Different *Trypanosoma brucei* guide RNA molecules associate with an identical complement of mitochondrial proteins *in vitro*

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ABSTRACT

kRNA editing is a mitochondrial transcript maturation process which evolved in kinetoplastid protozoa. It entails the insertion and deletion of exclusively uridine nucleotides directed by gRNAs into pre-mRNAs. Other participating components are not currently known. The aim of this study was to identify mitochondrial proteins that are in direct physical contact with gRNAs thereby possibly involved in the editing reaction. At low monovalent cation concentration (30 mM KCl) 8 polypeptides with apparent molecular weights ranging from 124 to 9 kDa specifically cross-linked to gRNAs. Three of the proteins, 90, 21, and 9 kDa in size, were able to bind at higher salt concentrations (≥ 100 mM) indicating an enhanced affinity to the gRNA molecules. No cross-links were identified at ≥ 250 mM KCl. Four gRNAs, specific for different editing domains of the ATPase 6 and ND7 pre-mRNAs, were in contact with the same set of mitochondrial polypeptides suggesting the assembly of an identical RNP complex that does not include pre-mRNA molecules. The binding of the 90 kDa protein was sensitive to the presence of U-nucleotides at the 3'-end of the gRNAs and could specifically be blocked by modifying free sulfhydryl groups. The interaction with the 124 kDa polypeptide was inhibited by vanadyl ribonucleosides, implicating a role for 2', 3' hydroxyl groups in the gRNA – protein interaction.

INTRODUCTION

Kinetoplastid RNA (kRNA) editing is a messenger RNA (mRNA) processing event during which uridine nucleotides are inserted into and deleted from cryptic, non-translatable mRNA molecules. This series of biochemical reactions is required for the expression of several mitochondrially encoded genes in protozoan parasites such as *Trypanosoma, Leishmania* and *Crithidia*. The information for the insertion and deletion of the uridines resides in the primary sequence of other mitochondrial RNA transcripts, so called guide RNAs (gRNA). The molecules have a length of 50–70 nucleotides, contain posttranscriptionally added 3' oligo(U) extensions and function as templates in the editing reaction by base complementarity to editing domains of the mRNAs (reviewed in 1, 2).

The chemical mechanism of the editing reaction as well as the complete mitochondrial editing machinery are not currently known. Three lines of evidence suggest that mitochondrial components in addition to gRNAs and pre-edited mRNAs are involved. Firstly, gRNAs were identified as components of 19S and 35–40S mitochondrial complexes (3). Secondly, gRNAs form specific high molecular weight ribonucleoprotein (RNP) complexes when incubated with mitochondrial lysates (4, 5) and thirdly, mitochondrial protein extracts were found to be required for the formation of chimeric gRNA/pre-edited mRNA molecules presumed intermediates of the editing reaction (6–8).

Here we report the identification of eight proteins in mitochondrial extracts that directly interact with gRNA molecules. We show that different gRNAs, though specific for different editing domains of two pre-edited mRNAs bind an identical set of these proteins. The protein association is sensitive to increased monovalent cation concentrations and only three polypeptides are able to bind at 100 mM KCl. We further demonstrate that the presence of U-residues at the gRNAs 3'-end modulates the interaction to a 90 kDa polypeptide. Other nucleic acid components are not required for the protein association and chemical inhibition experiments identify sulfhydryl groups and possibly nucleoside 2', 3' cyclic phosphates as groups essential for the binding of two of the proteins to gRNAs.

MATERIALS AND METHODS

Biochemicals

All chemicals were reagent grade or better. Enzymes used for RNA, DNA and protein manipulation were obtained from either Boehringer Mannheim, Pharmacia or Stratagene and were used

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in accordance with the vendors recommendations. Radioactive compounds were purchased from Amersham Buchler. Synthetic oligodesoxynucleotides were synthesized automatically using β-cyanoethyl phosphoramidite chemistry.

Cell growth and mitochondria isolation

The procyclic developmental stage of *Trypanosoma brucei brucei* [IsTaR1.7 (9) and TREU 667 (10)] was grown at 27°C in SDM-79 medium supplemented with 10% (v/v) fetal bovine calf serum (11). Cells were grown to a cell density of approximately 10^7 cells/ml and were mechanically disrupted in a hypotonic buffer (1 mM Tris−HCl, pH 8, 1 mM Na$_2$EDTA). Mitochondrial vesicles were isolated from the cell lysate essentially as described (12). Vesicle preparations were stored at −80°C in 20 mM Tris−HCl, pH 8, 2 mM Na$_2$EDTA, 250 mM sucrose and 50% (v/v) glycerol. The preparations were checked for kDNA by phase contrast and fluorescence microscopy in the presence of ethidium bromide.

Construction of synthetic *T.brucei* gRNAs and RNA synthesis

Synthetic genes for guide RNAs gA6-14, gA6-48, gND7-506 and gND7-385 (for nomenclature see 13) encoding 3' oligo(T) extensions were constructed by self-assembly of overlapping synthetic oligodesoxynucleotides according to Reyes and Abelson, 1989 (14) and cloned into plasmid pBS- (Stratagene). Positive clones were identified by restriction enzyme digestion of isolated plasmid DNA and sequenced on an automated DNA sequencer using the dye terminator technology (15). Poly-uridylated ^{32}P-labeled gRNA molecules were synthesized by run off transcription from linearized plasmid DNA templates using T7 polymerase and [α-^{32}P]UTP or [α-^{32}P]ATP following standard procedures. Transcripts were purified by denaturing gel electrophoresis on 6% (w/v) polyacrylamide gels (acrylamide/methylene bisacrylamide 19/1) in 89 mM Tris−borate, pH 7.5, 2 mM EDTA. Guide RNAs lacking their U-tail were transcribed in two ways: (i) by abortive transcription from plasmid constructs as above but at limiting UTP concentrations (1 μM) or (ii) from plasmid constructs that did not encode an oligo(T) sequence. The complete absence of the 3' oligo(U) extension was confirmed in a RNase T1 fingerprint analysis. RNA secondary structures were calculated using the MFOLD subroutine of the GCG software package based on a free energy minimization algorithm (16, 17).

Preparation of mitochondrial and nuclear extracts

Mitochondrial vesicles were concentrated by centrifugation and resuspended in 0.1 ml 6 mM Hepes, pH 7.5, 30 mM KCl, 0.5 mM DTT. The lysis of the vesicles was performed at 4°C with 0.2% (v/v) Nonidet P-40 in the presence of 1 μg/ml leupeptin, 1% (w/v) phenylmethylsulfonyl fluoride (PMSF) and 10 μg/ml bovine trypsin inhibitor for 5 min. Extracts were cleared from insoluble material by centrifugation at 16000×g for 5 min at 4°C. Protein concentrations were determined in a dye-binding assay using bovine plasma gamma globulin as a standard (18). *T.brucei* nuclear extracts were prepared according to published procedures (19).

UV cross-linking and label transfer experiments

Transfer of radioactive label from substrate gRNAs to RNA-binding proteins was carried out as in (20) with modifications. Uniformly labeled gRNAs (15−100 nM) were incubated with inceasing amounts of mitochondrial lysate (0.1−25 μg total

protein) in a final volume of 15 ml binding buffer (6 mM Hepes, pH 7.5, 30 mM KCl, 2.1 mM MgCl$_2$, 0.5 mM DTT, 1 mM ATP, 0.1 mM Na$_2$EDTA, 10 μg/ml yeast tRNA, 6% glycerol). Incubation was at 27°C for 30 min. In competition experiments, competitor RNA was included prior to the addition of the mitochondrial extract. Following incubation the entire sample volume was placed onto a plastic membrane floating on ice water and irradiated at 254 nm with an energy dose of 0.12 J/cm^2 for 5 min up to 60 min. After incubation with 1.5 μg RNase A at 30°C for 30 min an equal volume of SDS gel-loading buffer was added, samples were heated to 95°C for 3 min and subjected to electrophoresis in discontinuous polyacrylamide gels (6% (w/v) spacer gel, 10% (w/v) separation gel) containing 0.1% (w/v) SDS (21). The gels were stained with Coomassie Brilliant Blue and dried. ^{32}P-Labeled proteins were visualized by autoradiography and non-saturated autoradiograms were scanned for quantitation (Howtek Scanmaster 3, pdi software version 2.2).

Extract inhibition studies

Mitochondrial extracts (30−50 μg mitochondrial protein) were treated with either 10 mM *N*-ethylmaleimide (NEM), 1 mM diethyl pyrocarbonate (DEPC) or 5 mM phenylglyoxal (PG) in 6 mM Hepes, pH 7.5, 30 mM KCl, 0.5 mM DTT for 10 min at 30°C. Excess inhibitor was inactivated by the addition of cysteine, histidine or arginine solutions (pH 8) respectively at final concentrations of 50 mM and further incubation for 10 min at 30°C. Similarly, inhibition with vanadyl ribonucleoside complexes (VRC) was performed at a final concentration of 10 mM (2.5 mM of each ribonucleoside, 10 mM VO^{2+}) in the same buffer for 10 min at 30°C. Protein digestion of mitochondrial extracts was performed with 0.2 mg/ml proteinase K for 60 min at RT. Nucleic acids were hydrolyzed with micrococcal nuclease at a final concentration of 127 U/ml in the presence of 1.5 mM CaCl$_2$ for 20 min at RT. The reaction was stopped by adding EGTA (4 mM). Poly(U) preparations used as non-homologous competitors were size fractionated on a Sephadex G50 superfine (Pharmacia) column in 50 mM Tris−HCl, pH 7.5, 150 mM KCl, 1 mM Na$_2$EDTA.

RESULTS

An identical set of mitochondrial proteins cross-link to different gRNAs

To identify mitochondrial proteins that form stable, direct contacts with gRNA molecules we used an ultraviolet (UV) light-induced cross-linking technique (20, 22). ^{32}P-Labeled gRNA molecules were incubated with a low salt, non-ionic detergent extract of *T.brucei* mitochondria and subsequently irradiated at 254 nm. The reaction was performed in the presence of a defined concentration of tRNA molecules to account for non-specific RNA-protein interactions. The four gRNA substrates used in this study (Fig. 1) are specific for different editing domains of the *T.brucei* ATPase 6 (A6) and NADH 7 (ND7) mRNAs (13), and were labeled with [α-^{32}P]UTP or [α-^{32}P]ATP by *in vitro* transcription. The molecules contain 10 vector-derived extra bases at their 5' ends and have 3' oligo(U) tails of 3−9 nucleotides thus mimicking the 3' end size variation found *in vivo* (23). The cross-linked samples were extensively digested with ribonuclease A, and the proteins separated on a discontinuous polyacrylamide gel containing SDS. Proteins cross-linked to the gRNAs were detected by the radioactive oligonucleotides which remained covalently attached after the ribonuclease treatment while the non-
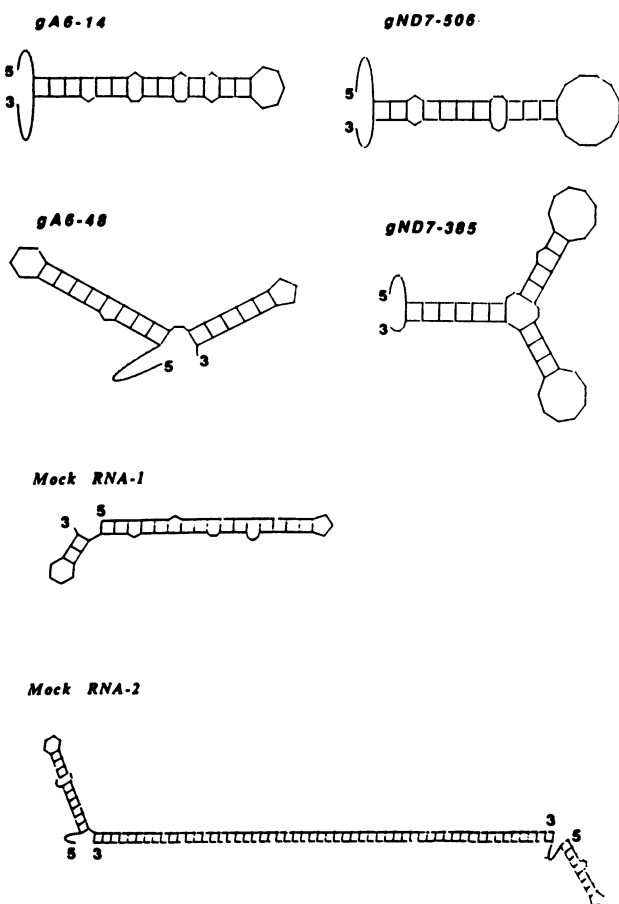
Figure 2. Analysis of UV-cross-linked proteins. ^{32}P-Labeled gA6-14 RNA was incubated with increasing amounts (0.1−1.3 mg/ml, left to right) of *T.brucei* mitochondrial extract (mt.-lysate) and irradiated with UV-light. After complete digestion with RNaseA samples were analyzed by SDS−PAGE followed by autoradiography. Cross-linked proteins are indicated by arrow heads. Lane '0' denotes a sample treated identical throughout the experiment with no mitochondrial extract added. Apparent molecular weights are given on the left margin and were calculated to vary within a range of ±10−15%.

Figure 1. Secondary structure predictions of RNA molecules used as substrates for cross-linking. The four gRNA molecules gA6-14, gA6-48, gND7-385 and gND7-506 have been identified in *T.brucei* mitochondria (13) and foldings were calculated including the 3' oligo(U) tails of the molecules for $T = 27°C$. Only the thermodynamically most stable structures are shown (16, 17). Free energies ($\Delta G°_{27}$) were as follows: gA614: −4.5 kcal/mol, gA6-48: −5.4 kcal/mol, gND7-385: −5.1 kcal/mol and gND7-506: −4.7 kcal/mol. Mock RNA-1 is a 56 nucleotides *in vitro* T7 transcript from *Hind*III linearized pBS- DNA (Stratagene). Mock RNA-2 is the annealed product of two *in vitro* transcripts from pBluescriptKS⁻ to yield an extended, 59 bp double stranded molecule.

Figure 3. Protein cross-linking of different gRNA substrates. Two gRNA molecules specific for the editing of different domains of the *T.brucei* ATPase 6 mRNA (gA6-14, gA6-48) and two gRNAs specific for the editing of two *T.brucei* ND7 mRNA regions (gND7-385, gND7-506) were cross-linked using identical conditions. Cross-linked proteins are annotated with arrow heads. Apparent molecular weights are given on the left (lane Ma.). Lane: tot., total *T.brucei* mitochondrial extract stained with Coomassie Brilliant Blue. Lane: no, control sample with no protein extract added.

cross-linked proteins were stripped from the RNA by the denaturing conditions.

At low extract concentrations (0.1 mg/ml) four mitochondrial proteins cross-linked to the gRNAs with apparent molecular weights of 90, 59, 36 and 21 kDa (Fig. 2). The addition of 10 times more extract (1 mg/ml) increased the yield for these four polypeptides and revealed additional weak cross-linking products with apparent molecular weights of 124, 64, 45 and 9 kDa. These eight cross-links were a constant feature of all extract preparations tested. Variations were found in the relative intensities of the protein bands from one preparation to another and in some cases we saw an additional product in the range of 52 kDa (see Fig. 4B). The four different gRNA substrates gave essentially an identical cross-linking pattern, indicating the assembly of the same RNP complex upon mitochondrial extract addition (Fig. 3). Cross-linking products of lower apparent molecular weight appeared to be more diffuse than those of higher molecular weight, most likely due to the size variation and hence, variable
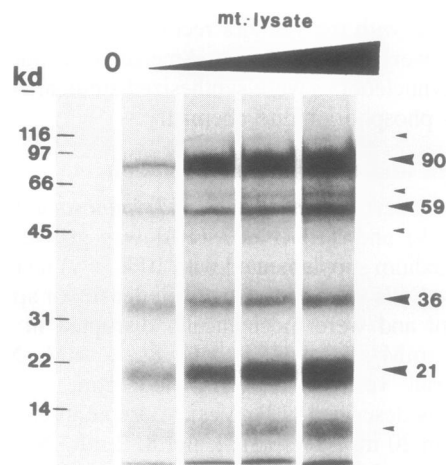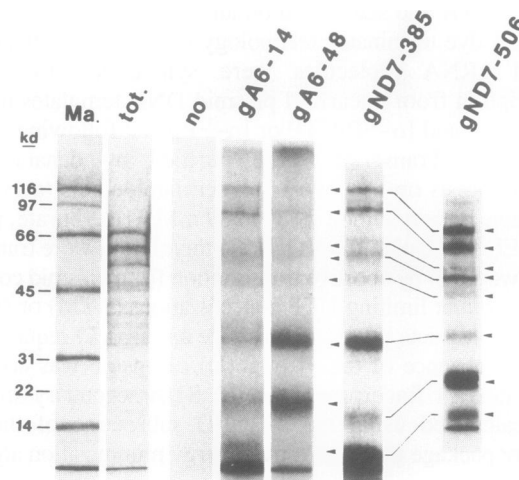
contribution of the covalently attached oligoribonucleotides to the proteins.

The specificity of the cross-linking reaction

The cross-linked proteins were specific for both the *T.brucei* mitochondrial extracts and the input gRNAs. Incubation of a *T.brucei* nuclear lysate with gRNAs using identical conditions as described above resulted in no specific cross-linking products (Fig. 4A). A synthetic RNA (see Figure 1 and description in the
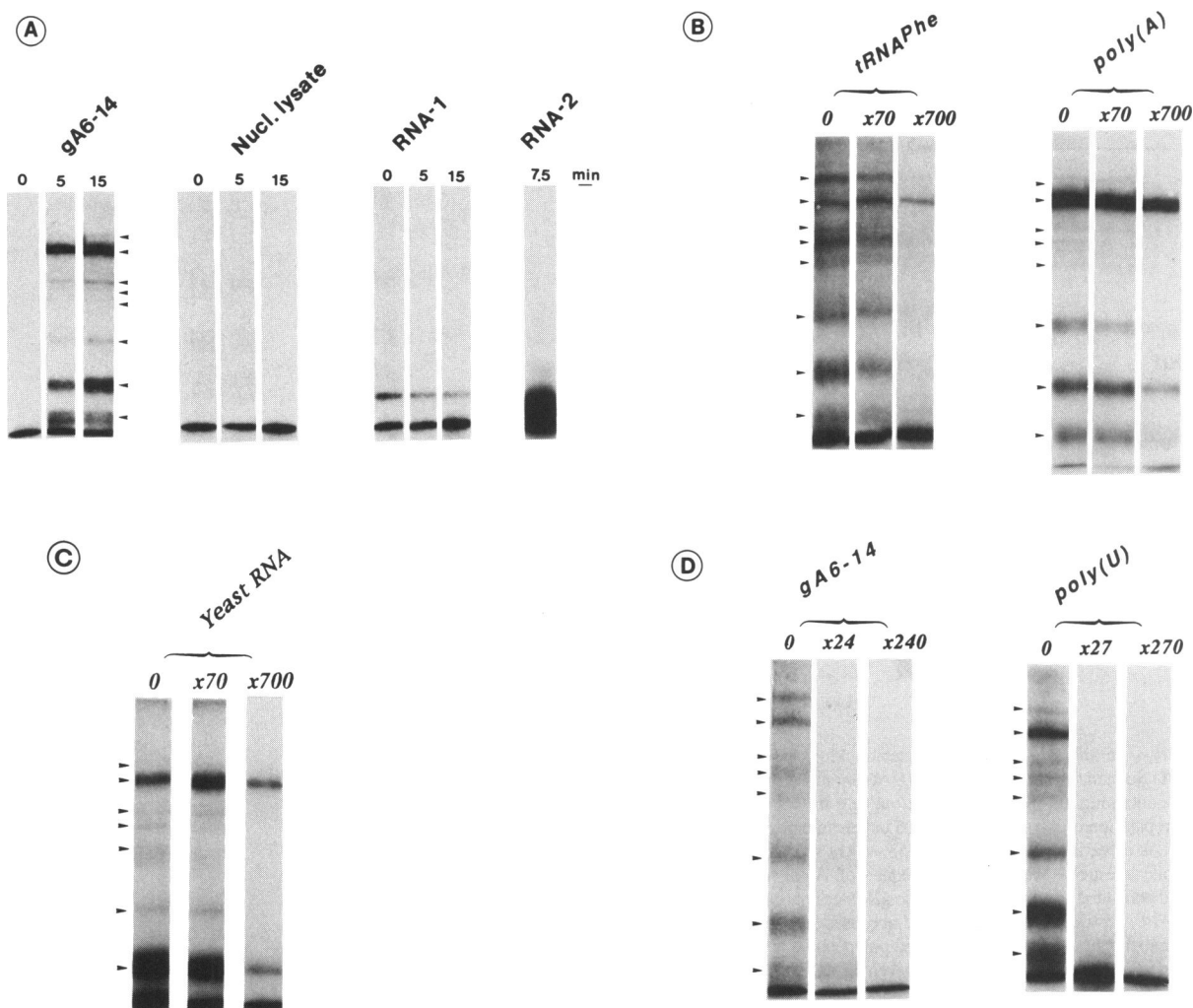
Figure 4. Testing the cross-linking specificity. (A) Different RNA substrates and *T.brucei* protein extracts were used in the cross-linking assay. gA6-14: Control sample of ^{32}P-labeled gA6-14 RNA lacking it's 3' terminal U-extension and incubated with *T.brucei* mitochondrial extract. Cross-linked proteins are marked by arrow heads. Nucl. lysate: The same gA6-14 RNA substrate but treated with *T.brucei* nuclear extract. RNA-1/RNA-2: Mock RNA substrates (see Fig.1) incubated with *T.brucei* mitochondrial extract. Numbers on top of the lanes indicate irradiation times with UV-light. (B) Competition of cross-linking with heterologous RNA molecules. ^{32}P-Labeled gA6-14 RNA was used as the cross-linking substrate. Incubation with mitochondrial extract was done in the presence of increasing amounts of tRNA[Phe] (70- and 700-fold molar excess) or poly(A) in a 70- and 700-fold excess on a mass basis. (C) Competition of cross-linking with total yeast RNA in a 70- and 700-fold excess on a mass basis. (D) Competition of cross-linking with a homologous RNA competitor (gA6-14) in a 24- and 240-fold molar excess and with poly(U) in a 27- or 270-fold molar excess. Cross-linked proteins are annotated by arrow heads.

legend to the figure) of similar length to gRNA molecules (RNA-1) could not be cross-linked to *T.brucei* mitochondria components nor did an extensively double stranded RNA molecule (RNA-2) (Fig. 4A).

The cross-link specificity was additionally confirmed in competition experiments with homologous and heterologous RNA competitors. Unlabeled preparations of tRNA[Phe] in a 70-fold molar excess over input ^{32}P-gRNA did not compete. At a 700-fold molar excess all cross-links were significantly reduced, although not to zero, with the exception of the 90 kDa protein. Similarly, an excess of the homopolymer poly(A) in the reaction mix had no effect. Even at a very high poly(A) concentration (700-fold excess on a mass basis) the 90 and 21 kDa proteins, and to some extend the 9 kDa polypeptide were still able to bind (Fig.4B). An identical result was obtained using a total RNA preparation from yeast cells as a competitor including all major RNA classes. A 700-fold excess on a mass basis had no influence

on the 90 and 21 kDa cross-links indicating high affinities of these two polypeptides for the gRNA molecules (Fig. 4C). Unlabeled preparations of gRNAs at only a 20−25-fold molar excess, however, totally abolished the cross-linking reaction (Fig. 4D). Interestingly, the homopolymer poly(U) was a potent competitor as well. A 30-fold molar excess resulted in a total inhibition of protein binding, very similar to the aforementioned gRNA effect (Fig. 4D). This behavior was dependent on the length of the oligo(U) preparation since the three U-nucleotides, the corresponding nucleoside and the free base had no effect even at concentration up to 1 mM. However, oligo(U) isolates above 35 nucleotides in length completely abolished the cross-linking reaction (data not shown).

Cross-linking with 3'-end truncated gRNAs

In order to yield a first picture of the gRNA domain structure that is required for protein binding we used gRNA substrates
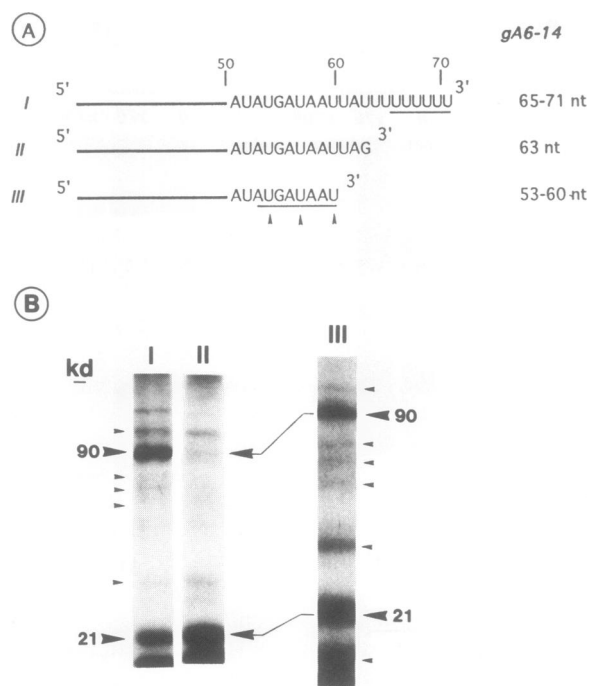
Figure 5. Cross-linking of gRNA substrates lacking 3' terminal oligo(U) extensions. (A) Sequences of the three tested gA6-14 substrates at their 3' ends. I: Molecules containing an oligo(U) tail and varying in length between 65 and 71 nt. II: A preparation of the exact length of 63 nt terminating in a G nucleotide. III: Guide RNAs of variable length between 53–60 nt some of which terminate in a U nucleotide (see arrow heads below the sequence). A bar below the sequences annotates the determined size variation of these gRNA preparation. (B) The three different gA6-14 substrates were gel purified and used as substrates for protein binding with mitochondrial extract. Cross-linked polypeptides are marked by arrow heads and apparent molecular weights are given in kDa.

of both, variable and defined length, lacking their 3' oligo(U) extensions. The molecules were synthesized either from DNA templates not encoding a 3' oligo(T) tail or by abortive RNA transcription at limiting UTP concentration from plasmids encoding an oligo(T) extension. Figure 5 summarizes the results of such an experiment using gA6-14 as a substrate. The standard cross-linking pattern was obtained using a gA6-14 population ranging in size between 65–71 nt with 3–9 U's at the 3' end. The exact deletion of the U-tail (length of the RNA substrate: 63 nt) almost completely abolished the interaction with the 9 kDa polypeptide and slightly increased the yield for the 21 kDa cross-link. However, a further reduction of the length of the RNA molecules to 53–60 nt increased the yield for both polypeptides again, indicative of either a structural rearrangement of the gRNAs or of selectively binding to those substrate gRNA molecules that terminate in a U-nucleotide (see Fig. 5A). Lastly, the truncated gRNA molecules behaved identical in all the competition experiments described in the previous paragraph (Fig. 4).

Inhibition of cross-linking

Cross-linking was completely disrupted by pre-digesting the mitochondrial extract with proteinase K. In contrast, hydrolysis with micrococcal nuclease had no effect, indicating that no other nucleic acid molecules are required for the assembly reaction (Fig. 6A).
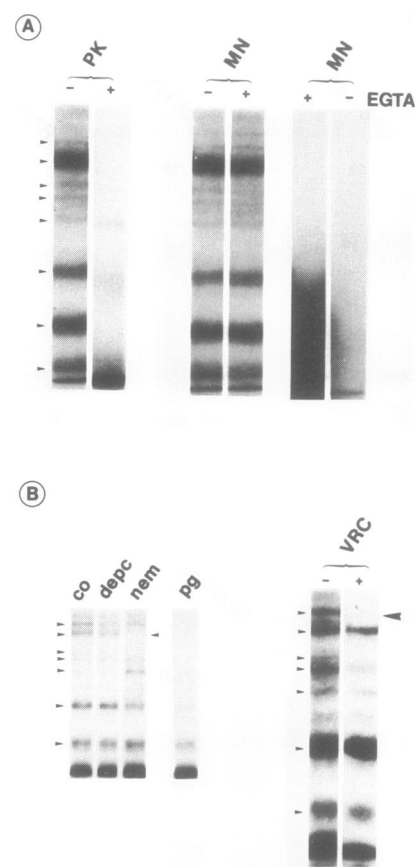

Figure 6. Cross-linking inhibition studies. (A) Standard cross-linking reactions were performed with 3' end truncated gA6-14 RNA and mitochondrial extracts either untreated (lane −) or pretreated (lane +) with proteinase K (PK) or micrococcal nuclease (MN). The two lanes on the right are control samples with gA6-14 RNA digested with micrococcal nuclease in the presence (+) or absence (−) of EGTA. (B) Inhibition reaction with diethyl pyrocarbonate (depc), N-ethylmaleimide (nem), phenylglyoxal (pg) and vanadyl ribonucleoside complex (VRC). Lanes: 'co' and '−' annotate control reaction with no inhibitor added. Cross-linked polypeptides are marked by arrows.

Several specific inhibitory effects were found. Irreversible alkylation of free sulfhydryl groups with N-ethylmalimide completely abolished the 90 kDa cross-linking product (Fig. 6B). The addition of vanadyl-ribonucleoside complexes (VRC) prevented the formation of the 124 kDa cross-link and the modification of free amino groups with phenylglyoxal reduced the yield of all cross-linking products significantly. Carbethoxylation of imidazole rings with diethylpyrocarbonate had no effect excluding the possibility of salt bridges between phosphates and protonated histidines (24) (Fig. 6B).

Cross-linking was completely abolished at KCl concentrations ≥ 250 mM (Fig. 7a). An increase in the monovalent salt concentration from the standard condition of 30 mM to 100 mM K$^+$ caused a significant decrease of all cross-linking products except for the 90, 21 and 9 kDa polypeptides. The latter three proteins remained constant or even increased their yield at higher salt concentrations, indicating enhanced affinities to the gRNAs (Fig. 7b).

Figure 7. Standard cross-linking conditions were used with ^{32}P-labeled gA6-14 RNA and 0.3 mg/ml mitochondrial protein extract at varying KCl concentrations as indicated. (a)The different densitometer tracings of such an experiment with the cross-linked proteins marked by small arrow heads. Large arrow heads below the densitometer profiles point to the 90, 21 and 9 kDa cross-linked polypeptides which remain constant up to 100 mM KCl. Abs. stands for absorbance and mig. for migration. (b) Bar graphs showing the relative cross-linking yields of the 90, 36, 21 and 9 kDa proteins at KCl concentrations between 30−500 mM. Please note the almost linear decrease of the 36 kDa polypeptide which is representative for the salt dependence of all other cross-linked proteins except the 90, 21 and 9 kDa products as shown.

DISCUSSION

Currently guide RNAs and pre-edited mRNAs are the only identified components of the mitochondrial editing reaction. However, it's very likely that additional mitochondrial molecules are also involved in the process since gRNAs comigrate as components of two large mitochondrial complexes in sedimentation experiments (3) and furthermore, associate specifically with mitochondrial proteins to form high molecular weight ribonucleoprotein (RNP) complexes *in vitro* (4, 5). It has also been demonstrated that chimeric gRNA-pre-edited mRNA molecules, which are presumed intermediates of the editing reaction, require mitochondrial proteins for their formation (6−8). These data and the analogy to other RNP particle directed biochemical reactions like splicing or protein synthesis support the hypothesis that RNA editing occurs in a mitochondrial RNP complex provisionally termed the 'editosome' (25−27). The present study identifies mitochondrial proteins that directly interact with gRNAs and thus are potential editing components.

We used four different gRNAs as substrates which fold into different secondary structures calculated by a nearest neighbor free energy minimization algorithm (17). Despite the predicted structural differences all four molecules were able to bind an identical set of mitochondrial proteins, which excludes overall secondary structure recognition as the primary interaction motif. However, it is very likely that the actual solution structures of gRNA molecules deviate substantially from the thermodynamically most stable conformations (Fig. 1) and that the binding domains are simple secondary structure motifs like the small stem loops present in every gRNA. Similar situations have been characterized for a variety of other ribonucleoprotein particles like ribosomal proteins, proteins involved in splicing, the HIV Tat and Rev proteins (all reviewed in 28) and the iron responsive element binding protein (29).

Two of the gRNAs (gA6-14 and gND7-506) specify editing of the first editing domain of their pre-mRNAs and thus can be considered 'initiator' gRNAs. The other two gRNAs can be viewed as 'elongator' molecules since upstream editing is required for the binding of these gRNAs to the pre-edited mRNAs (13). Clearly, the cross-linking patterns for the four gRNA substrates are identical, indicating no difference for the formation of potentially 'early' versus 'late' acting gRNA−RNPs.

Guide RNAs lacking their 3' oligo(U) extensions had a dramatically reduced affinity for the 90 kDa protein possibly indicating the recognition of the U-tail by the polypeptide. However, in the case of gRNA gA6-14, a further reduction of the 3'-end beyond the U-tail substantially increased the yield for this cross-link again. This can be interpreted as due to a selective binding of the protein to those gRNA molecules in the population terminating in a U-nucleotide. This would imply, that a single U-residue can be sufficient for the binding of the 90 kDa protein (see Fig. 5A) and that the affinity between the polypeptide and the gRNA slightly decreases with an increasing length of the U-tail. A tempting interpretation for this result would be that the 90 kDa protein is involved in the regeneration of the U-tail during the editing reaction. If the gRNA's U-tail is the donor respectively the acceptor for the U-nucleotides during editing it might be important to constantly regenerate the 3'-end in order to allow a reentry of gRNAs into the reaction. The gRNA binding site for the uridylating activity would be a domain at the 3'-end of the molecule possibly monitoring the number of U's at the 3'-end and displaying a differential affinity for the length of the U-tail.
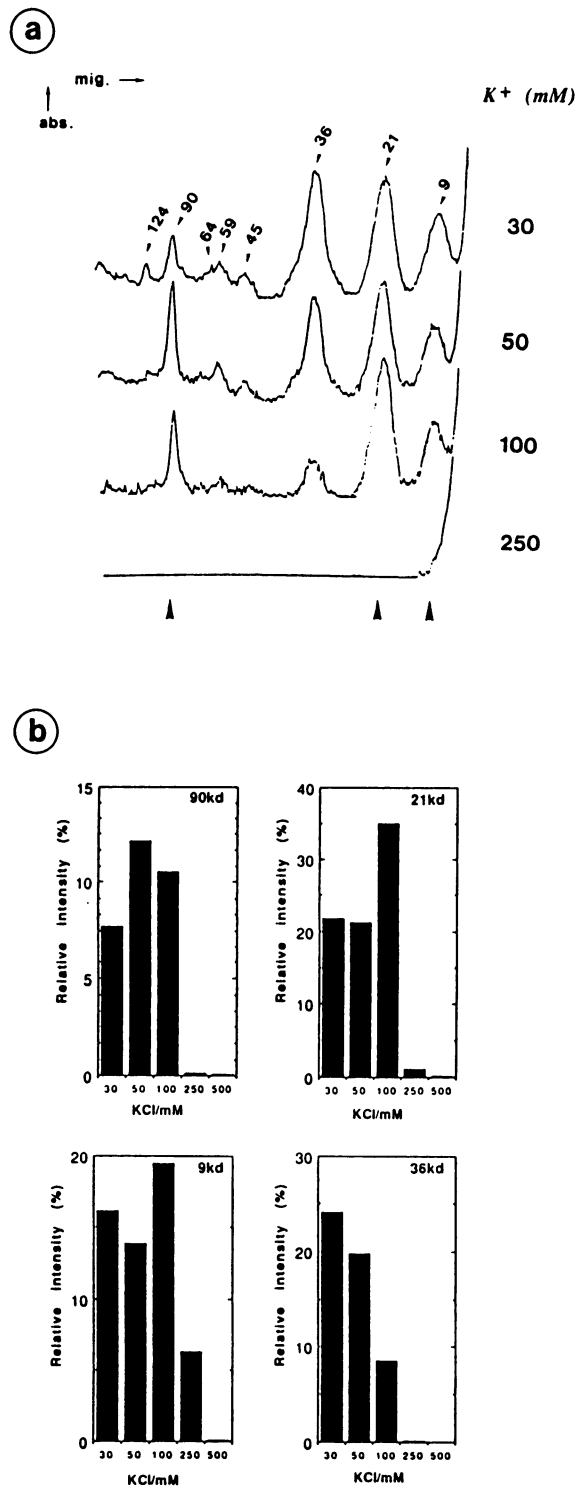
Interestingly, a terminal uridylyltransferase (TUTase) activity has been found in kinetoplastid mitochondria (30) and was also characterized to be a component of the 19S gRNA-containing RNP complex in *T.brucei* mitochondria (3). However, we can not exclude that a refolding of the gRNA molecules as a consequence of deleting the 3'-end contributes to the above described phenomenon as well. Again, secondary structure predictions based on free energy minimization are not very helpful in this case because no experimental data are yet available to confirm the predicted foldings.

That no other nucleic acids are required for the interaction with the proteins, excludes pre-edited mRNAs as part of the presumed complexes. With respect to the editing reaction, this can be interpreted as the formation of a gRNA—protein precursor complex that transiently binds pre-edited mRNAs and possibly other components very much like the aforementioned splicing and translational machineries which associate with RNA substrates and co-factor molecules while they function. The two gRNA complexes identified by Harris and Hajduk (7)in a sedimentation analysis have been interpreted similarly as two complexes trapped at different modes of action (3). Which of the identified polypeptides actually contributes as a binding component to the different RNP particles needs to be shown in a different experimental approach combining RNP separation techniques with the UV cross-linking technology (37).

The specific inhibition reactions of the 90 kDa protein with *N*-ethylmaleimide suggests a role for sulfhydryl groups in its RNA binding. Similar situations have been identified in other RNA—protein binding systems (31, 32) and characterized as an interaction based on the nucleophilic attack of a SH-group on the C6 atom of a uracil nucleotide to form a transient covalent Michael adduct (33). In the case of the iron responsive element binding protein (IRE-BP) this reversible covalent modification was found to be essential for the interaction and hence, for the function of the protein (32).

The vanadyl ribonucleoside inhibition of the 124 kDa cross-link suggests the following: Oxovanadium(IV) ion (VO^{2+}) ribonucleoside complexes are competitive inhibitors for ribonucleases. The complexes have the structure of a trigonal bipyramid with a pentacovalently coordinated vanadium atom thus acting as transition state analogs resembling nucleoside 2',3'-cyclic phosphates (34). Since the formation of the 124 kDa cross-link can be specifically inhibited with VRC, the RNA—protein interaction might be based on a similar structure, again possibly indicating a transient covalent interaction. Without temporarily hydrolyzing the phosphate backbone of the substrate RNA only the 3'-ends of the gRNAs seem to be plausible domains for this type of interaction. VRC inhibition of protein cross-linking to substrate mRNA and of RNP complex formation has also been found in the case of apolipoprotein B mRNA editing which was paralleled by the inhibition of apoB editing activity (35, 36).

The complete inhibition of protein binding at ≥250 mM monovalent cation concentration suggests a strong contribution of electrostatic interaction in the association of the proteins. A similar salt dependence was found for the assembly of the apoB editosome (35) and for the two gRNA-containing complexes in *T.brucei* mitochondria (3), although kinetoplastid RNA editing and apoB mRNA editing are mechanistically thought to be different. The increased salt stability of the 90, 21 and 9 kDa polypeptides suggests higher affinities of these proteins to the gRNAs. This is further substantiated by the appearance of these cross-links at very low mitochondrial extract concentration (Fig. 2) as well as the binding of these proteins in the presence of very high non-specific RNA competitor concentrations (Fig. 4B, 4C).

We have no satisfactory explanation for poly(U) acting for all proteins as a potent competitor for cross-linking. A titration effect of protein binding sites on the oligo(U) tail can be ruled out since the tail seem to be a recognition element only for the 90 kDa polypeptide. However, since the length of the oligo(U) was crucial for the competition effect, the molecules might have functioned as poly-anions, thus interfering with protein binding by charge compensation, in line with the salt inhibition data discussed above. The difference to the competition data with tRNA and poly(A), which also are poly-anions, might be due to the different dipole moments of the individual bases (38) combined with the different structures of the molecules.

It is important to emphasize that we have no evidence to correlate the cross-linked polypeptides directly to the kRNA editing process. It is likely that some of the proteins are also involved in other mitochondrial reactions. However, the so far only demonstrated biochemical function of the gRNA moiety is its involvement in RNA editing, which make at least some of the identified proteins good candidates for participants in the editing reaction.

ACKNOWLEDGEMENTS

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