min. After extraction with phenol–chloroform, followed by chloroform, the RNAs were precipitated and then run on a 6% polyacrylamide–8 M urea gel. The gels were autoradiographed and the specific cleavage products quantified by densitometry. Ligase reactions were performed essentially as described by Bakalara *et al.* (1989). Yeast tRNA (1 μg) or mitochondrial RNA (3 μg) was incubated with 10 μl of each fraction, 10 μCi pCp and buffer (final concentrations: 25 mM HEPES pH 7.9, 10 mM magnesium acetate, 0.5 mM DTT, 50 mM KCl, 1 mM ATP, 15% glycerol, 10 U RNAsin (Promega) and 10% DMSO) in a final volume of 20 μl at 26°C for 3 h. Reactions were terminated by the addition of stop buffer and incubated at 26°C for 10 min. After extraction with phenol–chloroform, followed by chloroform, the RNAs were precipitated and run on a 6% polyacrylamide–8 M urea gel. The gels were autoradiographed and the labelled products quantified by densitometry. Chimera formation assays were performed as described by Harris and Hajduk (1992). Synthetic cytochrome *b* gRNA, uniformly labelled with [α-^{32}P]UTP, was incubated with unlabelled synthetic pre-edited cytochrome *b* mRNA in the presence of 20 μl extract in buffer (final concentrations: 25 mM HEPES, 10 mM magnesium acetate, 1 mM EDTA, 60 mM KCl, 0.5 mM DTT, 10% glycerol, 1 mM ATP and 1 mM UTP) in a total volume of 30 μl for 3 h at 26°C. Reactions were terminated with stop buffer, the RNAs extracted and precipitated as above and electrophoresed on an 8% polyacrylamide–8 M urea gel. The gel was autoradiographed. Chimera bands were quantified by densitometry.

Acknowledgements

We would like to thank Dr Kathleen Fearon and Dr Brian Adler for many helpful discussions, and Ms Karen Bertrand for criticisms of the manuscript. We acknowledge the support of the Center for AIDS Research at the University of Alabama at Birmingham (P30 AI27767) in providing the University of Wisconsin Genetics Computer Group programs for our use. This work was supported by P.H.S. grant AI 21401 to S.L.H. S.L.H. is a Burroughs Wellcome Fund Scholar in Molecular Parasitology.

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Received on May 14, 1992; revised on August 21, 1992