MOLECULAR AND CELLULAR BIOLOGY, July 1997, p. 3915–3923 Vol. 17, No. 7
0270-7306/97/$04.00+0

Mitochondrial Glutamate Dehydrogenase from *Leishmania tarentolae* Is a Guide RNA-Binding Protein

FRÉDÉRIC BRINGAUD,[1]† RENATA STRIPECKE,[2]‡ GEORGES C. FRECH,[1,2]§ STEPHEN FREEDLAND,[2] CHRISTOPH TURCK,[3] ELAINE M. BYRNE,[1,2] AND LARRY SIMPSON[1,2,4]*

Howard Hughes Medical Institute,[1] Department of Molecular, Cellular and Developmental Biology,[2] and Department of Medical Microbiology and Immunology,[4] UCLA School of Medicine, University of California Los Angeles, Los Angeles, California 90024, and Howard Hughes Medical Institute, University of California, San Francisco, San Francisco, California 94143-0724[3]

Received 10 December 1996/Returned for modification 11 February 1997/Accepted 2 April 1997

To identify specific proteins interacting with guide RNAs (gRNAs) in mitochondrial ribonucleoprotein complexes from *Leishmania tarentolae*, fractionated and unfractionated mitochondrial extracts were subjected to UV cross-linking with added labeled gRNA and also with [α-^{32}P]UTP-labeled endogenous RNA. An abundant 110-kDa protein (p110) localized in the T-V complex, which sediments in glycerol gradients at the leading edge of the 10S terminal uridylyltransferase peak, was found to interact with both types of labeled RNAs. The p110 protein was gel isolated and subjected to microsequence analysis, and the gene was cloned. The sequence revealed significant similarity with mitochondrial glutamate dehydrogenases. A polyclonal antiserum was raised against a recombinant fragment of the p110 gene and was used to demonstrate a stable and specific gRNA-binding activity by coimmunoprecipitation and competitive gel shift analyses. Complex formation was strongly inhibited by competition with poly(U) or by deletion or substitution of the gRNA 3′ oligo(U) tail. Also, addition of a 3′ oligo(U) tail to an unrelated transcript was sufficient for p110 binding. Both the gRNA-binding activity of the p110 protein and in vitro gRNA-independent and gRNA-dependent uridine insertion activities in the mitochondrial extract were inhibited by high concentrations of dinucleotides.

RNA editing in trypanosomatid mitochondria involves the insertion and deletion of uridine (U) residues at multiple sites mainly within the coding regions of transcripts of more than half of the structural genes in the maxicircle genome (2, 24, 53, 57). The sequence information required for editing is provided by guide RNAs (gRNAs), which are small RNAs (40 to 70 nucleotides) that are complementary (if G:U and, rarely, C:A pairs are allowed) to edited blocks of the mature RNA (3, 42). The gRNAs also possess 3′-terminal nonencoded oligo(U) tails (4) that are created by the action of a mitochondrial terminal uridylyltransferase (TUTase) (1).

Several models for the mechanism of the U insertion-deletion type of RNA editing have been proposed (3, 5, 12). Recent results from partial in vitro systems with both *Trypanosoma brucei* (28, 48, 49) and *Leishmania tarentolae* (10, 15, 20, 21) suggest that the original enzyme cascade model is essentially correct. This model postulated an initial cleavage of the preedited mRNA at the first editing site, followed by an addition of U residues to the 3′ end of the mRNA 5′ fragment. It was initially proposed that the number of U's added was directly determined by base pairing with the guide A or G nucleotides in the cognate gRNA (3), but recent evidence suggests that multiple U's are first added to the 5′ fragment and those terminal U's not base paired to the gRNA are then deleted by a 3′ exonuclease (10, 18, 28, 48). The final step is a ligation of the 5′ fragment and the 3′ fragment. Guide RNA-dependent U deletions would involve the removal of all of the added U's and the encoded U's due to the lack of guiding nucleotides in the gRNA at that site. Religation of the fragments without complete removal of non-base-paired 3′-terminal U's from the mRNA 5′ fragment results in the formation of gRNA-mediated misedited sequences (10, 28).

Several enzymatic activities required by the enzyme cascade model have been identified in mitochondrial extracts from kinetoplastid protozoa. These include the mitochondrial TUTase (1), a preedited sequence-specific endoribonuclease (26, 51), an RNA ligase (1, 39, 45), an RNA helicase activity (36), and a 3′ U-specific exonuclease (18). To date, however, only the RNA helicase gene has been cloned (37), although two adenylated polypeptides have been identified as putative components of the RNA ligase (39, 45). Several of these activities have been shown to comigrate in glycerol gradients and in native gels with gRNAs and in some cases mRNAs, in the form of complexes ranging from 10S to 35S in size (22, 30, 40, 41, 44, 50).

In mitochondrial extracts from *L. tarentolae*, several classes of RNP complexes have been identified. (i) The 10S T complexes contain RNA components which can be labeled in vitro by incubation with [α-^{32}P]UTP (9, 38). The approximately 200-kDa T-IV complexes exhibit TUTase activity in an in situ assay and contain gRNA (38). A 70-kDa polypeptide component of T-IV has been identified by Western analysis (39). Other classes are (ii) a 20S complex that contains the p45 and p50 adenylated polypeptides which are putative RNA ligase components (39) and (iii) a set of heterodisperse high-molecular-weight complexes that may represent oligomers of T-IV complexes bound to mRNAs (39).

A gRNA-independent U insertion activity from *L. tarentolae* mitochondrial extract (15, 20, 21) was shown to sediment at 20S in glycerol gradients (38), and gRNA-dependent U deletion (48, 49) and U insertion (28) activities from mitochondrial

* Corresponding author. Mailing address: Howard Hughes Medical Institute, University of California at Los Angeles, 6-780 MacDonald Research Laboratory, Los Angeles, CA 90095-1662. Phone: (310) 825-4215. Fax: (310) 206-8967. E-mail: simpson@hhmi.ucla.edu.

† Present address: Laboratoire de Biologie Moléculaire et d'Immunologie de Parasites Protozoaires, UPRESA-5016 CNRS, Université de Bordeaux II, 33076 Bordeaux cédex, France.

‡ Present address: Children's Hospital, Los Angeles, Calif. 9002.

§ Present address: Myriad Genetics, Inc., Salt Lake City, UT 84108.

extracts of *T. brucei* were shown to also sediment at approximately 20S (17). Peris et al. (38) have proposed that the ligase-containing 20S complex and the p70-gRNA-containing heterodisperse complexes interact in the editing process. Clearly a detailed knowledge of the individual components of the editing machinery and biochemical reconstitution of activities in vitro is required to understand this process in detail.

In this study, we isolated and cloned the gene for a 110-kDa polypeptide from the T-V complex of *L. tarentolae*, which was shown previously by UV cross-linking experiments to interact with added gRNA (7), and we provide evidence for a stable and specific gRNA-binding activity of the p110 protein, which may prove to be biologically relevant.

MATERIALS AND METHODS

Cell culture, mitochondrial isolation, and preparation of mitochondrial and cytosol extracts. *L. tarentolae* (UC strain) was grown to late log phase (1×10^8 to 2×10^8 cells/ml) (52). The kinetoplast-mitochondrial fraction was isolated by flotation in Renografin density gradients (6). The extent of cytosolic contamination of the mitochondrial fraction was measured to be 1 to 5% in various preparations, as determined by Northern blot detection of 18S rRNA in the mitochondrial fraction, as described previously (33). A clarified mitochondrial Triton extract (TS) was prepared as described previously (1). Fractionation of the mitochondrial extract by Superose 6 gel filtration and by sedimentation in 36-ml 10 to 30% glycerol gradients in an SW28 rotor was performed as described previously (38). A cytosolic fraction was obtained from the initial supernatant after cell disruption. This fraction also contained the contents of nuclei ruptured in the hypotonic lysis conditions but had little contamination with mitochondria, since these remained intact under the cell rupture conditions and were pelleted in the initial centrifugation step. The proteins were precipitated overnight at −20°C in the presence of 4 volumes of acetone. After centrifugation, the pellet was resuspended in 0.1% sodium dodecyl sulfate (SDS). The quantity and quality of the precipitated proteins were checked before the Western blot analysis was performed.

Construction and transcription of synthetic gRNAs. The synthetic gRNA genes gCybII, gND7-II+polyC, and gND7-II − polyU were obtained from PCR-amplified DNA. The gCybII construct was designed so that in vitro transcription from the T7 promoter (35) yielded RNA molecules with 13 U's at the 3′ terminus. The construct gND7-II+polyC encoded RNA transcripts with 13 C's at the 3′ terminus, while the construct gND7-II-polyU did not encode a poly(U) tail. To obtain synthetic gND7-II gRNA, a PCR construct, designed to contain a *Dra*I site after 13 T residues at the 3′ end of the gRNA, was cloned into pUC19. The resultant plasmid, pUC19-gND7-II, was linearized with *Dra*I prior to in vitro transcription. The negative control transcripts were obtained from pU1AmutCAT (56) and pGem-7Zf(−) (Promega), linearized with *Xba*I and *Cla*I, respectively. The pGem transcript was 66 nucleotides long and had 41% A+T; the pU1AmutCAT transcript was 66 nucleotides long and had 39% A+T. Plasmid pGem-poly[U] was constructed by using the pGem-7Zf(−) vector. Labeled RNAs were obtained by transcription with T7 RNA polymerase and [α-^{32}P]ATP and purified by denaturing acrylamide gel electrophoresis following standard procedures.

UV cross-linking. For UV cross-linking of the TS extract, aliquots (10 μl) of TS were incubated for 40 min at 27°C in a mixture containing 1 mM ATP, 1 mM GTP, 20 mM dithiothreitol (DTT), 12.5 mM HEPES-KOH (pH 7.5), 40 mM KCl, 6 mM magnesium acetate, 3 mM KPO_4 (pH 7.5), and 10 μCi of [α-^{32}P]UTP or 5×10^5 cpm of [α-^{32}P]ATP-labeled synthetic gND7-II gRNA. The samples were irradiated with 254-nm UV light for 30 min at 4°C (Stratalinker UV box [Stratagene]; 5 cm from the UV source), incubated with RNase A (100 μg/ml) for 30 min at 37°C, and then boiled for 2 min in 50 mM Tris (pH 6.8)–100 mM DTT–2% SDS–0.1% bromophenol blue. The samples were loaded on an 8% acrylamide–0.1% SDS-gel for electrophoresis (47), and the gels were vacuum dried before autoradiography either on X-ray film or on a PhosphorImager cassette (Molecular Dynamics).

For UV cross-linking of native gel bands (7), aliquots (10 μl) of TS were incubated for 40 min at 27°C in 1 mM ATP–1 mM GTP–20 mM DTT–12.5 mM HEPES-KOH (pH 7.5)–40 mM KCl–6 mM magnesium acetate–3 mM KPO_4 (pH 7.5)–10 μCi of [α-^{32}P]UTP (800 Ci/mmol). The samples were loaded on a native acrylamide gradient (4 to 16%) gel, which contained 0.1% Tween 20 and was stabilized with a 10 to 30% glycerol gradient (38, 55). The gels (14 by 14 cm) were run at 125 V for 24 h at 4°C in 40 mM Tris-acetate–1 mM EDTA (pH 8). The wet gels were exposed to X-ray film, and the experimental lanes were excised. The excised acrylamide blocks containing labeled nucleoprotein complexes were irradiated with 254-nm UV light for 5 min at 25°C as described above, incubated with RNase A (10 μg/ml) for 30 min at 37°C, and then incubated with 50 mM Tris (pH 6.8)–100 mM DTT–2% SDS–0.1% bromophenol blue for 30 min at 25°C. The gel blocks were layered on a 12% acrylamide–0.1% SDS gel. After electrophoresis, the gel was processed for autoradiography.

Two-dimensional separation of proteins and peptide sequencing. An aliquot (10 μl) of the TS extract was first separated in a native gradient gel as described above. The lane was excised and incubated with 50 mM Tris (pH 6.8)–100 mM DTT–2% SDS–0.1% bromophenol blue for 30 min at 25°C. The gel slice was layered on a 10% acrylamide–0.1% SDS gel for the second-dimension electrophoresis. The proteins were electroblotted onto Immobilon-P^{SQ} filters (Millipore) as described previously (25) and stained with Coomassie brilliant blue. The protein comigrating with the UV cross-linked p110 spot was eluted and digested with trypsin, and peptides were isolated by high-pressure liquid chromatography. N-terminal sequences of the major peptides were determined by standard procedures. See Fig. 1D in reference 7 for a photograph of the two-dimensional gel used for this experiment.

Cloning and analysis of the gene encoding the p110 protein. Total RNA from *L. tarentolae* was prepared as described previously (34), and poly(A)$^+$ RNA was purified by using a poly[A] Quick kit from Stratagene. Single-stranded cDNA primed by random hexanucleotides was synthesized from poly(A)$^+$ RNA and used as a substrate for PCR amplification. The Sp110-5 (5′-AT<ATC>AT<ATC>GT<GATC>GA<GA>GG<GATC>GC<GATC>AA<TC><TC>T-3′) and Sp110-6 (5′-<GAT>AT<GATC>GC<GA>TT<GA>AA<TC>TC<GATC>A-3′) degenerate primers were constructed by reverse translation of the amino acid sequences of the p110 tryptic peptides, pp59A (IIVEGA NLFISQDA) and pp59B (LEFNAI), and used as 5′ and 3′ PCR primers, respectively. The cDNA and primers were denatured for 10 min at 95°C, and then 30 cycles of PCR (10 s at 95°C, 30 s at 60°C, and 30 s at 72°C) were performed in 50-μl reaction volumes (46). The amplified fragment was gel isolated and cloned into the pT7blue T-vector (Novagene). Both strands of four independent clones were sequenced by dideoxy-chain termination (U.S. Biochemical Sequenase kit), using a 19-mer oligo(U) primer and the T7 promoter primer (Novagene). The PCR clone pPCR110-16, containing a 275-bp fragment of the p110 gene, was used for further analysis.

By screening an *L. tarentolae* (UC strain) cosmid genomic DNA library (kindly provided by D. Campbell) (11) with a cloned α-^{32}P-labeled PCR fragment, a clone containing a large genomic DNA fragment containing the p110 gene was obtained. Several DNA fragments containing portions of the p110 gene were subcloned into the pUC18 vector and sequenced by the dideoxy-chain termination method (U.S. Biochemical Sequenase kit), using specific oligonucleotide primers.

For Southern analysis of the p110 gene, 10 μg of genomic *L. tarentolae* DNA was digested with 15 to 20 U of restriction enzymes and separated through a 0.6% agarose-Tris-borate-EDTA gel. The gel was blotted onto a Magna-graph nylon membrane (MSI), which was UV cross-linked and prehybridized in hybridization buffer (4% formamide, 5× Denhardt's solution, 2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% SDS, 20 mM Tris-HCl [pH 7.5], 200 μg of salmon sperm DNA per ml) for 6 h at 45°C. The membrane was hybridized in the same buffer against a p110-specific [α-^{32}P]dATP-labeled probe (2×10^7 cpm) at 45°C overnight. The membrane was washed at 60°C in 2× SSC–0.1% SDS and exposed for densitometry by using a PhosphorImager.

DNA and amino acid sequences were analyzed by using the DNA STRIDER program and the Genetics Computer Group package, and database homology searching was performed by using BLAST.

Production of p110 antiserum and Western blot analysis. A *Bam*HI/*Eco*RI-digested PCR fragment encoding 327 amino acids of the p110 protein (from amino acids 529 to 856) was inserted into the pGEX-5X-1 vector (Pharmacia) previously digested with *Bam*HI and *Eco*RI. The resultant plasmid, pGEX110-2, encodes a 70-kDa protein corresponding to the glutathione *S*-transferase (GST) open reading frame at the N-terminal extremity, two factor Xa recognition sites, the p110 fragment, and six histidine residues at the C-terminal extremity.

Escherichia coli DH5α transformed with plasmid pGEX110-2 was grown in Luria-Bertani medium at 37°C to an optical density at 600 nm of 0.6, and expression of the recombinant p110 protein was induced by incubation for 4 h in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside. Cells were disrupted in a French press, and the lysate was centrifuged at 4,000 rpm for 10 min to collect the inclusion bodies, which were solubilized in buffer S (7 M urea, 50 mM sodium phosphate [pH 8]). The solution was applied to an Ni^{2+}-nitriloacetic acid-agarose column (Qiagen), which was then washed with 10 column volumes of buffer S. The recombinant p110 fragment was eluted into buffer S containing 250 mM imidazole and was separated by SDS-polyacrylamide gel electrophoresis. The 70-kDa protein was electroeluted from the polyacrylamide gel for 15 h at 50 V in 0.1% SDS–50 mM NH_4HCO_3, using a Little Blue Tank electroelutor (ISCO, Inc.). A rabbit was repeatedly injected with 100 to 150 μg of the 70-kDa fusion protein mixed with Freund's incomplete adjuvant. A specific antigenic response against p110 was obtained after three injections within 6 weeks.

Immunoblotting from SDS-acrylamide gels was carried out as described previously (25). Filters were blocked for 15 min with TPBS-milk (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4, 0.05% Tween 20, 5% low-fat milk), incubated for 2 h with serum against the p110 protein (1:1,000 in TPBS-milk), washed with TPBS, blocked again for 15 min with TPBS-milk, and incubated for 1 h with goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Bio-Rad) (1:1,000 in TPBS-milk). After being washed with buffer A (50 mM Tris-HCl [pH 7.5], 20 mM NaCl), the filters were incubated with buffer A containing 0.05% H_2O_2 and 2.8 mM 4-chloro-1-naphthol.

Gel retardation assays. Aliquots (10 μl) of glycerol gradient fractions of the TS extract were mixed with 0.05 to 0.5 pmol of heat-denatured labeled RNAs in an 11- to 13-μl final volume and incubated at 27°C for 30 min. Competition

assays were performed by coincubating the unlabeled competitor RNAs with the labeled RNA and then adding the glycerol gradient fraction, unless otherwise indicated. Polyribonucleotides were purchased from Boehringer Mannheim. After the binding reaction, 3.5 μl of gel shift loading buffer (90% [vol/vol] glycerol, 0.05% [wt/vol] bromophenol blue) was added to each sample, and the samples were loaded onto 4% native polyacrylamide gels (acrylamide/bisacrylamide ratio, 19:1). Electrophoresis was performed at 4°C at 8 V/cm. The gels were fixed in 10% (vol/vol) acetic acid–10% (vol/vol) methanol, dried, and autoradiographed. Quantitative analysis of gel shifts was performed with a PhosphorImager.

Immunoprecipitation of UV cross-linked gRNA-p110. A 100-μl aliquot of fraction 5 from the glycerol gradient fractionation was mixed with 5 pmol of labeled gND7-II and incubated at 27°C for 30 min. The sample was irradiated with 254-nm UV light for 15 min on ice and incubated with 20 μl of RNase A (2 mg/ml) for 15 min at 37°C. Five to 10 μl of polyclonal serum was mixed with immunoprecipitation buffer (50 mM Tris-HCl [pH 7.5], 300 mM NaCl, 1% [vol/vol] Triton X-100, 1 mM phenylmethylsulfonyl fluoride, leupeptin [2 mg/ml]) in a 490-μl final volume, and 10 μl of the UV-cross-linked and RNase A-digested sample was added. After rotation of the samples at 4°C overnight, 50 μl of a 50% (vol/vol) suspension of protein A-Sepharose in phosphate-buffered saline was added to each sample, which was further incubated with rotation at 4°C for 1 h. The resin was washed three times with wash buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 0.1% Triton X-100) and boiled in 30 μl of protein loading buffer (250 mM Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.1% bromophenol blue) for 15 min. Aliquots (20 μl) of the samples were loaded onto SDS–8% acrylamide gels. The gels were fixed in 10% (vol/vol) acetic acid–10% (vol/vol) methanol, dried, and autoradiographed.

Electroblotting of native gels and immunodetection. Native gels were electroblotted onto a Nytran-ECL nitrocellulose membrane (Amersham) in blotting buffer (25 mM Tris, 192 mM glycine, 20% [vol/vol] methanol [pH 8.3]) at 30 V and 4°C overnight. Immunodetection was performed with an enhanced chemiluminescence kit (Amersham) or the SuperSignal CL-HRP substrate system (Pierce) as instructed by the manufacturer, using a 1:1,000 dilution of primary antiserum. Stripping of the Western blots for further immunodetection was achieved by incubating the membranes in 100 mM 2-mercaptoethanol–2% SDS–62.5 mM Tris-HCl (pH 6.8) at 50°C for 30 min with agitation.

In vitro gRNA-independent and gRNA-dependent U insertion activity. One assay involved the use of a T7-transcribed 5′ fragment of preedited cytochrome *b* mRNA (pNB2 RNA) as a substrate as described previously (20). The lysate (Triton lysate) was preincubated with 0.5 or 5 mM dinucleotide at 27°C for 30 min before addition of substrate RNA and [α-^{32}P]UTP, and the reaction mixture was then incubated at 27°C for 1.5 h. The intact labeled pNB2 RNA was gel isolated and digested with RNase H, using the S-165 oligomer, which is complementary to the RNA downstream of the editing domain (20). The 5′ and 3′ fragments were then separated on an acrylamide-urea gel, and the dried gel was exposed for quantitation in a PhosphorImager.

Another assay allowed the detection of the precise number of gRNA-independent or gRNA-dependent U insertions into site 1 of a preedited ND7 mRNA substrate with a mutated anchor sequence. The mRNA substrates used and the assay were described previously (10). The ND7.1x 5′-extended substrate showed up to 13 U insertions in a gRNA-independent reaction. The ND7.2x 5′-truncated substrate showed little U insertion activity in the absence of added gRNA but showed a ladder of up to 13 U's and a predominant +3 band in the presence of added gND7x[+3] gRNA, which has three guiding nucleotides and an anchor sequence with compensatory mutations to preserve base pairing with the mRNA substrate. The assay involved asymmetric reverse transcription-PCR amplification, yielding a cDNA copy of the treated mRNA, which was subjected to primer extension through site 1. The presence of inserted U's at site 1 in the mRNA was detected by incorporation of [α-^{32}P]ATP in the primer extension products.

GDH assay. Glutamate dehydrogenase (GDH) activity was assayed by the conversion of 2-oxoglutarate to 1-glutamate in the presence of ammonium acetate and NADH. The reaction mixture contained 0.1 M Tris-HCl (pH 7.6), 14 mM 2-oxoglutarate, 220 mM ammonium acetate (pH 7.5), 0.15 mM NADH (Sigma), 1 mM EDTA, 1 mM ADP, 10 mM potassium cyanide, and 15 μl of gradient fraction. The oxidation of NADH was monitored spectrophotometrically at 340 nm.

Nucleotide sequence accession number. The nucleotide sequence of the DNA fragment encoding the p110 protein has GenBank accession no. U31177.

RESULTS

Identification of a 110-kDa gRNA-binding protein. A clarified mitochondrial detergent extract (TS) was fractionated by Superose 6 gel filtration (data not shown), and an aliquot of the fraction containing the peak of the TUTase activity was incubated with synthetic α-^{32}P-labeled gND7-II gRNA. A major labeled complex (T-V) gel migrating with a molecular mass of approximately 560 kDa (38) was detected on a native gel (Fig. 1A). This gel fragment was subjected to UV cross-linking plus RNase A digestion, and a single labeled protein component migrating at 110 kDa was detected by SDS-acrylamide gel electrophoresis (Fig. 1A). The p110 protein was also the major gRNA-binding component in the unfractionated TS mitochondrial extract, as shown by the UV cross-linking assay in Fig. 1B, lane 2 (see also reference 9).

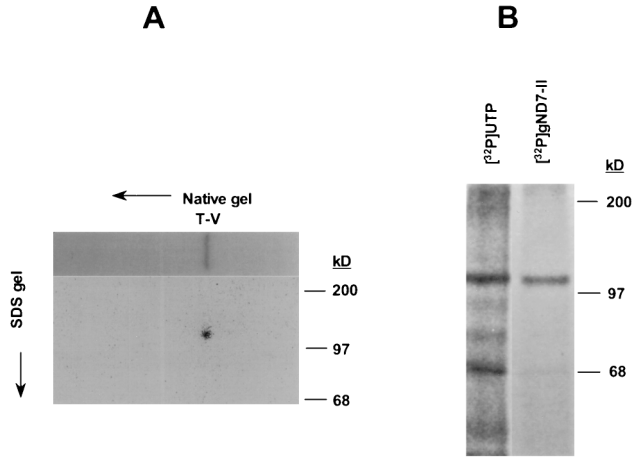

FIG. 1. Detection of p110 as a gRNA-binding protein. (A) Ten microliters of a Superose 6 gel filtration fraction of an *L. tarentolae* TS mitochondrial extract containing the peak of TUTase activity was incubated with [^{32}P]ATP-labeled gND7-II gRNA. The sample was electrophoresed in a 4 to 16% native acrylamide gradient gel, which was exposed wet. The lane was excised, UV cross-linked, and RNase A digested, and the labeled products were separated in an SDS-acrylamide gel. The dried gel was exposed for PhosphorImager analysis. (B) Aliquots (10 μl) of the TS mitochondrial extract were incubated for 40 min at 27°C with [^{32}P]UTP or with [^{32}P]ATP uniformly labeled synthetic gRNA (gND7-II). The material was then subjected to UV cross-linking and RNase A digestion, and the products were separated in an SDS-acrylamide gel. The gel was vacuum dried and exposed to film. Molecular weight markers are indicated.

We showed previously that the T complexes, especially T-II and T-IV, became labeled when the TS extract was incubated with [α-^{32}P]UTP (9, 38). The UV cross-linking experiment in Fig. 1B, lane 1, shows that the p110 protein can be cross-linked to an [α-^{32}P]UTP-labeled endogenous component of the TS extract. The UTP-labeled components in T-IV at this nucleotide concentration represent gRNAs which are labeled at the 3′ terminus with uridylyl residues by the endogenous TUTase activity, which has also been localized to this complex by an in situ gel assay (38). The UTP-labeled endogenous RNA component of the T-V complex was shown previously to represent mRNA fragments which were 3′ end labeled by the TUTase activity (38).

Cloning of the gene encoding the p110 protein and identification of p110 as a GDH. The TS extract was fractionated by two-dimensional native SDS gel electrophoresis, and the gel was electroblotted onto a membrane which was stained with Coomassie blue (data not shown). We had shown previously that a single prominent 110-kDa protein migrates in the T-V position in this fractionation (7). The protein was eluted from the filter and used for microsequence analysis of tryptic fragments and full-length protein.

N-terminal amino acid sequences were obtained for several tryptic peptides from the gel-isolated p110 protein (Fig. 2). The sequences were used to design degenerate PCR primers. A portion of the p110 cDNA was PCR amplified from total cell cDNA by use of degenerate oligonucleotides as 5′ and 3′ primers. The PCR fragment was cloned, sequenced, and used as a probe to clone a large genomic fragment from an *L. tarentolae* cosmid library. This fragment was partially se-

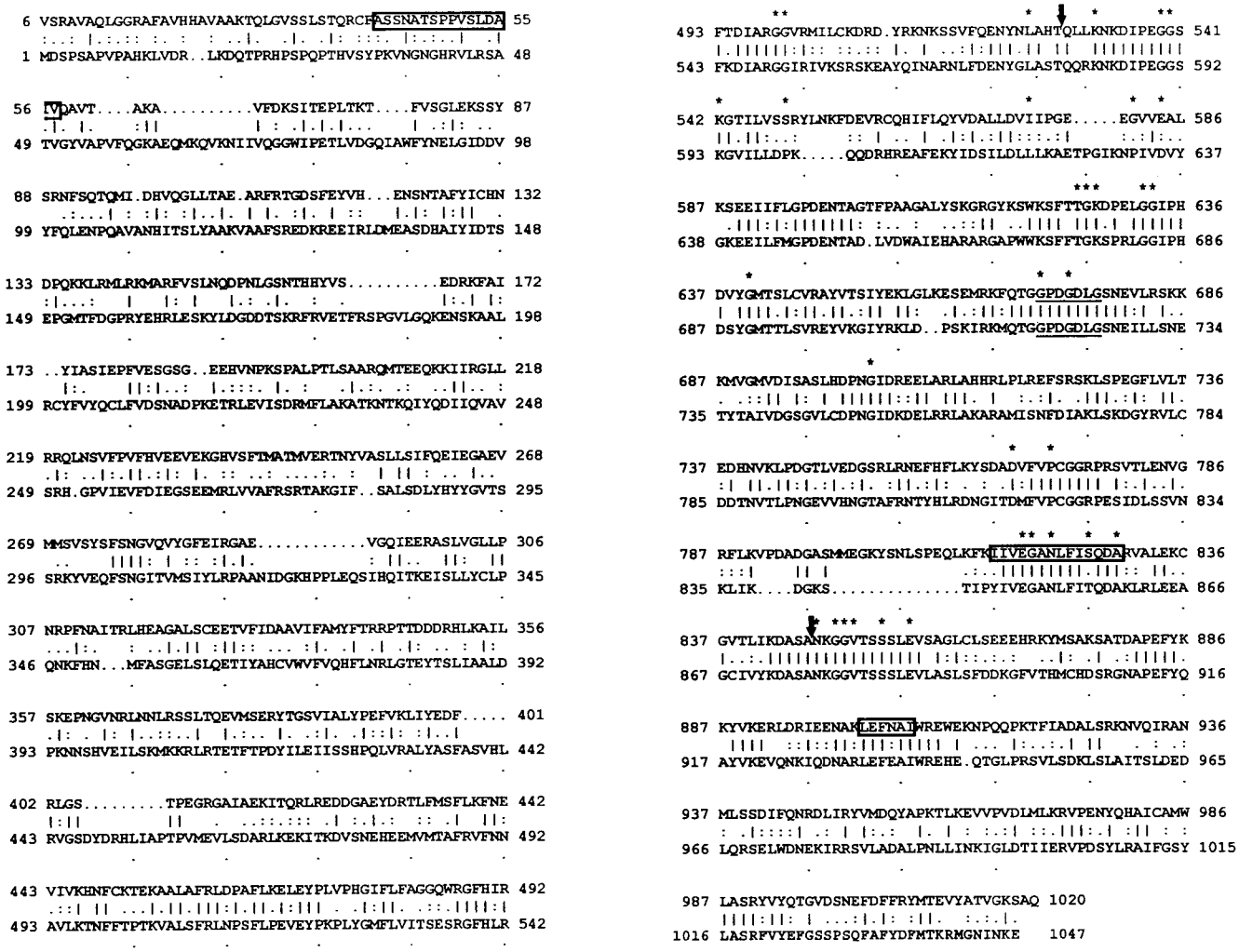

FIG. 2. Alignment of the p110 amino acid sequence (top line) with the *N. crassa* (29) mitochondrial GDH sequence (bottom line). The sequences were aligned by the Genetics Computer Group BESTFIT local alignment program. Gaps are indicated by dashes, identical amino acids are indicated by bars, and conservative amino acid substitutions are indicated by one or two dots. Asterisks indicate conserved residues found in both hexameric and tetrameric GDHs (8), and the consensus sequence for the dinucleotide-binding domain is underlined. The N-terminal sequence of the mature p110 protein (ASSNATSPPVSLDAIV) is boxed, as are the sequences of the two tryptic peptides pp59A (IIVEGANLFISQDA) and pp59B (LEFNAI) which were used to construct degenerate oligonucleotides for PCR. The fragment corresponding to the 327 amino acids expressed in *E. coli* as a fusion protein is demarcated by two lines. The N-terminal five amino acids of the p110 sequence (MMRHT) are not shown since they do not align with the *N. crassa* sequence.

quenced, and the sequence was found to contain an open reading frame coding for 1,020 amino acids (Fig. 2).

A BLAST homology search of this sequence against the Swiss Protein database showed that p110 has significant similarity with mitochondrial GDHs. The best alignment was with the *Neurospora crassa* enzyme (29), which exhibited 37% identity and 57% similarity with the p110 sequence (Fig. 2). Furthermore, the NAD-binding domain of the *N. crassa* GDH as well as many conserved residues in this protein showed a high degree of identity with the p110 sequence (Fig. 2). We conclude that the cloned *L. tarentolae* p110 protein represents a GDH homolog.

A Southern blot analysis using 13 restriction enzymes which do not cut within the gene sequence was performed. Single GDH-specific fragments were detected in all digestions, with the smallest fragment being approximately 7 kb in size (data not shown). This result suggests that the GDH gene is either single copy or no more than two tandem repeats at a single locus. The use of several enzymes which cut within the gene confirmed that the gene is single copy (29a).

Cellular localization of the GDH protein. Antibodies were raised against a GST fusion protein containing a 327-amino-acid fragment of the GDH protein which was overexpressed in *E. coli*. Polyclonal antiserum was obtained by immunization of a rabbit with the 70-kDa recombinant p110-GST fusion protein. As shown in Fig. 3, the immune serum specifically recognized a 110-kDa protein in a gradient-purified mitochondrial fraction, while no signal was obtained with a cytosolic fraction and a very small signal was obtained with a total cell lysate. These results suggest a mitochondrial localization of this protein. In addition, the release of the GDH protein from the mitochondrial membrane fraction by detergent lysis (Fig. 3A, lane TS) suggests that this protein is localized in the mitochondrial matrix.

Additional evidence for a mitochondrial localization of the GDH protein was the presence of a presequence which was en-

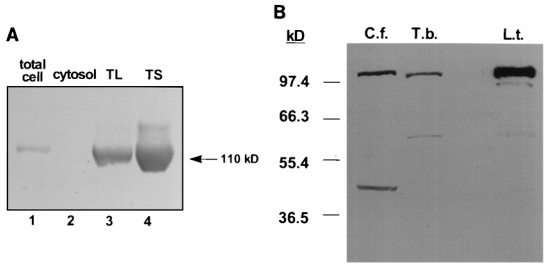

FIG. 3. Immunodetection of the p110 protein in mitochondrial extracts. (A) The indicated *L. tarentolae* extracts, after fractionation on SDS-acrylamide and transfer to a membrane, were probed with anti-p110 antiserum. Approximately 150 μg of protein was loaded in each lane. cytosol, the supernatant after removal of the mitochondrial pellet; TL, a Triton lysate of purified mitochondria; TS, a clarified Triton lysate of purified mitochondria. The p110 band is indicated. (B) Mitochondrial extracts from *C. fasciculata* (C.f.), procyclic *T. brucei* (T.b.), and *L. tarentolae* (L.t.) were fractionated on an SDS-acrylamide gel, which was electroblotted and probed with *L. tarentolae* p110 antiserum.

coded by the gene but was absent from the N-terminal sequence of the mature protein. The N-terminal sequence, ASXNAT SPXVXLDAIV, proved identical to the sequence of the GDH gene 41 amino acids downstream of the putative initiating methionine. The presequence may correspond to a mitochondrial signal sequence which is cleaved after the protein enters the mitochondrion (7, 58).

The *L. tarentolae* p110 antiserum recognized 110-kDa proteins in mitochondrial extracts from both *Crithidia fasciculata* and procyclic *T. brucei*, suggesting that homologous mitochondrial proteins are present in both trypanosomatid species (Fig. 3B). The 53-kDa fragment in the *C. fasciculata* lane represents a degradation product.

Characterization of p110-gRNA binding by gel shift assay. To decrease nonspecific interactions, the *L. tarentolae* mitochondrial TS extract was fractionated by sedimentation in a glycerol gradient prior to use for gel shift analysis. Each fraction was analyzed for the presence of p110 protein by Western blotting, TUTase activity, and GDH activity. The peak of TUTase activity was in fraction 4 (Fig. 4A), and the peak of GDH activity was in fraction 5. The antiserum recognized a single 110-kDa polypeptide which peaked in gradient fraction 5, together with the GDH activity. We showed previously that the [α-^{32}P]UTP-labeled T-V complex also sedimented at the leading edge of the TUTase peak and that a gel-shifted band comigrating in a native gel with this complex appeared when labeled gND7-II gRNA was incubated with the peak of TUTase activity from a Superose 6 fractionation (7, 38). It was also shown previously that a 110-kDa protein from this gel-isolated band was labeled after UV cross-linking and RNase digestion (7).

An identical glycerol gradient of the TS extract was used for gel shift analysis. In this gradient, the peak of TUTase activity also was in fraction 4 and the peak of p110 protein was in fraction 5 (data not shown). Gradient fractions 4 to 6 were utilized for the gel shift assay, using two different labeled gRNA probes and also a heterologous labeled RNA probe of similar size and specific activity (U1Amut). While several complexes formed between the gradient fractions and the gRNA probes (Fig. 5A, lanes 1 to 6), almost no signal could be detected when a heterologous RNA probe was used (Fig. 5A, lanes 7 to 9). The major high-molecular-weight complexes formed with the gRNA probes comigrated with T-V and T-VI. Similar results were obtained previously (7, 9). The formation of the gel-shifted band with labeled gND7-II gRNA was competed effectively by a 3-fold molar ratio of unlabeled gND7-II

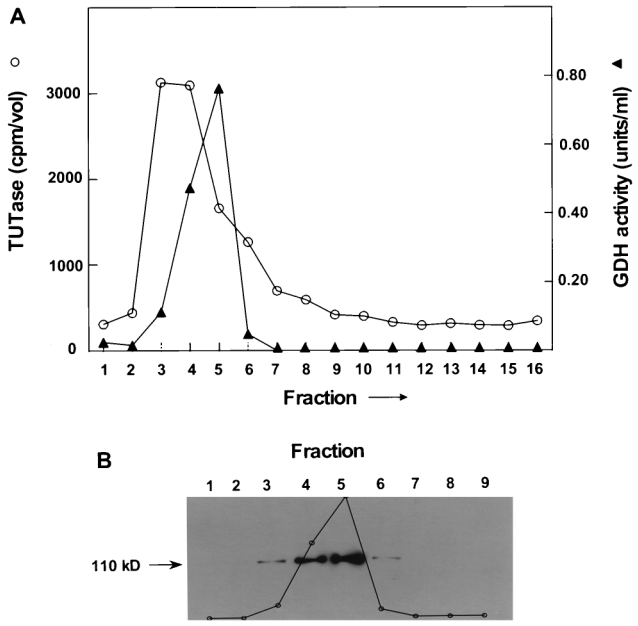

FIG. 4. Localization of p110 protein, TUTase activity, and GDH activity in glycerol gradient fractions of mitochondrial TS extract. (A) Aliquots (25 and 15 μl) of each gradient fraction were assayed for TUTase and GDH activities, respectively. (B) Aliquots (3 μl) of each gradient fraction were electrophoresed on SDS-acrylamide; the gel was blotted, and the filter was probed with p110 antiserum. Only fractions 1 to 9 are shown due to the absence of detectable bands in the remaining fractions. The superimposed graph shows the densitometric values of the bands. The gRNA-independent U insertion activity sedimented in fractions 8 to 10 at approximately 20S (data not shown) (38).

gRNA or gCyb-II gRNA added at time zero but unaffected by the same ratio of unlabeled pGem RNA or a 10-fold molar ratio of tRNA (data not shown). These complexes were analyzed by excision of the corresponding bands, UV and RNase A treatment, and SDS-acrylamide gel separation (data not

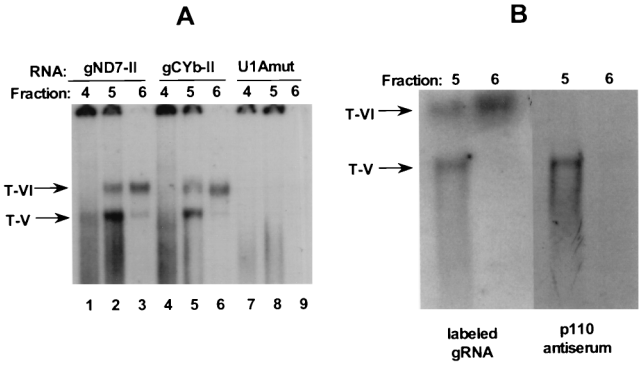

FIG. 5. Gel shift assay of labeled synthetic gRNAs and immunodetection of p110 in T-V. (A) Aliquots (10 μl) of glycerol gradient fractions 4, 5, and 6 of the TS mitochondrial extract were incubated with 0.5 pmol of [^{32}P]ATP-labeled synthetic gRNAs, and the resulting complexes were separated in a native 4% acrylamide gel. Two different labeled gRNAs, gND7-II and gCybII, and a short unrelated transcript, U1Amut, were used as probes. The T-V and T-VI complexes, which both bind exogenous labeled gRNA, are indicated by arrows. (B) Immunodetection of the p110-containing gel shift complex. One-half picomole of ^{32}P-labeled gND7-II gRNA was incubated with gradient fractions 5 and 6, and the complexes formed were analyzed in a native 4% acrylamide gel. The gel was electroblotted onto a nylon membrane, and the blot was exposed for autoradiography. The blot was then probed with anti-p110 antiserum. The position of the labeled T-V band is indicated.

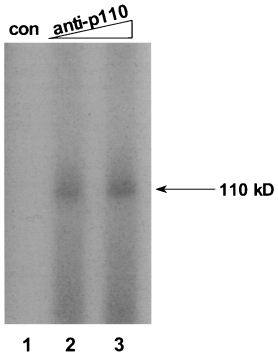

FIG. 6. Immunodetection of p110-gRNA complexes. Samples containing p110 UV cross-linked to labeled gND7-II gRNA were subjected to immunoprecipitation using preimmune serum (con) or p110 antiserum. After precipitation with protein A-Sepharose beads, the material was analyzed in an SDS-acrylamide gel.

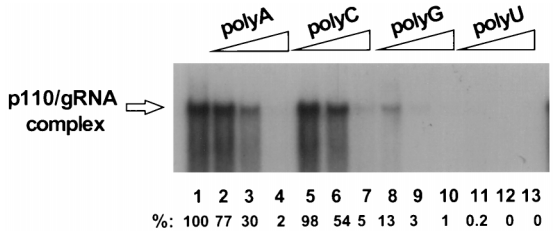

FIG. 7. Competition gel shift assays with polyribonucleotides. ^{32}P-labeled gND7-II RNA (0.05 pmol) was incubated in 12-μl reactions with glycerol gradient fraction 5 and increasing amounts (50-, 500-, and 5,000-fold molar excess) of unlabeled poly(U), poly(G), poly(C), and poly(A). After analysis of the samples on a native 4% acrylamide gel, formation of the p110-gRNA complex (arrow) was quantitated with a PhosphorImager. The values beneath the lanes represent the percentages of complex formed in presence of the competitors compared to that formed in control lane 1 with no competitors present.

shown), which showed that a 110-kDa protein in T-V was interacting with the bound labeled gRNA.

Immunodetection of the GDH protein in the complex detected by gel shifting of labeled gRNA was achieved by direct Western analysis of an electroblot of the native gel. Labeled synthetic gRNA was incubated with gradient fractions 5 and 6 (Fig. 5B), and the gel was then blotted and probed with p110 antiserum. The gel-shifted labeled complex contained the GDH protein (Fig. 5B).

The mitochondrial GDH is a gRNA-binding protein. The labeled gRNA-binding T-V complex obtained after UV cross-linking and RNase A digestion was reacted with p110 antiserum or preimmune serum. The immunoglobulins were then bound to protein A-Sepharose, and the bound proteins were eluted and fractionated in SDS-acrylamide. Preimmune serum did not precipitate any labeled protein, whereas immune serum precipitated a labeled 110-kDa protein (Fig. 6). This evidence, together with the Western blot analysis in Fig. 5B, indicates that the 110-kDa GDH protein in complex T-V is a gRNA-binding protein.

As shown in Fig. 5A, a heterologous RNA in the same size range (U1Amut) was not incorporated into the GDH-containing T-V complex. The specificity of the GDH-gRNA interaction was examined in more detail by competition experiments. Incorporation of ^{32}P-labeled gND7-II gRNA into the GDH complex was strongly competed by addition of unlabeled gND7-II gRNA added at time zero but not by addition of a similar-size transcript encoding unrelated pGem vector sequences (data not shown). However, when the gND7-II gRNA was added to preassembled GDH-gRNA complexes, little competition was observed (data not shown). The preformed GDH-gRNA complex was sensitive to high levels of heparin (greater than 100 μg/ml) and rRNA (250 μg/ml) (data not shown). These results indicate that GDH interacts with the gRNA in a specific manner and that the interaction is fairly stable.

The 3′ oligo(U) tail of the gRNA is critical for the GDH-gRNA interaction. Competition gel retardation experiments were performed with the four homopolyribonucleotides poly(A), poly(C), poly(G), and poly(U). Polyribonucleotides were added at 50-, 500-, and 5,000-fold molar excess over the labeled gND7-II transcript to the reaction prior to addition of the gradient fraction containing GDH. Even the highest titration points of poly(C) and poly(A) in the competition assay showed some complex formation (Fig. 7, lanes 4 and 7). Poly(G) showed an intermediate degree of competition (lanes 8 to 10), whereas a more dramatic competition was obtained with poly(U). A 50-fold molar excess of poly(U) over gRNA produced 99% inhibition of binding (lane 11), and higher levels of poly(U) virtually abolished p110-gRNA complex formation (lanes 12 and 13). These results suggest that the poly(U) tail of the gRNA could be a major target for the *L. tarentolae* GDH-gRNA interaction.

To directly investigate the relevance of the poly(U) tail in the GDH-gRNA complexes, an unrelated transcript synthesized from *Cla*I-digested plasmid pGem, with and without 13 3′-terminal U residues, was used. In the absence of the oligo(U) tail, this nonspecific transcript did not form a complex with GDH (Fig. 8A, lanes 4 to 6). However, the pGem RNA containing an oligo(U) tail was capable of forming a small amount of a complex comigrating with the GDH-gRNA complex (lanes 7 to 9). The presence of GDH in this complex was demonstrated by UV cross-linking and RNase A digestion (data not shown). However, this complex was more vulnerable to competition with other RNAs, indicating a lower-affinity interaction (data not shown).

Further evidence for the role of a 3′ oligo(U) tail is shown in Fig. 8B. Deletion of the U tail from the gRNA transcript or substitution with an oligo(C) tail significantly decreased, but did not totally abolish, the GDH-gRNA interaction. This result

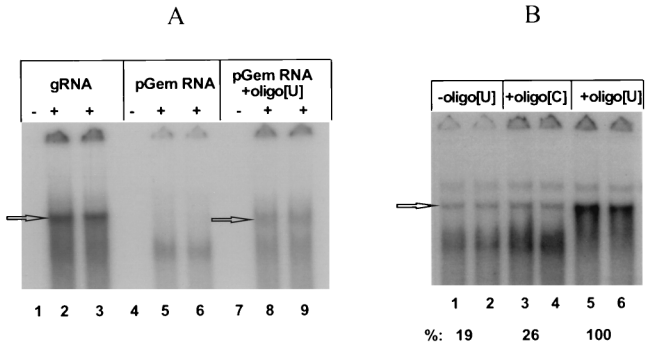

FIG. 8. The gRNA 3′ oligo(U) tail is the major target for gRNA-p110 complex formation. (A) The three indicated labeled synthetic RNAs were incubated with the TS mitochondrial extract, and the presence of a gel-shifted band was determined by native 4% acrylamide gel analysis. Lanes 1, 4, and 7 contain control samples with no extract. Lanes 2 and 3, 5 and 6, and 8 and 9 represent duplicate samples incubated with the TS extract. The gRNA used is gND7-II. pGem RNA+oligo[U] is a short pGem transcript with 13 U's at the 3′ end. The arrows indicate the p110-specific bands. (B) Aliquots of gND7-II gRNA without an oligo(U) tail (−oligo[U]), with an oligo(C) tail (+oligo[C]), and with an oligo(U) tail (+oligo[U]) were incubated with TS mitochondrial extract (in duplicate), and the relative amount of the gel-shifted band (arrow) was determined by native gel analysis. The percentages of label in the gel-shifted p110-specific bands in assays using the modified gRNAs compared to the amount of label in the band in assays using the normal gRNA are shown below the lanes.

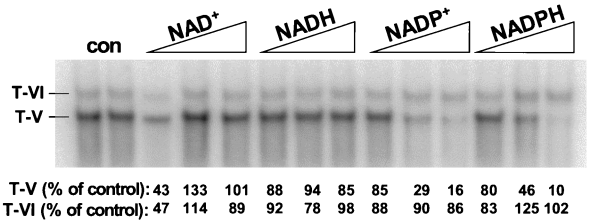

FIG. 9. $NADP^+$ and NADPH compete with gRNA for binding to p110. The gradient fraction containing p110 was incubated with NAD^+, NADH, $NADP^+$, and NADPH, as indicated, in various concentrations (50 μM, 500 μM, and 5 mM) for 10 min at 27°C. Afterwards, ^{32}P-labeled gND7-II gRNA was added, and the samples were incubated for 30 min at 27°C. After separation on a native 4% acrylamide gel, the formation of the labeled T-V complex containing p110 and the T-VI complex, which does not contain p110, was quantitated by PhosphorImager analysis. The two control lanes (con) represent duplicate samples. Below each experimental lane are the percentages of radioactivity in the T-V and T-VI bands relative to the amount in the control lanes.

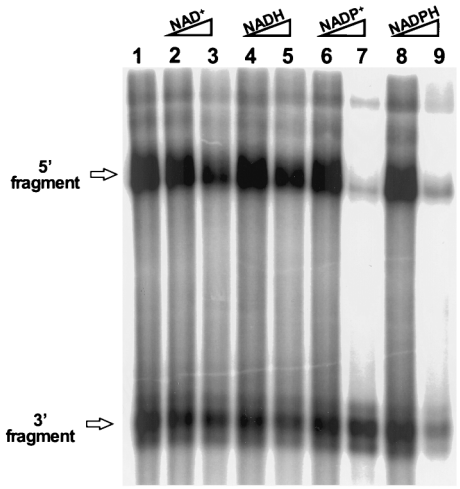

FIG. 10. Inhibition of in vitro gRNA-independent U insertions into preedited cytochrome *b* mRNA by NADP(H). The pNB2 substrate RNA was incubated with mitochondrial Triton lysate extract with [α-^{32}P]UTP as described in Materials and Methods. Different amounts of dinucleotides were added to the reaction mixtures prior to addition of the [α-^{32}P]UTP. The labeled intact RNA was gel isolated, annealed to an oligomer, and digested with RNase H. The 3′ and 5′ fragments (indicated by arrows) were separated by electrophoresis, and the relative amounts of label in the 5′ fragments were measured by PhosphorImager densitometry. Lane 1, control (no dinucleotide addition); lanes 2 to 9, 0.5 and 5 mM indicated dinucleotides added.

suggests that although a major determinant for GDH binding is the oligo(U) tail of the gRNA, additional regions may also be relevant for complex formation.

NADP dinucleotides inhibit binding of gRNA to GDH. The dinucleotides NAD^+, NADH, $NADP^+$, and NADPH were incubated with gradient fraction 5 prior to addition of labeled gND7-II gRNA, and the labeled complexes were resolved in a native gel. The relative amounts of T-V and T-VI complex formation were determined by PhosphorImager analysis. The formation of the labeled T-V complex was unaffected by addition of up to 5 mM NAD^+ or NADH but was strongly inhibited by 500 μM $NADP^+$ or NADPH, whereas the formation of the labeled T-VI complex was unaffected by these dinucleotides (Fig. 9). It should be noted that we showed previously that the formation of T-VI is highly dependent on the ratio of gRNA to mitochondrial extract and on the specific glycerol gradient fractions used, which accounts for the variable appearance of this band in different experiments (9).

$NADP^+$ specifically inhibits in vitro gRNA-independent and gRNA-dependent U insertion activity and does not affect 3′ TUTase activity. The experiment in Fig. 10 shows that 5 mM $NADP^+$ specifically inhibited the gRNA-independent (10, 15, 20) in vitro U insertion into the preedited domain of a cytochrome *b* mRNA substrate. There was little effect on the 3′ TUTase activity, as shown by the relative extent of labeling of the 3′ mRNA fragment. NAD^+ and NADH at the same concentration did not significantly affect either activity in this assay. However, the gRNA-independent U insertion activity of the glycerol gradient-purified 20S fraction was not affected by 5 mM $NADP^+$. It should be noted that gradient fractionation of the extract resulted in a substantial decrease in the absolute amount of U insertion activity from a 1:1 relative level compared to the 3′ TUTase activity with the same RNA in the same fractions to a 1:20 relative level, as was noted previously (38), and this may be relevant to the disappearance of the inhibitory effect of $NADP^+$.

Use of the primer extension assay of Byrne et al. (10) allowed the detection of the precise number of in vitro U insertions at site 1 of a preedited ND7 mRNA substrate. As shown in Fig. 11, both gRNA-independent U insertions in the ND7.1x preedited mRNA substrate and gRNA-dependent U insertions in the ND7.2x preedited mRNA substrate were inhibited by 1 to 5 mM NADP(H). NAD(H) at the same concentration produced an approximate 30 to 50% inhibition of these activities.

DISCUSSION

We have shown that a major gRNA-binding protein present in the approximately 560-kDa T-V complex in a mitochondrial extract from *L. tarentolae* is the 110-kDa GDH. The identification of this protein as a mitochondrial GDH was by cosedimentation in a glycerol gradient of the GDH activity and the 110-kDa protein recognized by a polyclonal antiserum and by the significant similarity of the sequence of a cloned and expressed fragment with the sequences of GDH proteins from other organisms. A major determinant of the gRNA recognized by the 110-kDa GDH appears to be the 3′ oligo(U) tail and the interaction appears to involve the NADP(H)-binding pocket of the enzyme since it can be specifically competed with this dinucleotide. It is of some interest that an immune serum against the *Drosophila* La protein, which is known to be a poly(U)-binding protein (23, 59), specifically recognizes the 110-kDa GDH protein in the mitochondrial extract from *L. tarentolae* as well as the recombinant protein encoded by plasmid pGEX110-2 (data not shown).

GDH activity in the mitochondrial TS extract was shown to utilize NAD^+ as a cofactor, which is consistent with the similarity of the *L. tarentolae* p110 amino acid sequence to the tetrameric NAD^+-binding GDH from *N. crassa* (8). However, the fact that the gRNA-binding activity of the GDH is specifically inhibited by NADP(H) and not NAD(H) suggests that the gRNA interacts with an NADP(H)-binding site. We also showed recently by UV cross-linking experiments that the *L. tarentolae* mitochondrial GDH binds monomeric UTP and that this binding is also strongly inhibited by high concentrations of NADP(H) and less strongly inhibited by NAD(H) (53a).

The specific inhibition of gRNA-independent and gRNA-dependent in vitro U insertion activity by NADP(H) at approximately the same concentration which inhibits the binding of p110 to gRNA provides suggestive evidence for a role of GDH or another dinucleotide-binding protein in RNA editing. However, the relatively high dinucleotide concentrations required

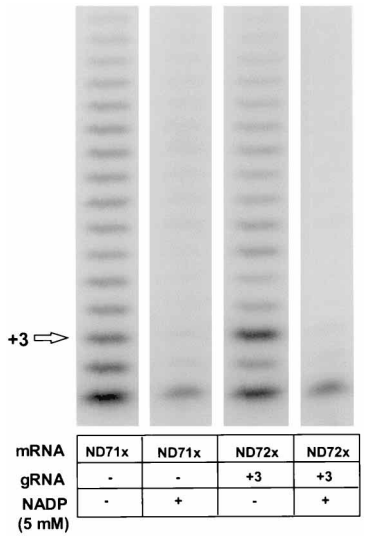

FIG. 11. Inhibition of in vitro gRNA-independent and gRNA-dependent U insertions into site 1 of preedited ND7x mRNA by NADP(H). The indirect primer extension assay of Byrne et al. (10) was used. The two first lanes show the ladder of gRNA-independent U insertions obtained with the ND7.1x mRNA substrate in the absence (lane 1) or in the presence (lane 2) of 5 mM NADP, and the two last lanes show the gRNA-dependent U insertions obtained with the ND7.2x mRNA substrate with added gND7x[+3] gRNA in the absence (lane 3) or in the presence (lane 4) of 5 mM NADP. The position of the +3 primer extension band is indicated on the left. The +1 band in all lanes is an artifact that occurs in the absence of treatment with mitochondrial lysate (10).

for both competition with gRNA 3′ oligo(U) binding and inhibition of in vitro editing-like activities argue against the specificity of this reaction. The loss of dinucleotide inhibition observed with a gradient-purified 20S fraction might be explained by the absence of GDH in the 20S fraction, as shown in Fig. 4. This remains to be investigated.

Both U insertion activities have been shown to require UTP in the in vitro reactions (10, 28). The gRNA[+3]-dependent U insertion reaction at site 1 of the ND7 mRNA substrate (10) was shown recently to require at least 30 μM UTP for maximum activity (9a), and it is likely that there is also a UTP requirement for U insertion editing in vivo. We speculate that one of the biological roles of the mitochondrial GDH may be to bind UTP and present it to the editing site. The binding of gRNA to the putative NADP(H) pocket may also play some role in this process, as evidenced by the selective effect of NADP(H) on in vitro editing. It is clear that further work is required to determine if GDH is actually involved in the mechanism of RNA editing.

In previous studies, several gRNA-binding proteins from *T. brucei* and *C. fasciculata* were identified on the basis of UV cross-linking experiments (30, 32, 44). In *T. brucei*, the most prominent proteins have molecular masses of approximately 83 to 90, 64, and 21 to 26 kDa (30, 32, 44), while in *C. fasciculata*, three proteins of 88, 65, and 30 kDa were found (32). It was suggested that the *T. brucei* 90-kDa protein resembles the *C. fasciculata* 65-kDa protein, in that they both bind to 3′ oligo(U) sequences and only poly(U) strongly competes for the binding. We have shown in this report that the *L. tarentolae* p110 antiserum recognizes 110-kDa mitochondrial proteins in both *C. fasciculata* and *T. brucei*. Further work must be performed to determine if these proteins correspond to any of the previously identified gRNA-binding proteins in these species.

Metabolic enzymes containing dinucleotide-binding domains have been proposed as a novel class of RNA-binding proteins (27, 31). Some of the enzymes that fall into this expanding group are glyceraldehyde-3-phosphate dehydrogenase, which by binding to tRNA may be involved in nuclear export (54), the human thymidylate synthase and dihydrofolate reductase proteins, which show binding to their own mRNAs and may function as translational autoregulators (13, 14), and the yeast mitochondrial NAD^+-dependent isocitrate dehydrogenase, which binds specifically to 5′ untranslated leaders of mitochondrial mRNAs (19). GDH from bovine liver mitochondria was also previously reported to be an RNA-binding protein (43). However, the bovine GDH interacted with mitochondrial cytochrome oxidase transcripts, and the interaction was not competed by poly(U) but was competed by cytosolic RNA. The bovine GDH is also smaller (50 kDa) than the 110-kDa *L. tarentolae* GDH.

It has been proposed that metabolic RNA-binding enzymes participate in general regulatory circuits linking a metabolic function to a regulatory mechanism, similar to the situation of the metabolic enzyme aconitase, which also functions as an iron-responsive RNA-binding regulatory element (16, 27). However, some workers have cautioned that some of these types of enzymes may merely represent "molecular fossils" of the transition from an RNA to a protein world and that the RNA-binding properties may not have a functional significance (31). Determination of the role, if any, of the mitochondrial GDH in RNA editing must await further experimentation.

ACKNOWLEDGMENTS

This project was supported in part by research grant AI-09102 from the National Institutes of Health to L.S. We thank Xuan Liu for participating in the isolation of the p110 protein for microsequence analysis, S. Wolin and C. Yoo for the gift of the La antiserum, and D. Campbell for supplying the *L. tarentolae* cosmid library. We thank Margarita Tayag and Shirley Wang for technical assistance.

R.S. was supported by a postdoctoral fellowship from the Deutsche Forschungsgemeinshaft.

REFERENCES

1. **Bakalara, N., A. M. Simpson, and L. Simpson.** 1989. The *Leishmania* kinetoplast-mitochondrion contains terminal uridylyltransferase and RNA ligase activities. J. Biol. Chem. **264:**18679–18686.
2. **Benne, R.** 1994. RNA editing in trypanosomes. Eur. J. Biochem. **221:**9–23.
3. **Blum, B., N. Bakalara, and L. Simpson.** 1990. A model for RNA editing in kinetoplastid mitochondria: "guide" RNA molecules transcribed from maxicircle DNA provide the edited information. Cell **60:**189–198.
4. **Blum, B., and L. Simpson.** 1990. Guide RNAs in kinetoplastid mitochondria have a nonencoded 3′ oligo-(U) tail involved in recognition of the pre-edited region. Cell **62:**391–397.
5. **Blum, B., N. R. Sturm, A. M. Simpson, and L. Simpson.** 1991. Chimeric gRNA-mRNA molecules with oligo(U) tails covalently linked at sites of RNA editing suggest that U addition occurs by transesterification. Cell **65:**543–550.
6. **Braly, P., L. Simpson, and F. Kretzer.** 1974. Isolation of kinetoplast-mitochondrial complexes from *Leishmania tarentolae*. J. Protozool. **21:**782–790.
7. **Bringaud, F., M. Peris, K. H. Zen, and L. Simpson.** 1995. Characterization of two nuclear-encoded protein components of mitochondrial ribonucleoprotein complexes from *Leishmania tarentolae*. Mol. Biochem. Parasitol. **71:**65–79.
8. **Britton, K. L., P. J. Baker, D. W. Rice, and T. J. Stillman.** 1992. Structural relationship between the hexameric and tetrameric family of glutamate dehydrogenases. Eur. J. Biochem. **209:**851–859.
9. **Byrne, E., F. Bringaud, and L. Simpson.** 1995. RNA-protein interactions in the ribonucleoprotein T-complexes in a mitochondrial extract from *Leishmania tarentolae*. Mol. Biochem. Parasitol. **72:**65–76.

9a. **Byrne, E., and L. Simpson.** Unpublished results.

10. **Byrne, E. M., G. J. Connell, and L. Simpson.** 1996. Guide RNA-directed uridine insertion RNA editing in vitro. EMBO J. **15:**101–108.
11. **Campbell, D. A.** 1989. c2X75, a derivative of the cosmid vector c2XB. Nucleic Acids Res. **17:**458.
12. **Cech, T. R.** 1991. RNA editing: world's smallest introns. Cell **64:**667–669.
13. **Chu, E., D. M. Koeller, J. L. Casey, J. C. Drake, B. A. Chabner, P. C. Elwood, S. Zinn, and C. J. Allegra.** 1991. Autoregulation of human thymidylate synthase messenger RNA translation by thymidylate synthase. Proc. Natl. Acad. Sci. USA **88:**8977–8981.

14. **Chu, E., C. H. Takimoto, D. Voeller, J. L. Grem, and C. J. Allegra.** 1993. Specific binding of human dihydrofolate reductase protein to dihydrofolate reductase messenger RNA in vitro. Biochemistry **32:**4756–4760.
15. **Connell, G. J., E. M. Byrne, and L. Simpson.** 1997. Guide RNA-independent and guide RNA-dependent urdine insertion into cytochrome b mRNA in a mitochondrial extract from *Leishmania tarentolae*. J. Biol. Chem. **272:**4212–4218.
16. **Constable, A., S. Quick, N. K. Gray, and M. W. Hentze.** 1992. Modulation of the RNA-binding activity of a regulatory protein by iron in vitro: switching between enzymatic and genetic function? Proc. Natl. Acad. Sci. USA **89:** 4554–4558.
17. **Corell, R. A., L. K. Read, G. R. Riley, J. K. Nellissery, T. E. Allen, M. L. Kable, M. D. Wachal, S. D. Seiwert, P. J. Myler, and K. D. Stuart.** 1996. Complexes from *Trypanosoma brucei* that exhibit deletion editing and other editing-associated properties. Mol. Cell. Biol. **16:**1410–1418.
18. **Cruz-Reyes, J., and B. Sollner-Webb.** 1996. Trypanosome U-deletional RNA editing involves guide RNA-directed endonuclease cleavage, terminal U exonuclease, and RNA ligase activities. Proc. Natl. Acad. Sci. USA **93:**8901–8906.
19. **Elzinga, S. D., A. L. Bednarz, K. van Oosterum, P. J. Dekker, and L. A. Grivell.** 1993. Yeast mitochondrial NAD(+)-dependent isocitrate dehydrogenase is an RNA-binding protein. Nucleic Acids Res. **21:**5328–5331.
20. **Frech, G. C., N. Bakalara, L. Simpson, and A. M. Simpson.** 1995. In vitro RNA editing-like activity in a mitochondrial extract from *Leishmania tarentolae*. EMBO J. **14:**178–187.
21. **Frech, G. C., and L. Simpson.** 1996. Uridine insertion into preedited mRNA by a mitochondrial extract from *Leishmania tarentolae*: stereochemical evidence for the enzyme cascade model. Mol. Cell. Biol. **16:**4584–4589.
22. **Goringer, H. U., D. J. Koslowsky, T. H. Morales, and K. Stuart.** 1994. The formation of mitochondrial ribonucleoprotein complexes involving guide RNA molecules in *Trypanosoma brucei*. Proc. Natl. Acad. Sci. USA **91:**1776–1780.
23. **Gottlieb, E., and J. A. Steitz.** 1989. Function of the mammalian La protein: evidence for its action in transcription termination by RNA polymerase III. EMBO J. **8:**851–861.
24. **Hajduk, S. L., M. E. Harris, and V. W. Pollard.** 1993. RNA editing in kinetoplastid mitochondria. FASEB J. **7:**54–63.
25. **Harlow, E., and D. Lane.** 1988. Antibodies—a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
26. **Harris, M., C. Decker, B. Sollner-Webb, and S. Hajduk.** 1992. Specific cleavage of pre-edited mRNAs in trypanosome mitochondrial extracts. Mol. Cell. Biol. **12:**2591–2598.
27. **Hentze, M. W.** 1994. Enzymes as RNA-binding proteins: a role for (di)nucleotide-binding domains? Trends Biochem. Sci. **19:**101–103.
28. **Kable, M. L., S. D. Seiwert, S. Heidmann, and K. Stuart.** 1996. RNA editing: a mechanism for gRNA-specified uridylate insertion into precursor mRNA. Science **273:**1189–1195.
29. **Kapoor, M., Y. Vijayaraghavan, R. Kadonaga, and K. E. LaRue.** 1993. NAD(+)-specific glutamate dehydrogenase of *Neurospora crassa*: cloning, complete nucleotide sequence, and gene mapping. Biochem. Cell Biol. **71:** 205–219.

29a. **Kirszenbaum, F., and L. Simpson.** Unpublished results.

30. **Köller, J., G. Nörskau, A. S. Paul, K. Stuart, and H. U. Goringer.** 1994. Different *Trypanosoma brucei* guide RNA molecules associate with an identical complement of mitochondrial proteins *in vitro*. Nucleic Acids Res. **22:**1988–1995.
31. **Kyrpides, N. C., and C. A. Ouzounis.** 1995. Nucleic acid-binding metabolic enzymes: living fossils of stereochemical interactions. J. Mol. Evol. **40:**564–569.
32. **Leegwater, P., D. Speijer, and R. Benne.** 1995. Identification by UV cross-linking of oligo(U)-binding proteins in mitochondria of the insect trypanosomatid *Crithidia fasciculata*. Eur. J. Biochem. **227:**780–786.
33. **Lima, B. D., and L. Simpson.** 1996. Sequence-dependent in vivo importation of tRNAs into the mitochondrion of *Leishmania tarentolae*. RNA **2:**429–440.
34. **Miller, R. L., A. L. Wang, and C. C. Wang.** 1988. Identification of *Giardia lamblia* isolates susceptible and resistant to infection by the double-stranded RNA virus. Exp. Parasitol. **66:**118–123.
35. **Milligan, J. F., D. R. Groebe, G. W. Witherell, and O. C. Uhlenbeck.** 1987. Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. Nucleic Acids Res. **15:**8783–8798.
36. **Missel, A., and H. U. Goringer.** 1994. *Trypanosoma brucei* mitochondria contain RNA helicase activity. Nucleic Acids Res. **22:**4050–4056.
37. **Missel, A., G. Nörskau, H. H. Shu, and H. U. Göringer.** 1995. A putative RNA helicase of the DEAD box family from *Trypanosoma brucei*. Mol. Biochem. Parasitol. **75:**123–126.
38. **Peris, M., G. C. Frech, A. M. Simpson, F. Bringaud, E. Byrne, A. Bakker, and L. Simpson.** 1994. Characterization of two classes of ribonucleoprotein complexes possibly involved in RNA editing from *Leishmania tarentolae* mitochondria. EMBO J. **13:**1664–1672.
39. **Peris, M., A. M. Simpson, J. Grunstein, J. E. Lilienthal, G. C. Frech, and L. Simpson.** 1997. Native gel analysis of ribonucleoprotein complexes from a *Leishmania tarentolae* mitochondrial extract. Mol. Biochem. Parasitol. **85:**9–24.
40. **Piller, K. J., C. J. Decker, L. N. Rusche, and B. Sollner-Webb.** 1995. *Trypanosoma brucei* mitochondrial guide RNA-mRNA chimera-forming activity cofractionates with an editing-domain-specific endonuclease and RNA ligase and is mimicked by heterologous nuclease and RNA ligase. Mol. Cell. Biol. **15:**2925–2932.
41. **Pollard, V. W., M. E. Harris, and S. L. Hajduk.** 1992. Native mRNA editing complexes from Trypanosoma brucei mitochondria. EMBO J. **11:**4429–4438.
42. **Pollard, V. W., S. P. Rohrer, E. F. Michelotti, K. Hancock, and S. L. Hajduk.** 1990. Organization of minicircle genes for guide RNAs in Trypanosoma brucei. Cell **63:**783–790.
43. **Preiss, T., A. G. Hall, and R. N. Lightowlers.** 1993. Identification of bovine glutamate dehydrogenase as an RNA-binding protein. J. Biol. Chem. **268:** 24523–24526.
44. **Read, L. K., H. U. Goringer, and K. Stuart.** 1994. Assembly of mitochondrial ribonucleoprotein complexes involves specific guide RNA (gRNA)-binding proteins and gRNA domains but does not require preedited mRNA. Mol. Cell. Biol. **14:**2629–2639.
45. **Sabatini, R., and S. L. Hajduk.** 1995. RNA ligase and its involvement in guide RNA/mRNA chimera formation. J. Biol. Chem. **270:**1–8.
46. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:**487–491.
47. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
48. **Seiwert, S. D., S. Heidmann, and K. Stuart.** 1996. Direct visualization of uridylate deletion in vitro suggests a mechanism for kinetoplastid RNA editing. Cell **84:**1–20.
49. **Seiwert, S. D., and K. Stuart.** 1994. RNA editing: transfer of genetic information from gRNA to precursor mRNA in vitro. Science **266:**114–117.
50. **Shu, H. H., K. Stuart, and H. U. Goringer.** 1995. Guide RNA molecules not engaged in RNA editing form ribonucleoprotein complexes free of mRNA. Biochim. Biophys. Acta **1261:**349–359.
51. **Simpson, A. M., N. Bakalara, and L. Simpson.** 1992. A ribonuclease activity is activated by heparin or by digestion with proteinase K in mitochondrial extracts of *Leishmania tarentolae*. J. Biol. Chem. **267:**6782–6788.
52. **Simpson, L., and P. Braly.** 1970. Synchronization of *Leishmania tarentolae* by hydroxyurea. J. Protozool. **17:**511–517.
53. **Simpson, L., D. A. Maslov, and B. Blum.** 1993. RNA editing in *Leishmania* mitochondria, p. 53–85. *In* R. Benne (ed.), RNA editing—the alteration of protein coding sequences of RNA. Ellis Horwood, New York, N.Y.

53a. **Simpson, L., and M. Tayag.** Unpublished results.

54. **Singh, R., and M. R. Green.** 1993. Sequence-specific binding of transfer RNA by glyceraldehyde-3-phosphate dehydrogenase. Science **259:**365–368.
55. **Smith, H. O., K. Tabiti, G. Schaffner, D. Soldati, U. Albrecht, and M. L. Birnstiel.** 1991. Two-step affinity purification of U7 small nuclear ribonucleoprotein particles using complementary biotinylated 2′-*O*-methyl oligoribonucleotides. Proc. Natl. Acad. Sci. USA **88:**9784–9788.
56. **Stripecke, R., and M. W. Hentze.** 1992. Bacteriophage and spliceosomal proteins function as position-dependent cis/trans repressors of mRNA translation in vitro. Nucleic Acids Res. **20:**5555–5564.
57. **Stuart, K.** 1993. RNA editing in mitochondria of African trypanosomes, p. 25–52. *In* R. Benne (ed.), RNA editing—the alteration of protein coding sequences of RNA. Ellis Horwood, New York, N.Y.
58. **Xu, C., and D. S. Ray.** 1993. Isolation of proteins associated with kinetoplast DNA networks *in vivo*. Proc. Natl. Acad. Sci. USA **90:**1786–1789.
59. **Yoo, C. J., and S. L. Wolin.** 1994. La proteins from *Drosophila melanogaster* and *Saccharomyces cerevisiae*: a yeast homolog of the La autoantigen is dispensable for growth. Mol. Cell Biol. **14:**5412–5424.