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Purification of a functional enzymatic editing complex from *Trypanosoma brucei* mitochondria

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Kinetoplastid mitochondrial RNA editing, the insertion and deletion of U residues, is catalyzed by sequential cleavage, U addition or removal, and ligation reactions and is directed by complementary guide RNAs. We have purified a ~20S enzymatic complex from *Trypanosoma brucei* mitochondria that catalyzes a complete editing reaction *in vitro*. This complex possesses all four activities predicted to catalyze RNA editing: gRNA-directed endonuclease, terminal uridylyl transferase, 3′ U-specific exonuclease, and RNA ligase. However, it does not contain other putative editing complex components: gRNA-independent endonuclease, RNA helicase, endogenous gRNAs or pre-mRNAs, or a 25 kDa gRNA-binding protein. The complex is composed of eight major polypeptides, three of which represent RNA ligase. These findings identify polypeptides representing catalytic editing factors, reveal the nature of this ~20S editing complex, and suggest a new model of editosome assembly.

Keywords: editing complex/RNA editing/RNA lipase/ *Trypanosoma brucei*/trypanosome

Introduction

In kinetoplastid protozoans, mitochondrial pre-mRNAs are edited by the insertion and deletion of U residues (reviewed in Arts and Benne, 1996) at multiple, closely spaced sites. This editing is directed by guide RNAs (gRNAs), ~70 nucleotide mitochondrial transcripts complementary (using G-U and Watson–Crick base pairing) to the edited sequence. The 5′ end of the gRNA comprises the anchor sequence, which can anneal to substrate pre-mRNA just 3′ of the first editing site. The gRNA then directs editing in a 3′→5′ direction along the pre-mRNA. The 3′ end of the gRNA consists of a 10–20 nucleotides post-transcriptionally added oligo(U) tail.

For several years it had been unclear whether RNA editing occurs by a transesterification-based mechanism (Blum *et al.*, 1991; Cech, 1991) or by enzymatically catalyzed reactions (Blum *et al.*, 1990; Sollner-Webb, 1991). Although the transesterificaton scheme was very elegant and appealing, the enzymes for an enzymatic mechanism were present in trypanosome mitochondria and initial experiments favored an enzymatic-based mechanism (Rusché *et al.*, 1995; Sabatini and Hajduk, 1995; and references therein). Recently, the direct enzymatic mechanism (as originally proposed by Blum *et al.*, 1990) was demonstrated for both deletional (Cruz-Reyes and Sollner-Webb, 1996; Seiwert *et al.*, 1996) and insertional (Kable *et al.*, 1996) *in vitro* editing of ATPase 6 (A6) pre-mRNA (see also Byrne *et al.*, 1996). In the first step of this mechanism, the gRNA anchor anneals to the pre-mRNA, positioning the first editing site at the phosphodiester bond immediately 5′ of the gRNA–mRNA duplex. Next, an endonuclease cleaves the pre-mRNA at this mismatched position. Then, U residues are either added to or deleted from the 3′ end of the upstream cleavage product by a terminal uridylyl transferase (TUTase) or a U-specific exonuclease, respectively. The exonuclease is evidently not the reverse reaction of the TUTase (Cruz-Reyes and Sollner-Webb, 1996). Finally, the two pre-mRNA fragments are rejoined by RNA ligase. A gRNA–mRNA chimeric RNA, an intermediate in the other proposed editing mechanisms, is apparently an alternate reaction product (Kable *et al.*, 1996; Seiwert *et al.*, 1996).

It had been hypothesized early on that editing would occur in a multicomponent complex, or editosome (reviewed in Göringer *et al.*, 1995). If editing involved sequential enzymatic reactions, this complex minimally would contain the above-mentioned enzymatic activities and RNAs, but it could be quite large. For instance, splicing, another pre-mRNA processing event which involves cleavage, nucleotide removal and rejoining of the phosphodiester backbone, occurs in a complex containing over 100 different components (Sharp, 1994). However, the nature of trypanosome editing complex(es) has been only vaguely understood, and their protein composition(s) remain virtually unknown.

To look for potential editing complexes, Pollard *et al.* (1992) separated trypanosome mitochondrial extract by glycerol gradient sedimentation and examined fractions for putative components of an enzymatic editing mechanism. They found endogenous pre-mRNA and gRNA to sediment broadly from 25S to 50S and from 10S to 50S, respectively. TUTase, RNA ligase, and gRNA–mRNA chimera-forming activities sedimented as a major ~20S peak and generally as a less abundant 35–40S peak. These results were interpreted as evidence for two complexes: (i) a ~20S gRNA maturation complex (complex I) that contains gRNA, TUTase, ligase, and chimera-forming activity; and (ii) a 35–40S editing complex (complex II) that contains these components as well as pre-mRNA.

The associations of additional potential RNA editing activities were subsequently examined. Endonuclease activity, although not originally observed in the ~20S fraction (Pollard *et al.*, 1992), is expected to be present in this fraction since it is required for chimera formation (Rusché *et al.*, 1995; Piller *et al.*, 1996). Indeed, two endonuclease activities were subsequently found to co-sediment with RNA ligase, one which cleaves free CYb

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pre-mRNA at the 3′ end of the editing domain (Piller *et al.*, 1995b) and a second which accurately cleaves both A6 and CYb pre-mRNA directed by their cognate gRNAs (Cruz-Reyes and Sollner-Webb, 1996; Seiwert *et al.*, 1996; Piller *et al.*, 1997). A 3′ U-specific exonuclease and the complete A6 U-deletional activity also co-sediment with these activities (Cruz-Reyes and Sollner-Webb, 1996). Additionally, RNA helicase activity was found to co-sediment with the RNA ligase and TUTase activities (Corell *et al.*, 1996; however, in this particular study all the activities were in a broad peak at ~35S). These results suggest the existence of a complex (or complexes) containing gRNAs, RNA ligase, TUTase, chimera-forming activity, gRNA-independent endonuclease, gRNA-directed endonuclease, 3′ U-specific exonuclease, RNA helicase, and the complete U-deletional activity.

However, these ~20S glycerol gradient fractions contain a very large number of different polypeptides (see Pollard *et al.*, 1992; Corell *et al.*, 1996; Figure 6A, lane 4) and therefore many different ~20S complexes. Consequently, finding two activities in the same fraction provides only limited evidence for their physical association with one another in a single complex. In fact, under altered sedimentation conditions, the two ~20S endonuclease activities can be resolved one from another (Piller *et al.*, 1997), demonstrating that not all the originally identified co-sedimenting activities were part of the same complex.

A second approach to studying editing-related assemblies has been to identify gRNA- and mRNA-containing complexes and their constituent proteins. Complexes containing endogenous RNAs have been labeled with [α-^{32}P]UTP using the TUTase of the extract (Peris *et al.*, 1994; Byrne *et al.*, 1995). Alternatively, complexes have been assembled *in vitro* using radiolabeled RNAs and mitochondrial extracts (Göringer *et al.*, 1994; Read *et al.*, 1994; Bringaud *et al.*, 1995; Byrne *et al.*, 1995), but these are at best precursors to endogenous complexes since they have lower sedimentation values (Shu *et al.*, 1995; Corell *et al.*, 1996). A number of the protein constituents of such complexes have been visualized by UV cross-linking (Köller *et al.*, 1994; Read *et al.*, 1994; Bringaud *et al.*, 1995; Byrne *et al.*, 1995; Leegwater *et al.*, 1995), most notably a 25 kDa protein which specifically binds gRNAs but not oligo(U) (Köller *et al.*, 1994; H.U.Göringer, personal communication). Unfortunately, the relationships of these RNA-associated complexes to the enzymatic complex(es) and RNA editing remain unclear.

Clearly, a major advance in the study of RNA editing would be the purification of editing activities in their native complexes. Such a purification would reveal whether multiple editing activities are part of the same enzymatic complex and, if so, whether this complex exists in the absence of gRNA and/or pre-mRNA. Furthermore, the protein composition and *in vitro* editing ability of such a complex could be determined. Towards this goal, we purified the mitochondrial RNA ligase and examined which activities and polypeptides co-purify. RNA ligase was chosen because it is required for RNA editing *in vitro*, is part of a larger complex, and can be visualized by adenylylation with [α-^{32}P]ATP (Sabatini and Hajduk, 1995).

By following RNA ligase, we obtained a fraction that consists of eight major, physically associated polypeptides, three of which are the adenylylatable ligase polypeptides. Other co-purifying activities are a gRNA-directed endonuclease, TUTase and 3′ U-specific exonuclease, all the activities of the enzymatic mechanism of editing. Indeed, the purified complex catalyzes a full round of U-deletional editing. Additionally, this editing complex appears to not lose initially associated components during the purification. However, the gRNA-independent endonuclease, RNA helicase, endogenous gRNAs and pre-mRNAs, and the 25 kDa gRNA-specific binding protein purify away from this complex.

Results

Co-purification of RNA ligase and other editing-related activities

To purify the RNA ligase, we first selected Q-Sepharose over other examined matrices because it binds RNA ligase under conditions where most of the major *Trypanosoma brucei* mitochondrial extract proteins are not bound (Figure 1A, compare load L and flow-through FT). RNA ligase activity, assayed by the dimerization and circularization of a 5′ end-labeled RNA (Rusché *et al.*, 1995), elutes from Q-Sepharose in a single peak between 170 and 200 mM KCl in fractions 15 and 16 (Figure 1B) with ~1/300 of the mitochondrial extract protein and ~1/3000 of total cellular protein (Table I; see also Figure 6A). RNA ligase activity, measured under conditions where activity varied linearly with sample amount, was found to be purified ~50-fold relative to the mitochondrial extract by passage over Q-Sepharose (Table I).

The trypanosome RNA ligase polypeptides can be directly labeled with [α-^{32}P]ATP since, like most ligases, they are activated by adenylylation (the covalent binding of AMP, the first step in the RNA ligase reaction mechanism); they are then deadenylylated in the presence of active substrate during productive ligation or in the presence of excess pyrophosphate by reversal of the initial reaction (Sabatini and Hajduk, 1995). Our unfractionated extract contains three adenylylatable polypeptides, two ~57 kDa and one ~50 kDa (Figure 1D). These represent RNA ligases since they specifically deadenylylate when incubated with ligatable RNA substrate (lane 3) but not when incubated with non-ligatable RNA or with ligatable DNA substrates (lanes 4 and 5) [see also Sabatini and Hajduk (1995) who reported a single 57 kDa band rather than a doublet]. These three polypeptides elute in fractions 15 and 16, consistent with being RNA ligase (Figure 1C).

Although the adenylylatable 50 kDa polypeptide appears under-represented in these fractions compared with the adenylylatable 57 kDa doublet, this is due to its becoming fully adenylylated by unlabeled endogenous ATP during fractionation. When the sample is first deadenylylated with pyrophosphate and then readenylylated in the presence of [α-^{32}P]ATP and pyrophosphatase, the 50 kDa polypeptide is adenylylated at the expected level (Figure 1E) and is seen to peak in fractions 15 and 16, co-eluting with the 57 kDa proteins (data not shown).

We next examined the Q-Sepharose fractions for the two endonuclease activities previously observed to co-sediment with RNA ligase at ~20S, one gRNA-directed (Cruz-Reyes and Sollner-Webb, 1996; Seiwert *et al.*, 1996; Piller *et al.*, 1997) and the other gRNA-independent (Harris

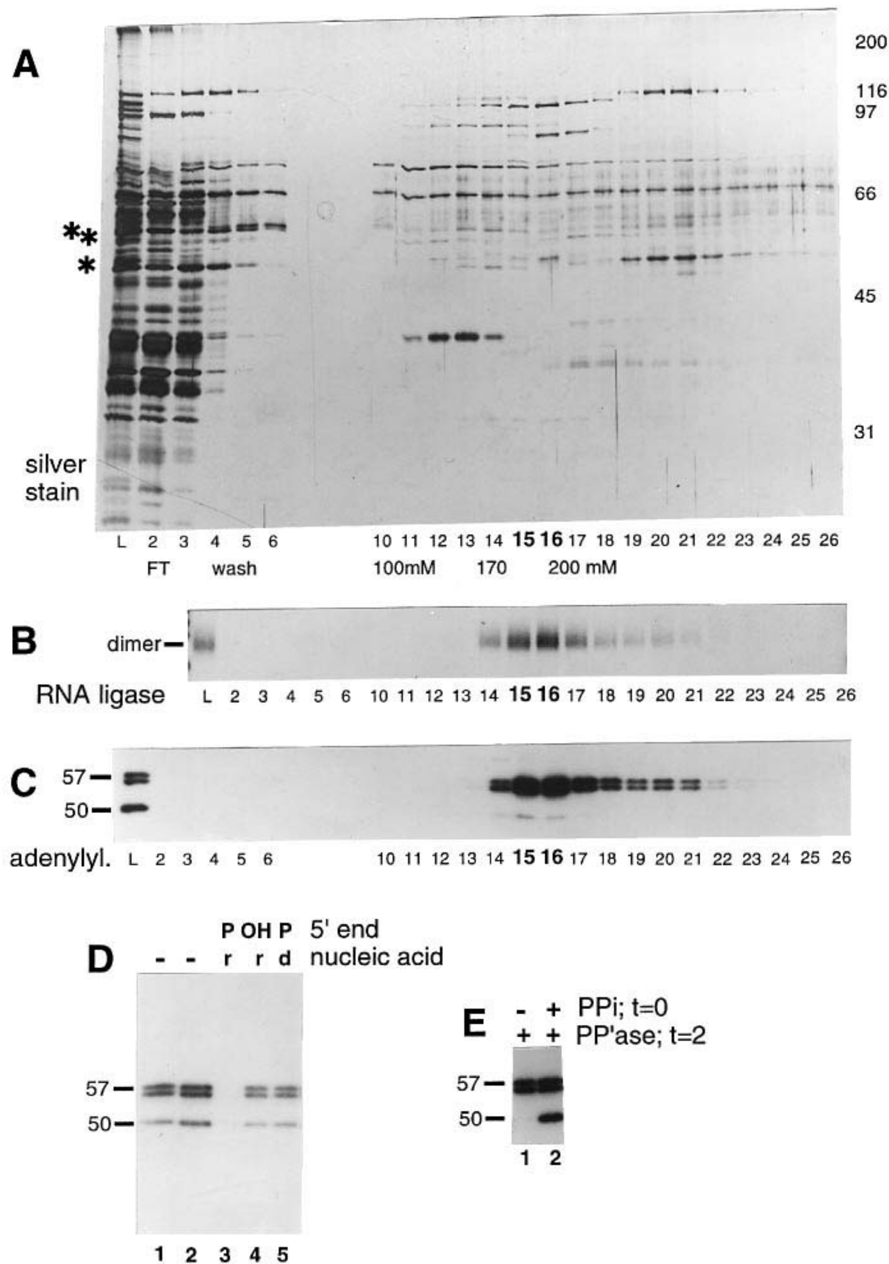

Fig. 1. RNA ligase activity binds to Q-Sepharose. (**A**) Protein silver stain of 2 µl of each numbered fraction or 0.5 µl of the column load (L) resolved on 10% SDS–PAGE. Fractions were adenylylated before electrophoresis. Protein molecular weight markers (Bio-Rad) are indicated. FT indicates flow-through, and approximate KCl concentrations are shown. The asterisks indicate the approximate position of the adenylylatable polypeptides, which do not coincide with major bands detectable in the extract. (**B**) RNA ligase assays using 2 µl of each numbered fraction or 0.5 µl of the column load (L) and ~0.7 pmol substrate RNA per reaction. The dimerized ligation product is shown. In this and future figures, the fractions with peak ligase activity are indicated in bold. (**C**) Autoradiograph of the gel in part (A). Approximate weight in kDa of the adenylylated species are shown. (**D**) Adenylylation of 0.25 µl of unfractionated extract (lane 1) and of this extract following incubation at 28°C with no addition (lane 2) or with 5′ phosphorylated pLL RNA (lane 3), phosphatase-treated pLL RNA (lane 4), or *Hin*dIII-digested DNA (lane 5). The P or OH 5′ end of these added nucleic acids and whether they are RNA (r) or DNA (d) is indicated. (**E**) Adenylylation assays using 0.5 µl of fraction 15 following mock treatment (lane 1) or deadenylylation with pyrophosphate (lane 2). 2 min later, pyrophosphatase was added to both reactions to allow the re-adenylylation in lane 2.

et al., 1992; Simpson *et al.*, 1992; Piller *et al.*, 1995b). Assays were performed using a 5′ end-labeled CYb pre-mRNA substrate with or without CYb gRNA (Figure 2A). Fractions 15 and 16 contain the single peak of gRNA-directed cleavage activity (left), which has been shown to cleave CYb pre-mRNA precisely at the site of gRNA–mRNA mismatch (Figure 2B and C; Piller *et al.*, 1997) and catalyze the first step in RNA editing (Cruz-Reyes and Sollner-Webb, 1996; Kable *et al.*, 1996; Seiwert *et al.*, 1996). These fractions show no cleavage of the editing region in the absence of CYb gRNA (Figure 2A, right). Thus, this gRNA-dependent endonuclease activity co-fractionates with RNA ligase activity.

The Q-Sepharose fractions 20–23 contain the gRNA-independent endonuclease activity (Figure 2A) which cleaves CYb pre-edited mRNA at editing site two in the

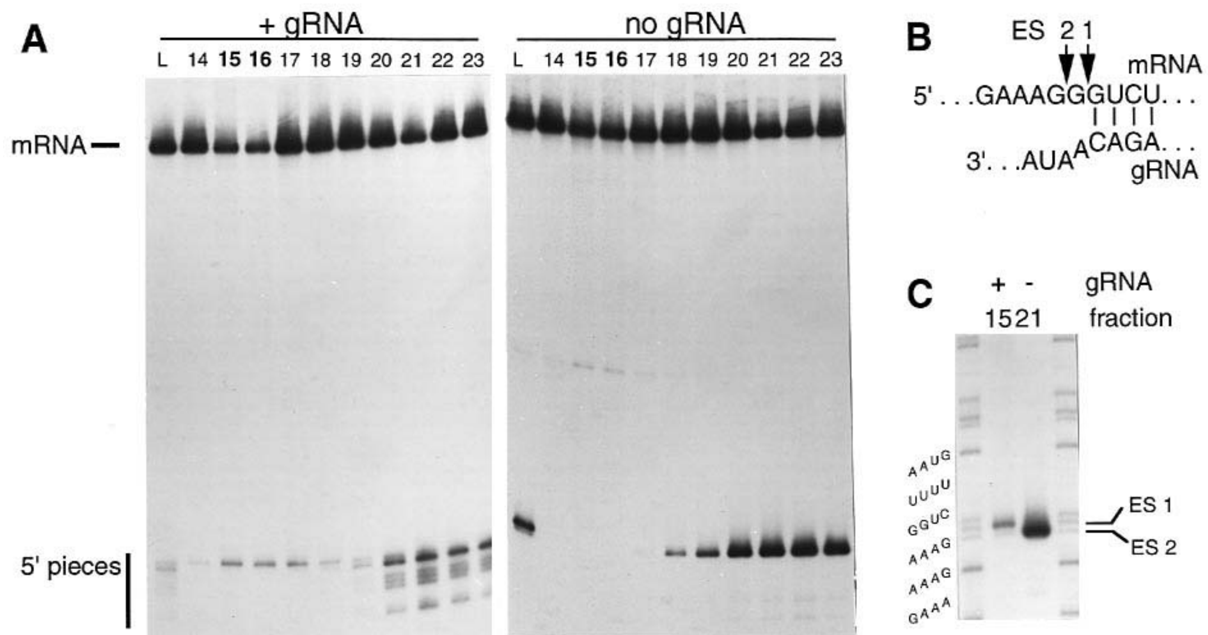

Fig. 2. gRNA-directed endonuclease activity co-purifies with RNA ligase. (**A**) Endonuclease assays of 5′ labeled Cyb pre-mRNA using 2 μl of the indicated Q-Sepharose fraction or 0.5 μl of the column load in the presence (left) or absence (right) of CYb gRNA. (**B**) Partial sequence of CYb pre-mRNA base-paired to the gRNA, indicating the positions of the first two editing sites, ES1 and ES2. (**C**) Endonuclease assays using 2 μl of fraction 15 or 21 in the presence or absence of gRNA as indicated. The two flanking lanes show sequence markers, generated with nuclease P1, which cleaves after G and leaves a 3′ OH (J.Cruz-Reyes *et al.*, manuscript in preparation) the end created by these endonucleases (Piller *et al.*, 1997). The pre-mRNA cleaved at ES1 (by fraction 15) and at ES2 (by fraction 21) are indicated.

Table I. Recovery and purification of RNA ligase activity

Step	Total protein	Ligase activity	Relative specific activity
T.brucei cell	10×	ND	ND
Mitochondrial extract	1	1	1
Q-Sepharose 15,16	1/300	1/6	50
Q-Sepharose 14–20		1/3	
DNA–cellulose 19,20[a]	1/9000	1/18	500
DNA–cellulose 18–22[a]		1/12	

Because this fractionation was analytical and yielded very low amounts of material, the total protein in each sample was estimated by silver staining. (Bradford assays were also used for whole-cell and mitochondrial extracts.) RNA ligase activity was determined as in Figure 3, using samples that had been titrated such that activity varied linearly with sample amount. Values are reported relative to the mitochondrial extract (2–4 μg protein/10^7 cells).
[a]Normalized for loading the entire Q-Sepharose peak.

absence of gRNA (Figure 2C) and at multiple adjoining sites when annealed to gRNA (Piller *et al.*, 1997). The fact that this nuclease fractionates away from the RNA ligase and gRNA-directed nuclease both on Q-Sepharose (Figure 2A) and under modified sedimentation conditions (Piller *et al.*, 1997) confirms that it is not stably associated with them.

We also examined the Q-Sepharose fractions for activities involved in the next step of editing, a TUTase (for the U insertion reaction) and a 3′ exonuclease (for the U-deletion reaction). TUTase activity was assayed by incubating each fraction with unlabeled CYb pre-mRNA and [α-^{32}P]UTP (Figure 3A). Consistent with the labeling representing a terminal U transferase, U residues are incorporated primarily at the 3′ end (>90%, data not shown), as determined by localizing the addition site using RNase H digestion (Frech *et al.*, 1995). The TUTase activity also peaks in fractions 15 and 16, coincident with RNA ligase, although an additional minor peak is found in fractions 19 and 20.

To assay for U-specific exonuclease activity, we chose as a substrate *in vitro*-synthesized CYb gRNA[558] (Riley *et al.*, 1994) which has a ~16 nucleotide oligo(U) tail. When this RNA is 5′ end-labeled and incubated in an exonuclease-containing fraction, it becomes shorter, demonstrating nucleotide removal from the 3′ end (Figure 3B, lanes 1, 2 and 6). Sizing relative to sequencing standards (Figure 3B, lanes 3–5; Cruz-Reyes and Sollner-Webb, 1996) shows this 3′ nucleotide removal to be specific for U residues, since shortening of the 5′ labeled substrate extends precisely through the U tail but no further (Figure 3B). (The larger bands observed in these reactions are not consistently seen and may represent U addition to the gRNA by an as yet uncharacterized activity.) The 3′ U removal activity was further examined using a substrate gRNA which is instead internally labeled with [α-^{32}P]UTP (Figure 3C). Incubation in active fractions results in the release of UMP and not oligonucleotides (Figure 3C, lanes 1–4; S.Seiwert and K.Stuart, personal communication), indicating that the activity removes individual mononucleotides, as expected for an exonuclease but not an endonuclease. The observation that UTP is not released confirms our previous evidence that this activity is not a reversal of the TUTase activity (Cruz-Reyes and Sollner-Webb, 1996). To demonstrate more rigorously that this activity is a U-specific 3′ mononucleotide exonuclease, the gRNA was extended at its 3′ end by one non-U residue (by adding pCp and then removing the terminal phosphate); nucleotides are no longer released when using this gRNA where the 3′ oligo(U) tail is 'blocked' by one 3′ C residue (Figure 3C, lanes 5–8). Thus, this is a

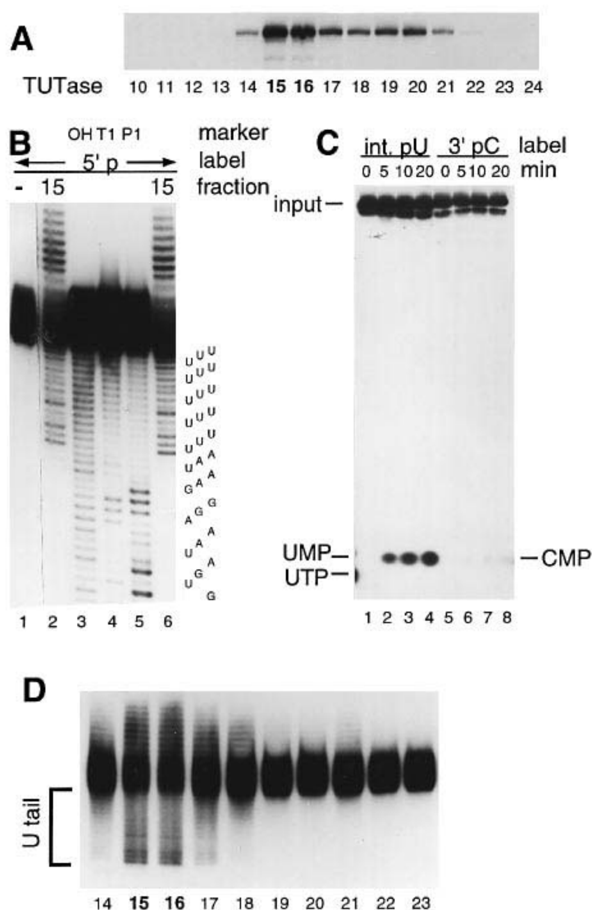

Fig. 3. Terminal uridylyl transferase (TUTase) and 3′ U-specific exonuclease activities co-purify with RNA ligase. (**A**) TUTase assays using 2 µl of the indicated Q-Sepharose fraction. (**B**) 5′ labeled gRNA[558] was incubated with (lanes 2 and 6) or without (lane 1) 2 µl fraction 15 under exonuclease assay conditions. Markers were generated by partial digestion of 4-fold more of the same RNA by alkaline hydrolysis (which cuts after any nucleotide leaving a 3′ OH; lane 3), T1 RNase (which cuts after G, leaving a 3′ P; lane 4), or nuclease P1 (with cutting after G and less after A, leaving a 3′ OH; lane 5). (The larger amount of RNA used in the sequencing lanes favored detection of the initial size heterogeneity of the starting gRNA; lane 1 is from the same experiment and same gel as lanes 2–6.) (**C**) CYb gRNA[558] was internally U-labeled (lanes 1–4) or 3′ end labeled and phosphatase-treated (lanes 5–8), incubated with 0.5 µl DNA–cellulose fraction (preparation 5; see Figure 4) for the indicated time, and electrophoresed on a 24% polyacrylamide gel. Markers were [α-^{32}P]UTP and completely nuclease P1-digested U-labeled RNA (UMP) or similarly digested C-labeled RNA (CMP). (**D**) Exonuclease assays as in (B) except using 1.5 µl of the indicated Q-Sepharose fractions.

U-specific exonuclease. We have assayed the Q-Sepharose fractions and found that the exonuclease activity has a single peak in fractions 15 and 16 (Figure 3D) co-eluting with RNA ligase, gRNA-directed nuclease, and TUTase. Because these four enzymatic activities elute in single coinciding peaks and because only 1/300 of the extract protein is present in these fractions, it is likely that these activities are physically associated.

Further purification demonstrates that the four enzymatic editing activities are physically associated

To test the association of the four editing-related activities, we subjected the peak Q-Sepharose fractions to further chromatographic separation. We selected a DNA–cellulose matrix because it does not bind most of the proteins from the Q-Sepharose peak (see Figure 6B) but does bind the adenylylatable polypeptides (Figure 4A and B). These polypeptides and RNA ligase activity (Figure 4C) co-elute between 85 and 120 mM KCl in fractions 19–21 along with ~1/30 of the loaded protein. As expected, the 50 kDa polypeptide is barely detectable by direct adenylylation but is revealed after prior deadenylylation with pyrophosphate (Figure 4B; see also Figure 1E). Quantification demonstrates that this DNA–cellulose step achieves an additional ~10-fold purification of RNA ligase activity, resulting in a ~500-fold purification relative to starting mitochondrial extract (Table I; see also Figure 6A).

The DNA–cellulose fractions were also assayed for the other enzymatic activities. Once again, the gRNA-directed nuclease, TUTase and U-specific exonuclease all elute coincident with the RNA ligase (Figure 4C). Given that these fractions contained only 1/9000 of the original mitochondrial extract protein (Table I), the precise co-elution of these four activities indicates that they are most likely physically associated with each other.

To confirm this physical association, a peak DNA–cellulose fraction was subjected to velocity centrifugation under modified conditions (Piller *et al.*, 1997) which give better resolution than previous centrifugation conditions (see Introduction). All four activities co-sediment at ~20S (Figure 4D). This result provides strong confirmatory evidence that the activities are associated in a macromolecular complex. Furthermore, because this purified complex has the same sedimentation value as do its component editing activities in the unfractionated extract (data not shown; Pollard *et al.*, 1992; Cruz-Reyes and Sollner-Webb, 1996; Piller *et al.*, 1997), it is unlikely that any major components were lost from the complex during purification.

The major polypeptides of the DNA–cellulose fraction include RNA ligase and are all found in a stable complex

The fractions containing the purified enzymatic complex consist of eight major polypeptides (Figure 5A, lanes 6–8). These polypeptides have been seen in all our DNA–cellulose preparations (Figure 5A, lane 8; Figure 5B and data not shown) and they are highly enriched by the purification procedure, since they are not visible in crude extracts or in a ~20S glycerol gradient fraction and are barely visible in the Q-Sepharose fraction (Figure 5A, lanes 1–5). Notably, these eight polypeptides precisely co-elute both with one another (Figure 5B, upper) and with the enzymatic activities (lower), while the surrounding fractions are devoid of detectable polypeptides. For identification, these bands are numbered I–VII. [Bands IVa and IVb are variants of one another since they have identical tryptic peptide profiles (D.Reim, Wistar protein sequencing facility, personal communication); band III is often a single band (Figure 5A, lane 8), but at times comprises a somewhat variable cluster (lane 6).]

Interestingly, the silver-stained bands IVa, IVb and V exactly co-migrate with the three radiolabeled adenylylated polypeptides (Figure 5A, lower panel), and they also co-elute from DNA–cellulose with these adenylylated polypeptides (Figure 5B). Additionally, when the DNA–

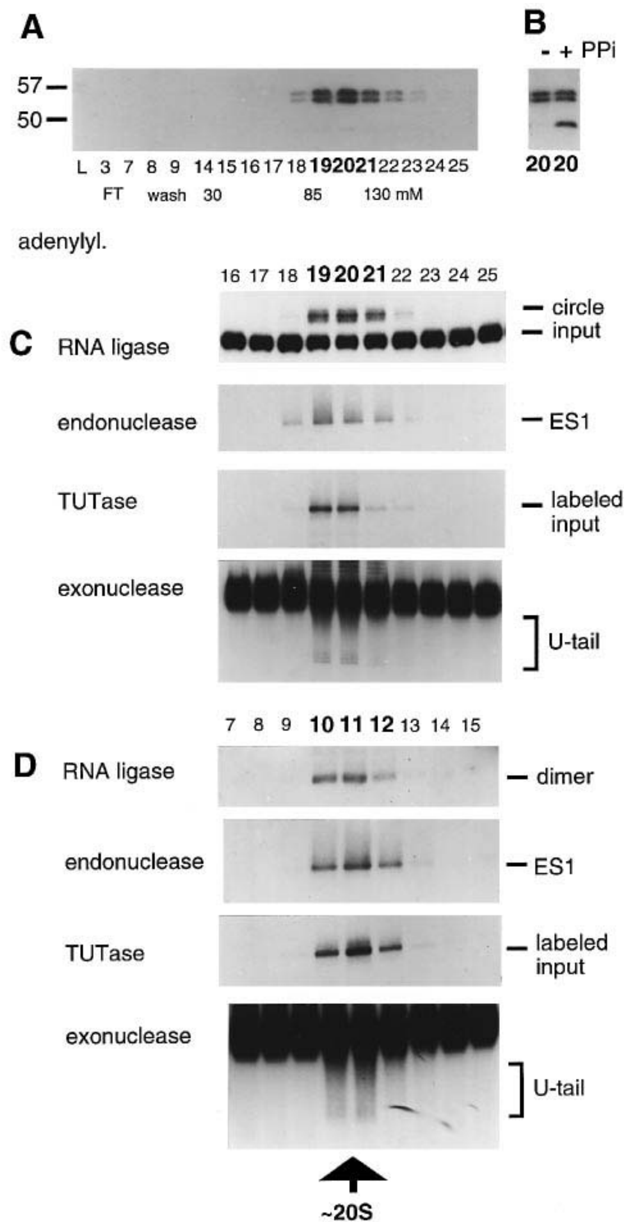

Fig. 4. RNA ligase, gRNA-directed endonuclease, TUTase, and 3′ U-specific exonuclease activities co-purify on DNA-cellulose and co-sediment on glycerol gradients. (**A**) Adenylylation reactions using 5 µl of the indicated DNA–cellulose fractions (preparation 1) or column load. The fractions with peak RNA ligase activity are indicated in bold. (**B**) Adenylylation assays of 1 µl of fraction 20 following deadenylylation with pyrophosphate (lane 2) or mock treatment (lane 1). As in Figure 1E, pyrophosphatase was added to both reactions following deadenylylation. (**C**) Each indicated fraction was used in: (line 1) RNA ligase assays containing 2 µl of fraction, ~0.01 pmol substrate RNA per reaction and supplemented to 10% glycerol; (line 2) gRNA-directed endonuclease assays containing 8 µl of fraction and gRNA, showing the 5′ product of the CYb pre-mRNA cleaved at ES1; (line 3) TUTase assays containing 2 µl of fraction and supplemented to 10% glycerol, showing the product of the input pre-mRNA labeled by addition of one U residue; or (line 4) exonuclease assays containing 10 µl fraction, showing the 3′ nucleotide removal stopping precisely at the end of the oligo(U) tail of the gRNA. (**D**) 8 µl of each glycerol gradient fraction (generated from DNA–cellulose preparation 3) was used in: (line 1) RNA ligase assays containing ~0.01 pmol substrate RNA; (line 2) gRNA-directed endonuclease assays; (line 3) TUTase assays; or (line 4) exonuclease assays. The 20S region was identified relative to thyroglobulin (19S) and catalase (11S) markers run in parallel.

cellulose fraction was separated by isoelectric focusing followed by SDS–PAGE, bands IVa, IVb and V continued to co-migrate with the radiolabeled polypeptides (Figure 5C). Furthermore, band V showed altered mobility when the sample was deadenylylated with pyrophosphate (Figure 5A, compare lanes 6 and 7), consistent with its being adenylylatable. On lower-percentage gels, bands IVa and IVb also shift slightly upon adenylylation/de-adenylylation (data not shown). Finally, a cDNA encoding band IVa has been cloned, and its expressed protein auto-adenylylates and catalyzes RNA ligation (L.N.Rusché *et al.*, manuscript in preparation). Thus, these three silver-stained polypeptides are the adenylylatable polypeptides, which in turn are RNA ligase molecules (Figure 1D). Therefore, the enzymatic complex is a major component of the DNA–cellulose purified fraction.

The fact that all eight polypeptides co-elute from DNA–cellulose, while the surrounding fractions have very little or no detectable protein (Figure 5B), suggests that these polypeptides are associated in a complex. To visualize this complex, we radiolabeled samples by adenylylation and subjected them to native gel electrophoresis. A single adenylylated complex was observed (Figure 6A), and a complex of this mobility was found in both the Q-Sepharose and DNA–cellulose fractions with peak enzymatic activities (Figure 6A; fractions 15, 16 and 19–21 respectively, compare with Figures 1B and 4C). This complex was then isolated from a native gel and analyzed by SDS–PAGE (Figure 6B, lane AC; data not shown). Comparison with the DNA–cellulose fraction (lane D) indicates that all eight major bands are present in the adenylylated complex from the native gel, a result that has been observed in three separate experiments. As a control, the region of the gel immediately below the adenylylated complex was similarly analyzed, and no proteins were detected (data not shown). Therefore, these eight polypeptides are physically associated and, since three of the bands represent RNA ligase, they evidently catalyze the enzymatic activities associated with RNA ligase. Thus, polypeptides I–VII comprise the enzymatic editing complex.

The purified complex catalyzes a full round of U-deletion activity

To examine whether the complex can catalyze a complete editing reaction, we assayed for *in vitro* U-deletion activity using *in vitro*-synthesized pre-edited A6 pre-mRNA and a cognate A6 gRNA which directs the deletion of four U residues at editing site one (Seiwert and Stuart, 1994). The U-deletion activity that can be seen in unfractionated mitochondrial extract is observed in the Q-Sepharose fractions (Figure 7), and elutes in fractions 15 and 16, along with the RNA ligase (data not shown). Like the peak Q-Sepharose fraction, the peak DNA cellulose fraction also catalyzes the U-deletion reaction (Figure 7). Thus, all the protein components necessary to carry out a complete round of U-deletional editing are contained in the highly purified DNA–cellulose fraction. While quantitation of U-deletion activity is less precise than of RNA ligase, due to non-linearity of signal with the amount of complex added, the U-deletion activity also appears to be extensively purified on the Q-Sepharose and DNA–cellulose columns. Indeed, the same amount of U-deletion (Figure 7)

is catalyzed using only ~1/100 as much protein from the DNA cellulose fraction as from the whole mitochondrial extract (see Figure 5A).

The enzymatic complex does not detectably dissociate during purification and does not contain gRNA, pre-mRNA or RNA helicase

Although none of the assayed enzymatic activities or the components essential for catalyzing the U-deletion reaction have been separated one from another during purification, it was possible that additional components could have been associated with the complex in the starting extract, but subsequently dissociated upon purification. However, despite several methods of analysis, we have obtained no evidence for such a hypothetical dissociation. First, the enzymatic activities sediment at ~20S whether from the starting extract or after their purification on Q-Sepharose and DNA–cellulose (Figure 4D). This result demonstrates that if anything were lost from the complex upon purification, it cannot have appreciably affected the sedimentation. Second, when the enzymatic complex is labeled by adenylylation and analyzed by native gel electrophoresis, it is seen to sediment identically, whether using the ~20S material of the crude mitochondrial extract, the Q-Sepharose-purified material, or the additionally DNA–cellulose-purified complex (Figures 6A and 8A, lanes 3–4; data not shown). Therefore, if anything were lost from the complex upon purification, it also cannot appreciably affect its electrophoretic migration. Finally, we have observed that the CYb pre-mRNA substrate binds selectively to this enzymatic complex (even in the absence of gRNA; L.N.Rusché, manuscript in preparation) and significantly alters its electrophoretic mobility, which can be scored using either adenylylation-labeled enzymatic complex (Figure 8A, lane 2) or labeled input pre-mRNA (Figure 8A, lanes 5 and 6). Notably, the complex with the bound mRNA fragment migrates identically whether formed from the ~20S material of the crude mitochondrial extract, the Q-Sepharose-purified complex (Figure 8A, lanes 5 and 6), or the additionally DNA–cellulose-purified complex (data not shown). Thus, if any components were lost during purification, they must be minor enough to also not affect the electrophoretic mobility of the mRNA-bound complex. Together, these three lines of data provide strong evidence that the enzymatic complex is stably associated and that components present in this complex in the crude mitochondrial extract are not dissociated during its chromatographic purification.

From previous studies of crude extract, it was inferred that endogenous gRNAs are part of a ~20S editing complex and that pre-mRNAs associate with this complex (Pollard *et al.*, 1992). To determine whether these RNAs are truly associated with the enzymatic editing complex, RNA extracted from our Q-Sepharose fractions was blotted to

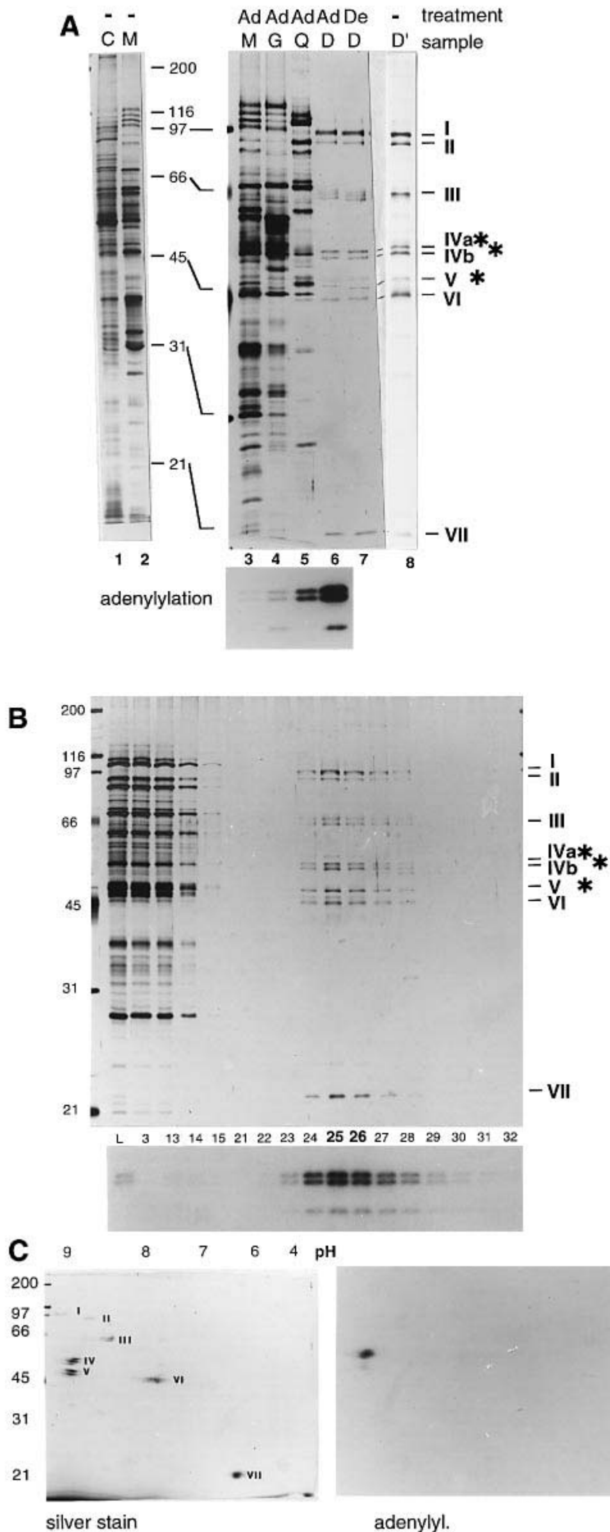

Fig. 5. The peak DNA–cellulose fraction contains eight major polypeptides, three of which are the adenylylatable polypeptides. (**A**) Protein silver stain (upper panel) or autoradiogram (lower panel) of 10^5 cell equivalents of whole-cell lysate (C), 10^6 cell equivalents (0.05 μl) mitochondrial extract (M), 5 μl ~20S standard glycerol gradient fraction (G), 5 μl Q-Sepharose fraction 15 (Q), 2.5 μl DNA–cellulose fraction (preparation 2, D), and 30 μl DNA–cellulose fraction (preparation 3, D'). Samples were adenylylated (Ad), deadenylylated (De) or not treated (–), as indicated, before electrophoresis. Major bands are identified by Roman numbers, and asterisks indicate the position of the adenylylated polypeptides. (Note that while the total protein concentration markedly decreases during purification, the ligase activity markedly increases; see Table I.) (**B**) Protein silver stain (upper) or autoradiogram of adenylylation reactions (lower, a different gel) containing 2 μl of the indicated DNA–cellulose fraction (preparation 4). (**C**) Silver stain (left) or autoradiogram (right) of an isoelectric focusing/SDS–PAGE 2D gel containing 30 μl adenylylation-labeled DNA–cellulose fraction (preparation 2). The pH is indicated. The silver-stained bands appear as doublets due to offset staining on the two gel faces, caused by a slightly diagonally running sample.

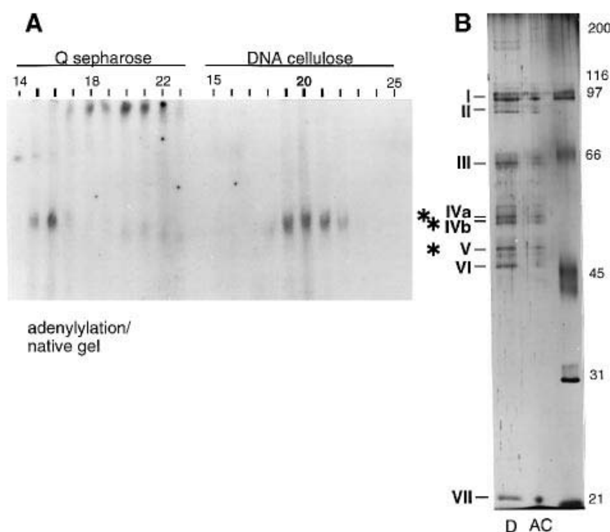

Fig. 6. The major polypeptides of the peak DNA–cellulose fraction are associated in an adenylylatable complex. (**A**) Adenylylation labeling and native gel electrophoresis of 3 μl of the indicated Q-Sepharose fractions (left) or 9 μl of the indicated DNA–cellulose fractions (preparation 1, right). Bold lines indicate the fractions with peak RNA ligase activity. The complexes from the Q-Sepharose and from the subsequently DNA–cellulose-fractionated material co-electrophoresed. (**B**) Protein silver stain of 6 μl DNA–cellulose preparation 4 (D) or of the adenylylated complex excised from a native gel (generated from 100 μl adenylylated DNA–cellulose fraction; preparation 3) (AC). The final lane shows protein molecular weight markers. An electrophoretic artifact on this gel caused the appearance of shadow bands, which do not represent additional polypeptides.

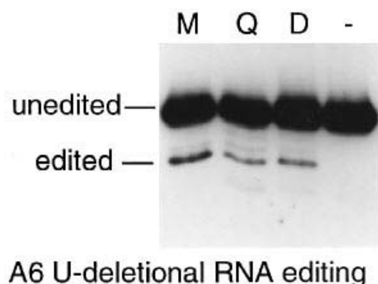

Fig. 7. The enzymatic editing complex catalyzes *in vitro* U-deletional editing. The gRNA-directed U-deletional editing of A6 pre-mRNA was assayed using 0.25 μl unfractionated mitochondrial extract (M), 2 μl Q-Sepharose fraction 15 (Q), 2 μl DNA–cellulose fraction (preparation 2, D), or no protein (–). The unedited input and edited product were distinguished by a terminated primer extension assay (Seiwert and Stuart, 1994).

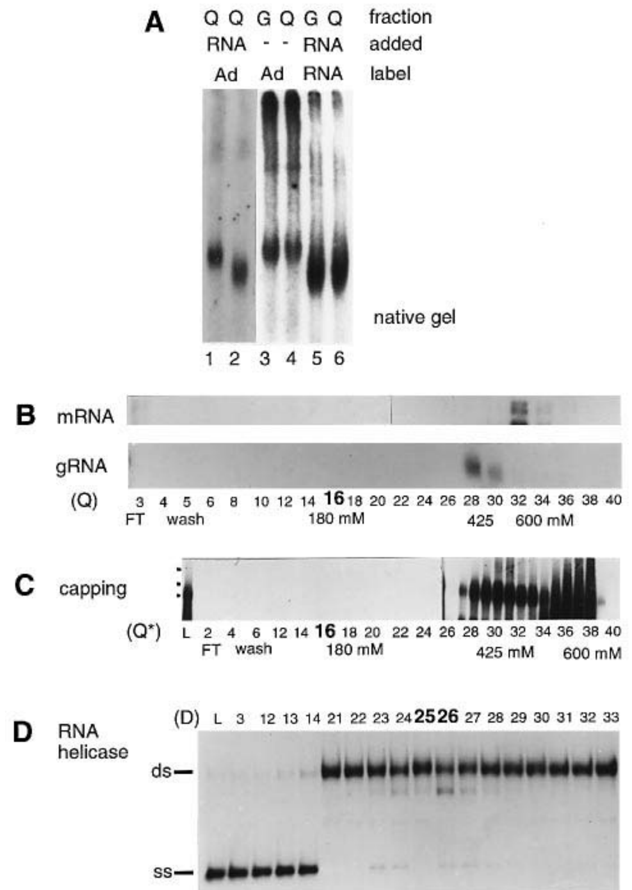

Fig. 8. The enzymatic editing complex does not dissociate and does not contain gRNA, mRNA, or RNA helicase activity. (**A**) Native gel electrophoresis of 3 μl (lanes 1–2) or 2 μl (lanes 4 and 6) of Q-Sepharose fraction 15 (Q) or 2 μl of 20S glycerol gradient fraction from whole extract (modified protocol, lanes 3 and 5, labeled G). The reactions were run without or with 10 fmol added preincubated CYb pre-mRNA and were labeled by using an adenylylated enzyme complex (Ad) or by using 5′ end-labeled CYb pre-mRNA (RNA). (Note that this RNA shift uses reaction conditions reminiscent of those in the TUTase assay; see Figure 3A). (**B**) RNAs extracted from 150 μl of the indicated Q-Sepharose fractions were electrophoresed, blotted, and probed for pre-edited CYb mRNA (upper) or CYb gRNA[558] and [560] simultaneously (lower) The enzymatic complex peaked in fraction 16, indicated in boldface. (**C**) RNAs extracted from 150 μl of the indicated Q-Sepharose fractions or column load [a different column from that of (A)] were capped with guanylyltransferase and [α-^{32}P]GTP and electrophoresed. The labeled products in fractions 27–34 are almost assuredly gRNAs for they coincide in size and elution with CYb gRNAs, while those in fractions 35–38 are primarily of smaller-sized material, evidently including degraded mRNAs (data not shown); a salt precipitate formed in fractions 39 and beyond may have interfered with their capping. (**D**) RNA helicase assays using 2 μl of each indicated DNA–cellulose fraction or column load (preparation 2). Partly double-stranded Y-branched input (ds) and single-stranded product (ss) are indicated. The band below the input in the lanes corresponding to the enzymatic complex evidently arises from cleavage of this Y-branched input RNA structure by the gRNA-dependent nuclease, whose structural requirements this substrate partially mimics (Piller *et al.*, 1997).

membranes and probed. For mRNAs, we assayed both unedited and edited CYb mRNA and saw that they elute at ~600 mM KCl, much beyond the enzymatic complex (Figure 8B and data not shown). These mRNAs therefore are not part of the enzymatic editing complex. Furthermore, because even short pre-mRNA fragments appreciably alter the electrophoretic mobility of the enzymatic complex (Figure 8A), the observation that the enzymatic complex has a fairly homogeneous electrophoretic mobility while cellular pre-mRNAs have very disperse sizes also makes it unlikely that the enzymatic complex of the extract is associated with cellular mRNA.

gRNAs were also assayed in the Q-Sepharose fractions. We probed for CYb gRNAs [558] and [560] (Riley *et al.*, 1994) and found that they elute from the column between 425 and 600 mM KCl (Figure 8B), some 300 mM beyond the enzymatic complex, but before the mRNA. Similar results (data not shown) were obtained by probing for COIII gRNA Tb1 (Pollard *et al.*, 1990). To detect total gRNA, we used a capping assay which labels the 5′ polyphosphate of gRNA molecules in a reaction with guanylyltransferase and [α-^{32}P]GTP (Blum end Simpson,

1990). This analysis produced a very intense signal in the same 425–600 mM KCl range (coincident with the CYb gRNA signal when assayed from the same column), and failed to detect any gRNAs eluting with the enzymatic complex (Figure 8C). The limits of our detection suggest that, at most, $<1\%$ of the gRNA and hence only a small fraction of the enzymatic complex could be in association (data not shown). Furthermore, the different elution profiles of the CYb mRNA and gRNA indicate that these components are also not stably associated with each other.

Although the enzymatic editing complex does not contain endogenous gRNAs, it could still contain the ~25 kDa binding protein that has been shown to bind gRNA specifically (Köller *et al.*, 1994). Indeed, it does contain one polypeptide, VII, of this approximate molecular weight. However, the gRNA-binding protein has a pI of 9–9.5 (H.U.Göringer, personal communication), while polypeptide VII has a pI of 6–7 (Figure 5C). Furthermore, partial peptide sequence from band VII (D.Reim and L.Rusché, unpublished data) is not contained in the sequence of the 25 kDa gRNA-binding protein (H.U.Göringer and L.Rusché, unpublished data). Thus, the 25 kDa gRNA-binding protein is not a part of the isolated enzymatic editing complex.

RNA helicase activity (Missel and Göringer, 1994) has also been reported to co-sediment with RNA ligase and TUTase (Corell *et al.*, 1996) and therefore may be part of the enzymatic complex. To assay for RNA helicase activity, a heterologous Y-branched RNA structure (Piller *et al.*, 1997) was incubated in fractions and then resolved on non-denaturing gels to separate the partly double-stranded input from the fully single-stranded product. The RNA helicase activity elutes on Q-Sepharose substantially displaced from RNA ligase activity, although their elution profiles overlap (data not shown). Furthermore, when the RNA ligase-containing Q-Sepharose fractions that contain the leading helicase activity are applied to DNA–cellulose, the helicase activity flows through the column, while the editing complex binds (Figure 8D). Thus, the RNA helicase is not associated with the other enzymatic editing activities.

Because purification of the enzymatic complex has not resulted in the detectable loss of components (Figure 8A and data above), we conclude that neither RNA helicase activity, nor mRNAs, gRNAs, or the 25 kDa gRNA-binding protein (Figure 8B–D) are associated with the enzymatic editing complex in the original extract.

Discussion

We report the chromatographic co-fractionation of the four activities shown to be required for RNA editing: gRNA-directed endonuclease, TUTase, 3′ U-specific exonuclease, and RNA ligase (Figures 1–4). The observation that on the first column (Q-Sepharose) the single endonuclease and the single exonuclease peaks and the main TUTase peak coincide precisely with the single RNA ligase peak indicates that these activities indeed co-purify (Figures 1–3). This indication is further demonstrated with the second column (DNA–cellulose), where the single peaks of these four enzymatic activities precisely co-elute (Figure 4A–C). Given that RNA ligase activity is enriched ~500-fold, that ~1/6000 of the starting mitochondrial protein is present after purification (Table I and Figure 6A), and that no protein is detectable in the flanking fractions from the DNA–cellulose column (Figure 5B), it seems virtually certain that these four activities co-purify not by chance but because they are physically associated. Further evidence for this association is that the four DNA–cellulose-purified activities co-sediment precisely at ~20S (Figure 4D) and that they function synergistically (L.N.Rusché *et al.*, manuscript in preparation). This work confirms previous suggestions, which were based only on single-round glycerol gradient centrifugation, that these enzymes may be associated in an enzymatic editing complex (Pollard *et al.*, 1992; Piller *et al.*, 1995b; Sabatini and Hajduk, 1995; Corell *et al.*, 1996; Cruz-Reyes and Sollner-Webb, 1996).

We also found that eight major polypeptides are present in the purified fractions (Figure 5A) and precisely co-elute with each other and with the enzymatic activities (Figure 5B). Furthermore, all of these eight polypeptides are associated in a complex (Figure 6). Three of these silver-stained polypeptides (IVa, IVb and V) represent RNA ligases since they co-migrate with the adenylylated polypeptides (Figure 5), display altered mobility upon deadenylylation (Figure 5A), and discharge specifically with ligatable RNA but not with non-ligatable RNA or with ligatable DNA (Figure 1D). [Polypeptides IVa and IVb are variants of one another (see Results).] Therefore, these eight polypeptides evidently catalyze the endonuclease, TUTase, and exonuclease activities as well as the associated RNA ligase. We have thus determined the protein composition of the enzymatic editing complex. With the exception of the adenylylated polypeptides, none of these polypeptides has been previously identified as an editing factor, and these results will now enable their cloning and characterization.

We further demonstrated that the enzymatic editing complex consisting of eight polypeptides catalyzes a complete U-deletional editing reaction *in vitro* when provided with pre-mRNA and gRNA (Figure 7C). It seems probable that the complex can also catalyze U-insertional editing since it can cleave at U-insertion sites (Figures 2A and 4C) and has TUTase activity (Figures 3A and 4C). The finding that the editing complex contains gRNA-directed endonuclease, U-specific exonuclease, TUTase and RNA ligase provides further support for the enzymatic mechanism of editing (Blum *et al.*, 1990; Cruz-Reyes and Sollner-Webb, 1996; Kable *et al.*, 1996; Seiwert *et al.*, 1996), since all its predicted enzymatic activities are present in the purified complex, while the two other major models of editing do not involve all four activities. The transesterification mechanism (Blum *et al.*, 1991; Cech, 1991) does not involve an endonuclease, exonuclease, or RNA ligase; the enzymatic chimera-based mechanism (Sollner-Webb, 1991) does not involve a 3′ U-specific exonuclease; and neither mechanism requires TUTase at each round.

We have also found that during purification, the enzymatic editing complex retains its sedimentation coefficient and its electrophoretic mobility, both alone and when bound to substrate pre-mRNA (Figures 4D and 8A) and therefore that it has not lost any detectable component. Interestingly, the combined molecular weights of bands I–VII is ~600 kDa (assuming a 1:1 stoichiometry for all

components except band I which appears more abundant by both silver staining and Coomassie staining and was counted twice). A globular ~20S protein complex is predicted to be 600–700 kDa, and thus, the observed proteins can account for the mass of the complex. Therefore, this enzymatic complex exists as a discrete module and evidently does not associate with other editing factors in our mitochondrial extracts.

In contrast to the four enzymatic activities and eight polypeptides found to constitute the enzymatic complex, two other enzymatic activities and two kinds of RNA previously thought to be part of the enzymatic complex based on their co-sedimentation with ligase are not part of the complex. First, a single-strand-specific, gRNA-independent nuclease that was proposed to be involved in editing (Harris *et al.*, 1992; Simpson *et al.*, 1992; Piller *et al.*, 1995a) fractionates away from the enzymatic complex on Q-Sepharose columns (Figure 2A) as well as under modified glycerol gradient sedimentation conditions (Piller *et al.*, 1997), indicating that it is neither part of the editing complex nor required to catalyze an editing cycle *in vitro*. Second, RNA helicase activity, which was reported to co-sediment with RNA ligase (Corell *et al.*, 1996), also fractionates away from the enzymatic complex on Q-Sepharose and DNA–cellulose chromatography (Figure 8D). This helicase is proposed to unwind fully complementary gRNA–mRNA duplexes, allowing the next gRNA to anchor and continue editing (Missel and Göringer, 1994). If this is indeed its function, helicase should not be required for editing a single site *in vitro*, and *in vivo* it could transiently join the editosome at a late stage. One simple model would be that the helicase arrives with the gRNA, but from our data this scenario appears unlikely since helicase and gRNAs fractionate differently on Q-Sepharose (Figure 8B and D; also data not shown).

Endogenous gRNAs and mRNAs are not part of the ~20S enzymatic complex either (Figure 8B and C; also data not shown). These findings may seem to contradict published conclusions (Pollard *et al.*, 1992), but re-examination of the original glycerol gradient data reveals that the sedimentation profiles of the RNAs and enzymes are substantially different—both the gRNA and mRNA profiles were very broad with virtually no perceptible peak at ~20S, while the ligase and TUTase both peaked at 20S. Therefore, the RNAs were probably not associated in these earlier studies but instead happened to have overlapping sedimentation profiles, much like the gRNA-independent nuclease and RNA helicase do with RNA ligase. The finding of others that certain of the enzymatic activities can also sediment at 35–40S (Pollard *et al.*, 1992; Corell *et al.*, 1996) could be due to their associating with endogenous mRNAs in those extracts, but we have observed neither a 35–40S complex (data not shown) nor an association of endogenous mRNAs with the enzymatic complex (Figure 8B). Further strengthening our observation that gRNAs are not part of the ~20S enzymatic complex, we have found that the ~25 kDa gRNA-specific binding protein is also not part of the enzymatic editing complex (see Results; also H.U.Göringer, D.Reim and L.Rusché, unpublished data). This result suggests that the enzymatic complex does not stably associate with gRNAs in the absence of pre-mRNAs, and it also demonstrates that the ~25 kDa gRNA-binding protein is not essential for the catalysis of an editing cycle *in vitro*. Instead, this protein may stabilize the gRNA and/or enhance its ability to associate with the pre-mRNA and editing complex. The finding that the ~20S enzymatic editing complex does not contain gRNAs or the ~25 kDa gRNA-specific binding protein also indicates that this complex is unlikely to play a role in gRNA maturation as had been originally proposed (Pollard *et al.*, 1992). Rather, the ~20S complex appears to be a core of enzymatic editing activities that catalyzes editing and editing-like reactions (Peris *et al.*, 1994; Corell *et al.*, 1996; Cruz-Reyes and Sollner-Webb, 1996).

Thus, of the six enzymatic activities and two kinds of RNA reported to co-sediment on glycerol gradients and thereby inferred to be part of a single complex, four are now shown by further purification not to associate. It therefore was not a forgone conclusion that the other four activities would co-purify, as we have shown they do.

Our results also provide insights into the assembly of the complete editing complex. The currently best accepted model of editosome formation (Pollard *et al.*, 1992; Göringer *et al.*, 1995; Correll *et al.*, 1996) suggests that an enzymatic complex is pre-assembled with gRNA and only then associates with pre-mRNA. However, our data instead suggest that the enzymatic editing complex is not prebound to a particular gRNA and therefore that it can act on any pre-mRNA. This would make sense for the trypanosome, which has hundreds of different gRNAs, for if gRNAs were stably pre-associated with editing complexes, only a small fraction of these complexes would be capable of editing any one pre-mRNA region. Furthermore, we have observed that the enzymatic editing complex binds CYb pre-mRNA at or near the editing domain in the absence of gRNA and that it has a higher affinity for pre-mRNA than for gRNA (L.N.Rusché, manuscript in preparation), suggesting that the enzymatic complex may associate with the pre-mRNA before binding the gRNA. This order of interaction could be favorable for the trypanosome, serving to increase the efficiency of editing in two additional ways. First, if the enzymatic complex can initially bind specifically to the structure of the editing domain (Piller *et al.*, 1995a), it could help to reduce the pre-mRNA sequence that gRNAs must search. Second, once the guiding capacity of a gRNA is fulfilled, the enzymatic editing complex could remain associated with partially edited mRNA during a gRNA exchange, enhancing the overall processivity of the editing reaction. In contrast, in the model in which the enzymes and gRNAs are part of a single, ~20S complex, the entire enzymatic machinery would have to dissociate from the pre-mRNA at each gRNA transition.

The above considerations suggest a modular assembly model for the formation of an active editosome. We propose that at least three separate components—the ~20S enzymatic editing complex, the unedited mRNA, and its cognate gRNA—come together to generate the editosome, that the enzymatic editing complex binds the pre-mRNA before its binding the gRNA, and that the pre-mRNA then remains bound through the sequential use of several gRNAs. Our data do not provide support for the alternate possible orders of association of these three components, neither that the complex first binds the gRNA (see above) nor that the mRNA first associates stably with the gRNA before binding the complex (Figure 8B and C; also data

not shown). Although these three components are the only requirements for one cycle of editing *in vitro*, additional factors may join the editosome *in vivo*. For example, RNAs edited *in vitro* are usually processed at only the 3′-most site (Seiwert *et al.*, 1996), suggesting that stimulatory factors may increase the processivity of the basal enzymatic editing complex. Additionally, other components such as RNA helicase, which is not found associated with gRNAs or the ~20S enzymatic complex, may associate at a later stage of editing. The nature of the *in vivo* functional editosome also remains to be elucidated.

In conclusion, we have obtained a highly purified complex from trypanosome mitochondria which catalyzes a full round of U-deletional editing when provided with pre-mRNA and gRNA. This complex possesses the four activities—gRNA-directed endonuclease, TUTase, 3′ U-specific exonuclease, and RNA ligase—predicted by the enzymatic model of RNA editing and is comprised of eight major polypeptides. We have therefore identified the individual polypeptides representing important enzymatic editing factors and elucidate the nature of a previously proposed ~20S editing complex.

Materials and methods

Cells, extract preparation and extract fractionation

Trypanosomes (strain TREU 667) were grown and mitochondria were isolated as described (Decker and Sollner-Webb, 1990). The mitochondrial vesicles were suspended at a density of 2×10^{10} cell equivalents per ml in buffer P (25 mM Tris–HCl, pH 8.4, 10 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 10% glycerol) supplemented with 100 mM KCl, 1 mg/ml pefabloc, 50 μg/ml antipain, 10 μg/ml E-64 (Sigma). They were lysed on ice with 0.5% Triton X-100 and cleared by centrifugation.

Q-Sepharose chromatography columns were first equilibrated at 4°C with buffer P containing 100 mM KCl. Extract (above) was loaded directly on the column at 1–5 mg protein/ml column volume and a flow rate adjusted to the column size to be 0.25 cm/min. Material was eluted with an 8-column volume linear 100–350 mM KCl gradient in buffer P (sometimes followed by an 8-column volume linear 350–1000 mM KCl gradient) and collected in 0.5-column volume fractions. The final KCl concentration of each fraction was determined by measuring conductivity.

DNA–cellulose chromatography columns were first equilibrated at 4°C with buffer P adjusted to pH 8.0 and containing 30 mM KCl. Q-Sepharose fractions were either diluted or dialyzed to 30 mM KCl before loading at 0.1–1 mg protein/ml column volume. Material was eluted with an 8-column volume linear 30–350 mM KCl gradient in buffer P adjusted to pH 8.0. Flow rates and fraction sizes were as for Q-Sepharose chromatography.

Standard glycerol gradients (used to isolate material for Figure 4A, lane 4) were performed as described (Pollard *et al.*, 1992). Modified gradients (Piller *et al.*, 1997) were generated using 125 μl unfractionated extract or 250 μl of DNA–cellulose preparation 3 (Figure 4A, lane 8), both brought to 100 mM KCl in 750 μl and applied to an 11.25 ml 10% to 30% glycerol gradient in buffer P with 100 mM KCl. Gradients were spun for 6 h at 38 000 r.p.m. in an SW41 rotor, and 15 fractions collected from the tube bottom.

Synthetic RNAs and RNA markers

CYb pre-edited mRNA (Decker and Sollner-Webb, 1990), CYb model gRNA (Piller *et al.*, 1995b), CYb gRNA[558] (Riley *et al.*, 1994; Piller *et al.*, 1996), pLL ligase substrate (Rusché *et al.*, 1995), and A6 (+1) pre-mRNA and A6ΔG gRNA (Seiwert and Stuart, 1994; Cruz-Reyes and Sollner-Webb, 1996) were transcribed as previously described. For the RNA helicase substrate, RNAs were transcribed from *Kpn*I-cut Bluescript II KS (Stratagene) with T7 RNA polymerase and from *Bsr*BI-cut 3′ ETS [M.Mukherjee, unpublished data; contains yeast rDNA (from –149 to +413 relative to the 3′ end of 25S) cloned into the *Eco*RV site of Bluescript II KS] with T3 RNA polymerase. All RNAs were transcribed, gel isolated and quantified as described (Piller *et al.*, 1995a). For 5′ end-labeling, dephosphorylated RNAs were labeled with T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP and gel isolated.

Sequencing markers were generated by incubating the 5′ end-labeled RNA plus 875 ng tRNA for 10 min at 50°C in 12 μl reactions containing: (i) 5 units nuclease P1 (in 6 M urea, 19 mM sodium citrate, pH 8.3, 0.32 mM $ZnCl_2$; J.Cruz-Reyes *et al.*, manuscript in preparation) for a G-ladder with 3′ OH termini; (ii) 5 units RNase T1 (in 6 M urea, 21 mM sodium citrate, pH 3.5, 1.5 mM EDTA) for a G-ladder with 3′ P termini; or (iii) 25 mM sodium phosphate, pH 12 for a nucleotide ladder with 3′ P termini. For approximate sizing, all denaturing RNA gels used end-labeled, *Hpa*II-cut pBR322 markers.

Enzyme assays

Assays, unless noted below, contained the indicated amount of fraction brought to the final volume with MRB (Piller *et al.*, 1995a; 25 mM Tris–HCl, pH 8.0, 60 mM KCl, 10 mM $Mg(OAc)_2$, 1 mM EDTA, 0.5 mM DTT, 5% glycerol) supplemented to 5 mM DTT. Concentrations of KCl ranged from 45 to 95 mM, glycerol from 5 to 0.5%, and Mg^{2+} was always 10 mM.

Adenylylation assays were performed in 10 μl with 1 μCi [α-^{32}P]ATP (3000 Ci/mmol). After a 5 min incubation on ice, reactions were stopped with 5 μl 3× loading buffer (30% glycerol, 15% β-mercaptoethanol, 0.2 M Tris–HCl, pH 6.8, 6% SDS, 0.3% bromophenol blue), heated for 3 min at 95°C, and run on 10% polyacrylamide (29:1 acrylamide:bis) SDS–PAGE gels in Tris–glycine–SDS buffer. For deadenylylation with pyrophosphate, samples were prepared as above but with 8 mM pyrophosphate added; after 2 min on ice, 1 unit pyrophosphatase (Sigma, I-1891) was added in 1 μl and the reaction was incubated another 5 min on ice. For deadenylylation by nucleic acid substrates, 0.25 μl unfractionated extract was adenylylated as above and then incubated 15 min at 28°C with 1.5 pmol pLL RNA (see above) containing either 5′ OH (phosphatase treated) or 5′ P (subsequently phosphorylated with polynucleotide kinase and excess ATP), or with 1.5 pmol *Hin*dIII-cut pUC8.

RNA ligase, endonuclease, TUTase and 3′ exonuclease assays were all incubated 30 min at 22°C in 20 μl containing 2–4 units RNasin and the following. Ligase assays: 1 mM ATP and the indicated amount of 5′ end-labeled pLL RNA. Endonuclease assays: ~10 fmol 5′ end-labeled CYb pre-mRNA and, when indicated, 900 fmol CYb model gRNA. TUTase assays: 100–250 fmol unlabeled CYb pre-mRNA and 5 μCi [α-^{32}P]UTP (3000 Ci/mmol). Exonuclease assays: ~30 fmol of 5′ end-labeled CYb gRNA[558], ~500 fmol of internally labeled CYb gRNA[558] or ~50 fmol of pCp 3′ end-labeled, phosphatase-treated CYb gRNA[558]. Separate control experiments examining gel mobility and using RNA ligase demonstrated the success of such phosphatase treatment. All reactions were stopped by addition of 10 μg tRNA and NaOAc to 0.3 M and phenol–chloroform extraction. Samples were precipitated before electrophoresis on 8% or 24% polyacrylamide (19:1 acrylamide:bis)–8.5 M urea gels (exonuclease assays) or 6% polyacrylamide (19:1)–8.5 M urea gels (other assays) in 1× TBE (100 mM Tris–HCl, 80 mM boric acid, 1 mM EDTA, pH 8.3).

U-deletion assays were performed as described using A6 (+1) pre-mRNA and A6ΔG gRNA and were analyzed using reverse transcriptase and the A6 RT primer (Seiwert and Stuart, 1994; Cruz-Reyes and Sollner-Webb, 1996). Products were run on 20% polyacrylamide (19:1)–8.5 M urea gels.

RNA helicase substrate formation and assays were modified from Missel and Göringer (1994). 1 pmol 5′ labeled Bluescript RNA and 10 pmol unlabeled 3′ ETS/Bluescript RNA in 20 μl containing 200 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA were heated to 80°C for 10 min and then cooled to 30°C at 1°C/min. The annealed Y-branched product was isolated from a 5% polyacrylamide (19:1) non-denaturing gel. Approximately 10 fmol of isolated substrate was incubated in 20 μl containing 5 mM Tris–HCl, pH 7.5, 30 mM KCl, 1 mM $MgCl_2$, 2 mM DTT, 2 mM ATP, 250 μg/ml tRNA, 50 μg/ml BSA and the indicated amount of fraction. After 1 h at 26°C, reactions were stopped by the addition of SDS to 0.4%, EDTA to 4 mM and glycerol to 5.6%, and were directly loaded on 5% polyacrylamide non-denaturing gels run in 0.5× TBE.

Electrophoretic methods

For 2D (isoelectric focusing-SDS–PAGE) gels, 30 μl DNA–cellulose fraction (preparation 2; Figure 4A, lane 6) was supplemented with 3 μCi [α-^{32}P]ATP and allowed to adenylylate on ice. The material was then precipitated with 8 vols acetone, suspended in 30 μl 9.8 M urea, 4% NP-40, 2% β-mercaptoethanol and 2% 3-10 Bio-Lyte ampholytes (Bio-Rad), applied to an 8-cm long, 3-mm diameter isoelectric focusing tube

gel (4% polyacrylamide, 9 M urea, 2% NP-40, 5% 3-10 ampholytes), and focused for 18 h at 400 V. The tube gel was then soaked in SDS equilibration buffer (60 mM Tris–HCl, pH 6.8, 2% SDS, 0.1 M DTT, 10% glycerol) for 5 min and laid across the top of a 1–1.5 mm thick 10% polyacrylamide SDS–PAGE gel.

To visualize the complex on native gels, both for adenylylation and RNA binding reactions, the indicated amount of fraction was incubated on ice for 5 min in 15 µl of buffer P. Adenylylation reactions contained 1 µCi [α-^{32}P]ATP, and RNA gel shift reactions contained 10 fmol 5′ labeled CYb pre-mRNA. 1 mm thick, 4% polyacrylamide (69:1 acrylamide:bis), 10% glycerol, 0.5× TBE and 0.1% Tween-20 gels were run for ~14 h at 5.5 V/cm.

To isolate the adenylylated complex for analysis on SDS–PAGE, 100 µl DNA–cellulose fraction (preparation 3; Figure 4A, lane 8) was adenylylated with 10 µCi [α-^{32}P]ATP and run on a native gel. The radiolabeled band was excised from the gel, soaked 25 min in SDS equilibration buffer (above), and placed in the well of a 10% SDS–PAGE gel.

RNA extraction, Northern blotting and capping

RNA was extracted from Q-Sepharose fractions with phenol–chloroform and precipitated with 0.3 M NaOAc, 44 µg/ml glycogen, and ethanol. For gRNA analysis, samples were electrophoresed on 6% polyacrylamide (19:1)–8.5 M urea gels and electroblotted to Zeta-probe membranes (Bio-Rad) in 0.5× TBE. For mRNA analysis, samples were run on 1% agarose–formaldehyde gels and transferred to Zeta-probe membranes by capillary action in 50 mM NaOH. Blots were hybridized in 160 µl/cm^2 with 10^6 c.p.m. probe/ml in 0.25 M sodium phosphate, 7% SDS at 42°C for 16 h and washed once with 2× SSC/0.1% SDS and twice with 0.5× SSC/0.1% SDS for 5 min each. DNA probes were 5′ labeled oligos (1–5×10^6 c.p.m./pmol) g[558] (5′-CCCTTTATCACCTAAAAATTCAC-ATTGTCT-3′) and g[560] (5′-CCTCCCCATTACTCAGAAATCTAC-ATTGTC-3′) for CYb gRNAs, CYb-pre (5′-CCCTTTCTTTTTTCT-CCGCTT-3′) for unedited mRNA, and CYb-ed (5′-AAAATAATA-AAAATCTACAACGAAACATAT-3′) for edited mRNA.

Capping reactions were performed according to the enzyme manufacturer's suggestions with RNA extracted from 150 µl Q-Sepharose fraction in 10 µl containing 50 mM Tris–HCl, 8.0, 1.2 mM $MgCl_2$, 6 mM KCl, 2.5 mM DTT, 6 units RNasin, 10 µCi [α-^{32}P]GTP (3000 Ci/mmol) and 1.3 units guanylyltransferase (BRL). They were incubated for 30 min at 37°C and analyzed as for ligase assays.

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