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**REPORT**

# Identification of candidate mitochondrial RNA editing ligases from *Trypanosoma brucei*

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## ABSTRACT

**Most mitochondrial genes of *Trypanosoma brucei* do not contain the necessary information to make translatable mRNAs. These transcripts must undergo RNA editing, a posttranscriptional process by which uridine residues are added and deleted from mitochondrial mRNAs. RNA editing is believed to be catalyzed by a ribonucleoprotein complex containing endonucleolytic, terminal uridylyl transferase (TUTase), 3′ uridine-specific exonucleolytic (U-exo), and ligase activities. None of the catalytic enzymes for RNA editing have been identified. Here we describe the identification of two candidate RNA ligases (48 and 52 kDa) that are core catalytic components of the *T. brucei* ribonucleoprotein editing complex. Both enzymes share homology to the covalent nucleotidyl transferase superfamily and contain five key signature motifs, including the active site KXXG. In this report, we present data on the proposed 48 kDa RNA editing ligase. We have prepared polyclonal antibodies against recombinant 48 kDa ligase that specifically recognize the trypanosome enzyme. When expressed in trypanosomes as an epitope-tagged fusion protein, the recombinant ligase localizes to the mitochondrion, associates with RNA editing complexes, and adenylates with ATP. These findings provide strong support for the enzymatic cascade model for kinetoplastid RNA editing.**

**Keywords: epitope tag; nucleotidyl transferase; PSI-BLAST; trypanosome**

## INTRODUCTION

Mitochondrial mRNAs in kinetoplastids undergo a remarkable process whereby uridine residues are added and/or deleted within the mRNAs (Benne et al., 1986; Stuart et al., 1997; Estevez & Simpson, 1999). This posttranscriptional process is termed RNA editing, and functionally serves to correct gene-encoded frameshifts, create new start codons, and even form complete open reading frames. In some cases, over 50% of an mRNA's content is added posttranscriptionally (Feagin et al., 1988). The mechanism for RNA editing remains unknown, and several different models for RNA editing have been proposed. One model proposes the involvement of multiple proteins in a series of enzymatic steps (the enzymatic cascade model; Blum et al., 1990). In this model, the pre-mRNA is endonucleolytically cleaved at a specific site to yield a 5′ and 3′ RNA fragment. At this cleavage site, the proper number of uridine residues are either added by a 3′ terminal uridylyl transferase (TUTase) or removed by a 3′ uridylyl-specific exonuclease (U-exo). An RNA ligase then ligates these two RNA fragments together to complete one round of RNA editing. In an alternate model, RNA editing would proceed through a series of transesterification steps, perhaps analogous to group I and II RNA splicing (Cech, 1991). The transesterification model suggests an evolutionary link between RNA editing and splicing.

Currently, the enzymatic cascade model is favored, based on in vitro studies (Kable et al., 1996; Seiwert et al., 1996), and several groups have shown that TUTase, U-exo, endonuclease, and ligase activities copurify as a stable 19S ribonucleoprotein complex (Pollard et al., 1992; Peris et al., 1994; Corell et al., 1996; Adler & Hajduk, 1997; Rusche et al., 1997; McManus et al., 2000). This 19S complex is considered to be the minimal editing complex, and a larger, less stable 35-40S

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complex is considered to be the active editing complex because it contains the preedited mRNA.

In *Trypanosoma brucei*, proteins of 50 and 57 kDa comigrate with ligase activity in editing complexes. These proteins are readily detected by adenylylation with [$\alpha$-$^{32}$P]-ATP, and deadenylylation occurs when they are incubated with added ligatable RNA (Sabatini & Hajduk, 1995). Ligases can be adenylylated because their catalytic mechanism involves the hydrolysis of ATP or NAD to yield an AMP covalently attached to a conserved active site lysine. The adenylylated enzyme then transfers the AMP moiety to the free 5′-phosphate at the end of the DNA or RNA strand. The final step is the phosphodiester bond formation with a 3′-hydroxyl concomitant with the release of the 5′-AMP.

ATP-dependent ligases belong to a superfamily of covalent nucleotidyl transferases that appear to have diverged from a common ancestral enzyme (Shuman & Schwer, 1995). Enzymes within this superfamily include mRNA capping enzymes, tRNA ligases, ATP- and NAD-dependent DNA ligases, and RNA ligases. Overall, the amino acid sequences among these different enzymes are quite different, but each enzyme contains at least five short sequence motifs that are conserved in the same order and with similar spacing (Shuman & Schwer, 1995; Aravind & Koonin, 1999). Several different covalent nucleotidyl transferases have been crystallized, and their structures determined. Elucidation of these protein structures has increased our understanding of the enzymatic mechanism of the covalent nucleotidyl transferase enzymes. All enzymes within this superfamily require a specific nucleotide for activity, and a comparison of the different structures revealed that the phylogenetically conserved motifs are located around the nucleotide bound at the active site KXXG motif.

Since the initial discovery of RNA editing, none of the proposed editing complex catalytic components have been identified. This is probably due to the difficulty in purifying mitochondrial editing complexes, and perhaps due to their presumed low abundance in mitochondrial extracts. Furthermore, the available in vitro editing assays are extremely inefficient and not sensitive enough to use effectively in purification. To identify potential editing proteins, we have taken a computational approach, because (1) RNA ligases are homologous, (2) there are a number of sensitive protein motif search programs readily available, and (3) sequencing of the genome of *T. brucei* is nearing completion.

In this article we describe the identification and cloning of two candidate RNA ligases (48 and 52 kDa) of *T. brucei*. When the 48-kDa protein is expressed in *T. brucei* lines as an epitope-tagged fusion protein, it localizes to the mitochondrion, associates with the 19S editing complex, and is adenylylated with ATP. These results suggest that the 48-kDa protein is a ligase and is physically associated with the RNA editing complexes of *T. brucei*.

## RESULTS AND DISCUSSION

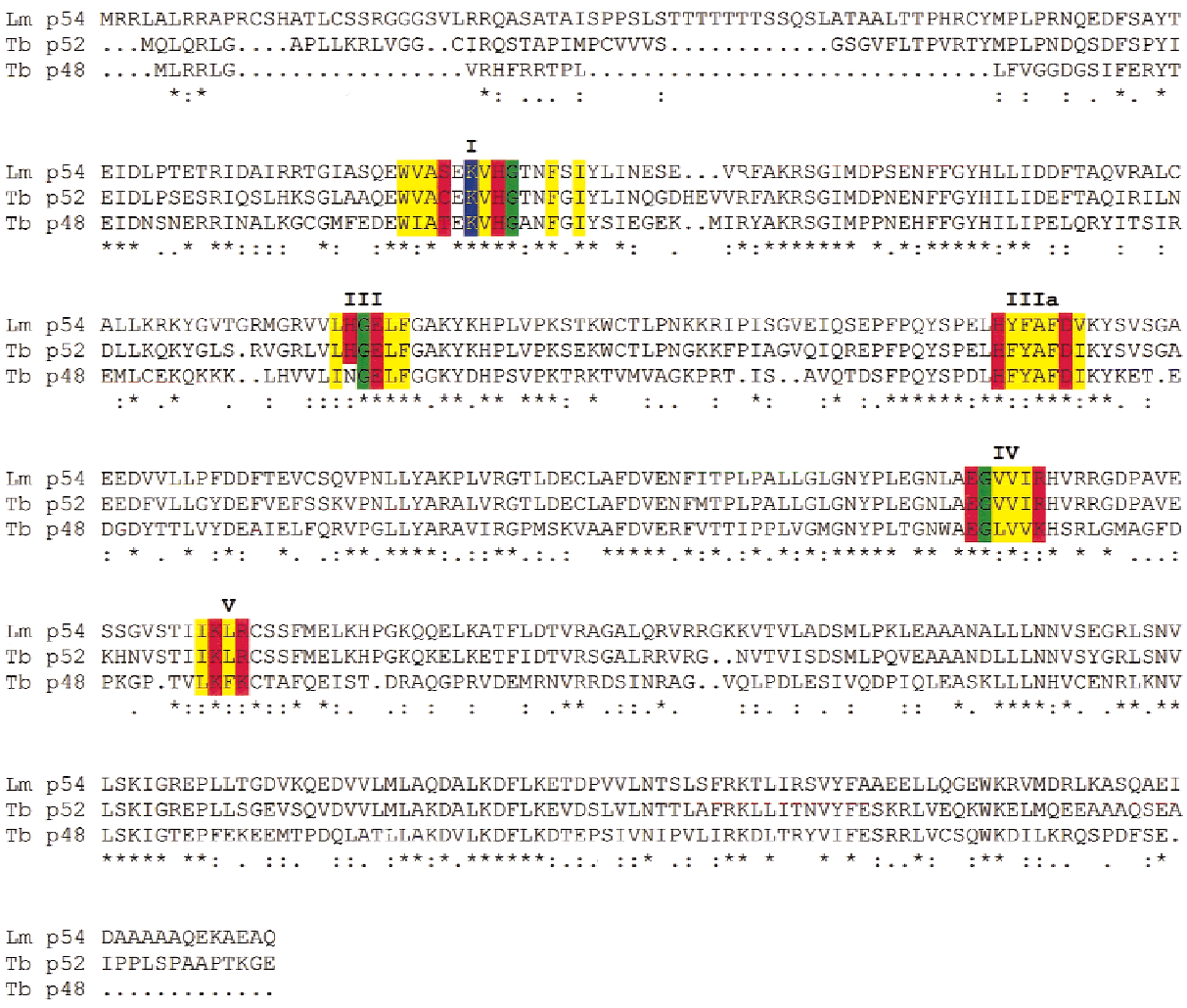
### Identification and sequence analysis

Because the overall sequence homology between ligases is quite weak, simple homology searches are not sufficient to easily identify members within this family. Recently, Avarind and Koonin investigated the ability of the iterative sequence database search method, PSI-BLAST, to identify subtle relationships among the covalent nucleotidyl transferase superfamily (Altschul et al., 1997; Aravind & Koonin, 1999). A 54-kDa candidate RNA ligase from *Leishmania major* was identified in this search, and was proposed to play a role in RNA editing. Using this sequence, we performed BLASTP searches on the TIGR and Sanger *T. brucei* genome databases, and found two homologous sequences (Fig. 1A). The first sequence contained a complete 48-kDa open reading frame and shared a 45% amino acid similarity to the *leishmania* sequence (Fig. 1A). The second sequence corresponded to a larger 52-kDa open reading frame and shared a 75% homology to the *L. major* 54-kDa candidate ligase. Because the 48-kDa and 52-kDa proteins only share a 39% homology to each other, the larger 52-kDa sequence is likely an ortholog of the *L. major* 54-kDa putative ligase. All three sequences share homology to the covalent nucleotidyl transferase superfamily and contain five key signature motifs, including the active site KXXG (Fig. 1B). The calculated 48- and 52-kDa protein sequences identified here are likely to correspond to the previously described 50- and 57-kDa RNA editing ligases of *T. brucei* (Sabatini & Hajduk, 1995; Rusche et al., 1997).

### Antibodies against *Escherichia coli* recombinant p48 recognize the trypanosome adenylylated ligase

To examine the relationship of p48 to the 50-kDa mitochondrial RNA ligase, recombinant p48 was expressed in *E. coli* and used to prepare rabbit polyclonal serum. These antibodies were used to examine the potential association of the endogenous trypanosome p48 protein to mitochondrial RNA editing complexes. Mitochondrial proteins were fractionated on glycerol gradients, and a portion of each fraction was analyzed on immunoblots using antibodies against the recombinant p48-kDa protein. To visualize the mitochondrial RNA ligases, the fractions were incubated with [$\alpha$-$^{32}$P]-ATP prior to separation on the SDS-gel. The gel was immunoblotted with anti-p48 and autoradiographed to detect the RNA editing complex associated ligases (Fig. 2A). The autoradiogram of the immunoblot showed that the 50-kDa adenylylated ligase overlaid exactly with the p48 band on the immunoblot. Adenylylation of the 50- and 57-kDa ligases decreased sharply in fraction 12, whereas the immunoreactive p48 band persists through

A

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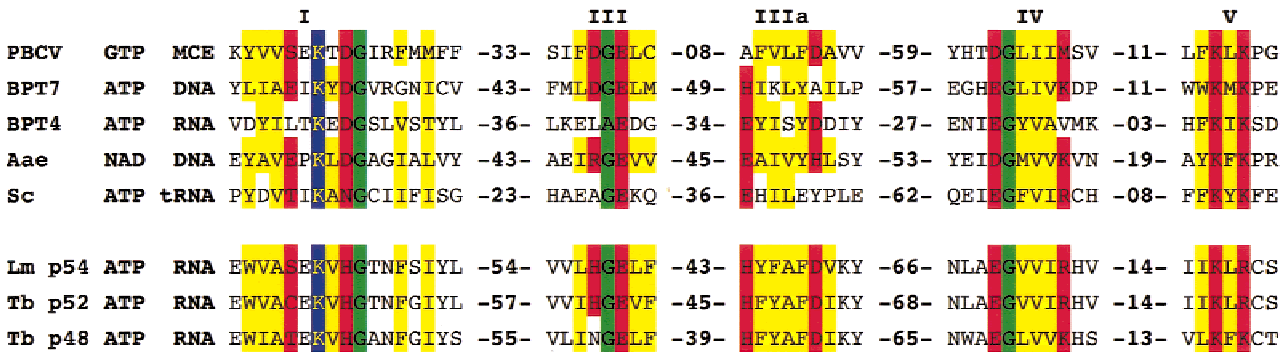


**FIGURE 1.** Sequences of three candidate kinetoplastid RNA editing ligases. **A**: Tb p48 and Tb p52 likely correspond to the previously described *T. brucei* 50- and 57-kDa protein sequences; Lm p54 corresponds to the *L. major* 54-kDa sequence. Conserved covalent nucleotidyl transferase superfamily motifs are color shaded and are labeled (with Roman numerals according to nucleotidyl transferase convention). The consensus is shown below, where asterisks represent fully conserved residues, and colons and periods represent strongly and weakly conserved residues, respectively. **B**: Tb48, Tb52, and Lm54 are members of the ligase superfamily. Motifs shown in **A** are aligned to various members of the covalent nucleotidyl transferase superfamily. PBCV: *Paramecium bursaria* Chlorella virus; BPT7: bacteriophage T7; BPT4: bacteriophageT4; Aae: *Aquifolius aeolius*; Sc: *Sacharomyces cerevisiae.* Lm p54, Tb p48, and Tb p52 are as above. GTP, ATP, or NAD is the nucleotide substrates for each respective enzyme. MCE: mRNA capping enzyme; DNA: DNA ligase; RNA: RNA ligase; tRNA: tRNA ligase. Shading is as follows: red: charged residues; yellow: hydrophobic residues; green: small residues; and blue: active site lysine residue.

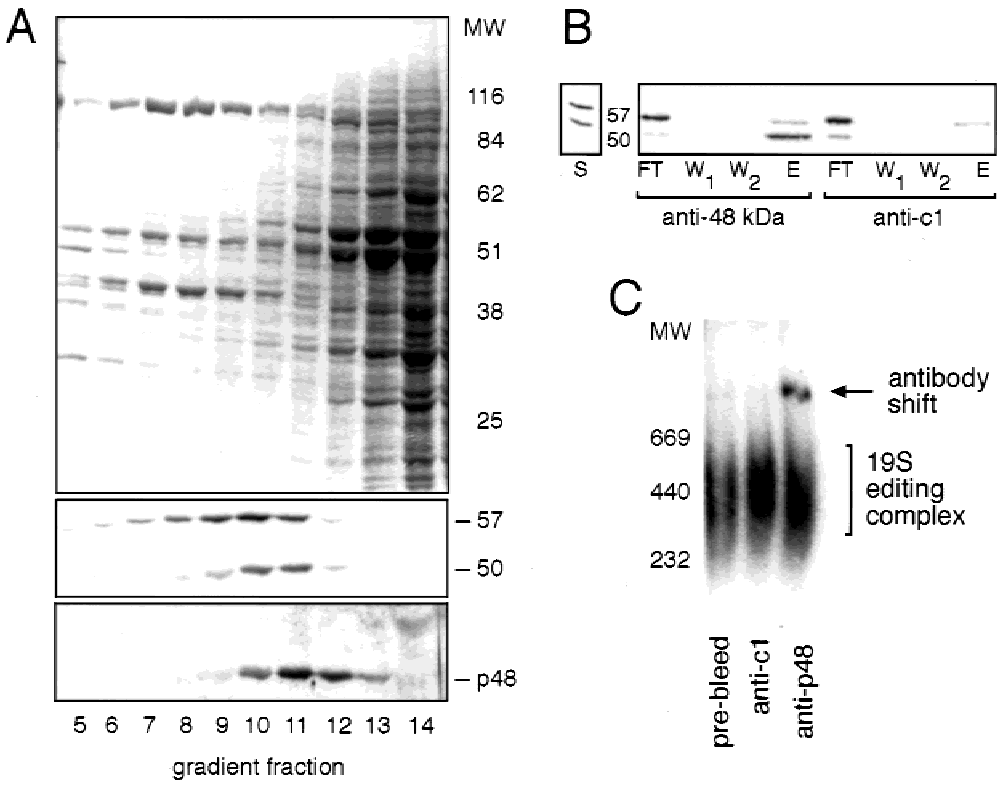


**FIGURE 2.** Polyclonal antibodies against the recombinant 48-kDa protein specifically recognize the previously described 50-kDa RNA editing ligase. **A**: Coomassie stain shows the total protein distribution of glycerol-gradient-sedimented mitochondrial extract. The RNA editing ligases present in these glycerol gradient fractions were radioadenylylated, separated on SDS-PAGE, and then transferred to a PVDF membrane. Middle panel is an autoradiograph of that PVDF membrane. Lower panel is a western immunoblot analysis of the membrane revealed that the immunoreactive band position corresponded to the adenylylated 50-kDa RNA editing ligase, apparent when the autoradiographic film was overlaid upon the immunoblot. MW is the molecular weight ladder in kilodaltons, and the positions of the 50- and 57-kDa ligases are designated on the right. **B**: Immunoprecipitation of adenylylated mitochondrial RNA editing complexes with anti-p48. S: starting glycerol gradient 19S fraction; FT: flow-through; W1 and W2: two consecutive washes of the protein A-coupled antibody beads; E: elution of the radiolabeled ligases off of the beads. Equal volumes of S, FT, W1, W2, and E were loaded; therefore this assay should not be visualized quantitatively. The adenylylated ligases are labeled 50 and 57. **C**: Anti-p48 supershift of adenylylated RNA editing complexes. Editing complexes were labeled with the addition of [$\alpha$-$^{32}$P]-ATP and incubated with anti-p48, anti-c1, or prebleed serum. Numbers on left correspond to a molecular weight ladder, and are in kilodaltons. The radioadenylylated 19S complex is indicated by a bracket and the anti-48-kDa specific shifted complex is shown by an arrow. Antibody against the recombinant 48-kDa ligase is labeled anti-48-kDa, and the antibody against cytochrome c1 is labeled anti-c1.

fraction 13 (Fig. 2A). We suspect the upper glycerol gradient fractions contain an inhibitor of ligase adenylylation. Another possibility is that the immunoreactive p48 band might represent another protein that coincidentally sedimented in the gradient, and comigrated with the 50-kDa ligase on SDS-PAGE. To address this possibility, ligase adenylylation activity and immunoreactivity were monitored through purification on both heparin and Q-sepharose chromatography. The 50-kDa adenylylated ligase copurified with the anti-p48 reactive band through these purification steps (data not shown).

To further examine the relationship of p48 to the 50-kDa editing complex RNA ligase, anti-p48 was tested for its ability to immunoprecipitate the adenylylated 50-kDa mitochondrial ligase from partially purified 19S RNA editing complexes (Fig. 2B). In these experiments, the editing complexes were incubated in [$\alpha$-$^{32}$P]-ATP to preadenylylate the editing ligases, and the resulting radiolabeled editing complexes were captured using anti-p48 bound to protein A sepharose. As a control for nonspecific precipitation, a polyclonal antibody against the *T. brucei* mitochondrial protein cytochrome c1 was tested. The adenylylated 50-kDa editing ligase was consistently and specifically immunoprecipitated by the anti-p48 polyclonal antibodies. A small amount of the 57-kDa ligase appeared to coimmunoprecipitate in these assays, which supported the possibility that both the 50-kDa and the 57-kDa ligase reside in the same editing complex. However, because an equivalent amount of 57-kDa ligase is present in the anti-c1 control antibody reaction, it likely represents nonspecific binding to the protein A sepharose. It is conceivable that either the antibodies or the stringent immunoprecipitation conditions could disrupt the complexes. This situation

complicates using the antibodies to immunoprecipitate enzyme activity, such as in vitro RNA editing. Thus far, with these antibodies, we have not found conditions that preserve either TUTase or RNA ligation activity (data not shown).

The presence of p48 within RNA editing complexes was also examined by antibody shift native gel analysis. Editing complexes are stable during nondenaturing PAGE analysis and can be visualized by radioadenylylation of the ligases prior to electrophoretic separation (Peris et al., 1997). The 19S complex typically migrates on native gels as a broad band of approximately 450 kDa. Addition of the anti-p48 shifts a portion of the radiolabeled complexes in the 19S fraction (Fig. 2C). Taken together, these in vitro studies demonstrate that antiserum raised against the recombinant p48 reacts with the radiolabeled protein within mitochondrial RNA editing complexes that was previously identified as a 50-kDa RNA ligase.

### Expression and analysis of epitope-tagged p48 in *T. brucei*

Recombinant p48 expressed in *E. coli* is largely insoluble and lacks ligase activity. Furthermore, no activity could be detected when p48 was in vitro translated using rabbit reticulolysates (data not shown). Some members of the covalent nucleotidyl transferase superfamily require posttranslational modifications or additional factors for activity (Prigent et al., 1992; Ho et al., 1998; Ahola et al., 1999). Because the RNA editing ligase is only found in large RNP complexes, we hypothesized that accessory trypanosomal proteins or cofactors might be necessary for activity. Therefore, to study activity, an epitope-tagged recombinant p48 was expressed in trypanosomes. We created two trypanosome expression vectors: one in which the p48 contained a C-terminal His(10) epitope, and a second in which the p48 contained a C-terminal HA epitope (Fig. 3A; McDowell et al., 1998). Both constructs contained sequences of the $\alpha$-$\beta$ tubulin intergenic region for site-specific integration, the PARP promoter for high-level expression, and the neomycin resistance gene for selecting stable lines (McDowell et al., 1998). The His(10)-tagged p48 cells were used in biochemical experiments and the HA-epitope-tagged cells were used in immunofluorescence studies.

Mitochondrial extracts were prepared from p48His and wild-type cells, and editing complexes were separated by glycerol gradient sedimentation. Gradient frac-

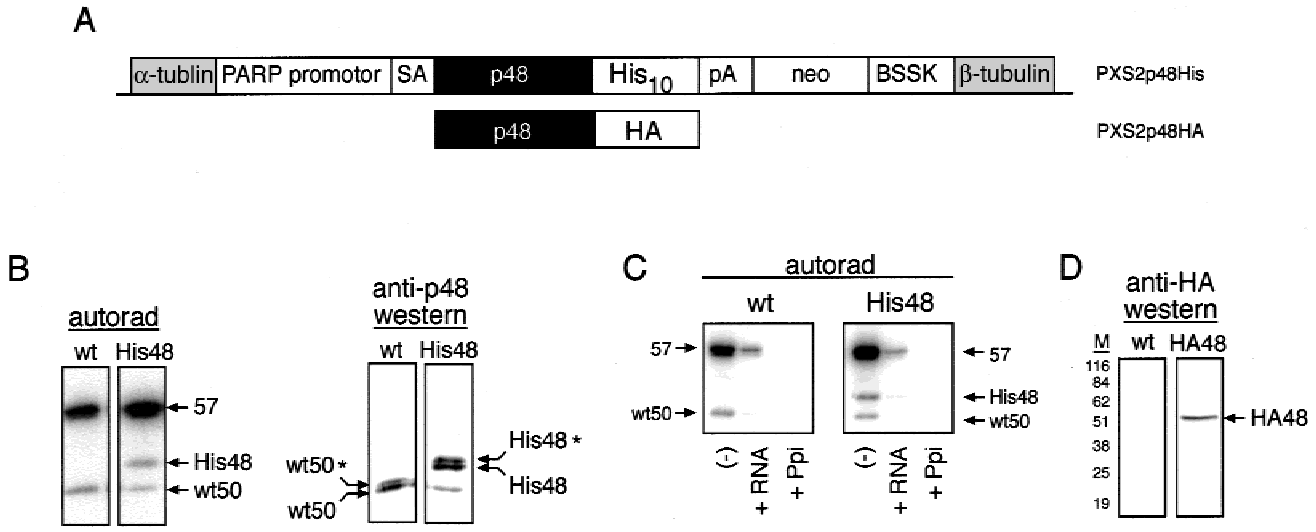


**FIGURE 3.** Construction of the homologous recombination vectors and analysis of the corresponding stable cell lines. **A**: Linearized layout of the pXS2p48His and pXS2p48HA constructs. The constructs were targeted to the $\alpha$ and $\beta$ tubulin array. Transcription is driven by a strong promoter (PARP), and 5′ *trans*-splicing (SA) and 3′ polyadenylation signals (pA) are provided for by the PARP intergenic sequences. The neomycin resistance gene (neo) is cotranscribed with the p48 epitope-tagged protein (either His10 or HA tagged), and is also processed for 5′ *trans*-splicing and 3′ polyadenylation (not shown). BSSK corresponds to Bluescript plasmid sequence. **B**: Transfected His-tagged p48 assembles in editing complexes and is adenylylated. Mitochondrial extract from wild-type trypanosomes and the pXS2p48His trypanosomes were prepared and sedimented on a glycerol gradient. Fractions corresponding to the 19S editing Complex I were used in this experiment. Autoradiogram depicts Complex I editing ligases that were radioadenylylated before separation on a SDS-PAGE gel. Western immunoblot analysis shows that the anti-48-kDa polyclonal antibodies detect both the wild-type 50-kDa ligase and the His-tagged 48-kDa protein. An asterisk denotes the adenylylated form, which migrates slightly above the unadenylylated form on low percentage acrylamide gels. **C**: Deadenylylation occurs in the presence of RNA or pyrophosphate. Ligases were preadenylylated prior to addition of 1 mg poly(A)15:poly(U)25 RNA or 12 mM pyrophosphate (Ppi) (Sabatini & Hajduk, 1995). **D**: Anti-HA antibody recognizes the HA-tagged 48-kDa ligase in the pXS2p48HA cells, and is not cross-reactive to proteins in the wild-type cells. Arrows point to the 57-kDa ligases (57), wild-type 50-kDa ligase (wt50), His-tagged 48-kDa protein (His48), and HA-tagged 48-kDa protein (HA48).

tions were radioadenylylated, run on SDS-PAGE, and transferred to an immunoblot. Analysis revealed that the His-tagged recombinant p48 was associated with both 19S and 35–40S editing complexes (data not shown). The His-tagged p48 runs approximately 2 kDa larger than the native p48 protein and was present only in transformed lines (Fig. 3B). On low percentage polyacrylamide gels, the covalent adenylyl moiety causes ligases to migrate slower in the SDS gel. In these experiments, this is visualized by the correspondence of the radioadenylylated band with the upper immunoreactive band, and not the lower immunoreactive band (in Fig. 3B, the upper adenylylated band is marked with asterisks).

Adenylylated ligases are considered reaction intermediates, and will readily deadenylylate in the presence of ligatable substrates. To determine whether the His-tagged p48 was competent for the next catalytic step, ligatable RNA was added to preadenylylated His-tagged p48 (Fig. 3C). This caused a rapid loss of the His-tagged p48 adenyl moiety, to a level comparable to the 50- and 57-kDa RNA ligases (Fig. 3C). The formation of an adenylylated ligase results in the release of pyrophosphate as a reaction byproduct. Because this reaction is reversible, high concentrations of pyrophosphate will lead to the deadenylylation of the ligase. To test this, 12 mM pyrophosphate was added to the preadenylylated His-tagged p48. Addition of pyrophosphate completely abolished the radiolabeled signal of the His-tagged p48, and the 50- and 57-kDa ligases. Therefore, when expressed in the trypanosome, the His-tagged recombinant p48 can associate in editing complexes and adenylylate with ATP. Taken together, these experiments support that the 48-kDa protein described here is the 50-kDa RNA ligase.

The HA-tagged 48-kDa ligase also is adenylylated, and is associated with editing complexes (data not shown). Antibodies against the HA epitope do not react with proteins from wild-type *T. brucei* on immunoblots (Fig. 3D). Trypanosome lines stably transformed with the HA-tagged p48 expressed an immunoreactive protein of ~50 kDa, consistent with the size of the epitope-tagged protein (Fig. 3D). On high percentage acrylamide gels, the adenylylated and nonadenylylated forms cannot be distinguished, and only a single band is present (Fig. 3D).

### Immunolocalization of epitope-tagged ligase in trypanosomes

Nuclear-encoded proteins that are imported into mitochondria often contain N-terminal targeting peptides. Both the *T. brucei* and *L. major* ligases contain N-terminal sequences homologous to kinetoplastid mitochondrial targeting signals (Fig. 4A). To determine subcellular localization of p48, we performed immunofluorescence microscopy on the C-terminal HA epitope cell line (Fig. 4B). Staining with the high affinity HA monoclonal antibody showed that the HA-tagged p48 colocalized with a known mitochondrial protein, the iron sulfur protein (ISP; Fig. 4B). In trypanosomes, the mitochondrion is a highly branched structure that extends the length of the cell. Thus, the observed immunofluorescence appears to display a pattern typical for a well-developed procyclic mitochondrion (Fig. 4B). These results are consistent with p48 being a mitochondrial protein.

Since the initial discovery of RNA editing by Benne and coworkers (1986), none of the editing complex catalytic components have been identified. Based on mitochondrial localization, editing complex association and adenylylation, our results establish that p48 corresponds to an RNA editing complex associated protein. The presence of covalent nucleotidyl transferase motifs and the ability of the enzyme to adenylylate and deadenylylate in the presence of pyrophosphate or RNA suggests that the protein is an RNA ligase. The characterization of p48 and the tentative identification of a second candidate RNA ligase (p52) provide additional support for the enzymatic cascade model for kinetoplastid RNA editing. The identification of these two candidate ligases should allow a number of important questions to be asked. For example, why does the editing complex contain two RNA ligases? Or is one ligase part of a uridine-addition editing complex and the other ligase part of a uridine-deletion editing complex? To shed light on these questions and others, we are in the process of creating cell lines in which either and both the 48- and 52-kDa enzymes are knocked out. These studies should help determine the exact role of these two proteins. Furthermore, we are utilizing the p48His transgenic trypanosomes and nickel affinity technology to purify p48His complexes. These purified complexes will be used to study the purported involvement of p48 in RNA editing.

We have not overlooked the evolutionary significance of the relationship of p48 RNA ligase to other members of the covalent nucleotidyl transferases. Conservation of the five sequence motifs and the active site KXXG clearly places the kinetoplastid p48 within this superfamily of proteins. Assuming that the p48 represents the RNA editing ligase, our findings are consistent with kinetoplastid RNA editing originating from preexisting trypanosome enzymes, such as the nucleotidyl transferases, perhaps as an RNA repair mechanism to compensate for frame-shift mutations within the mitochondrial genome (Covello & Gray, 1993).

## MATERIALS AND METHODS

### Antibody production

The 48-kDa protein was cloned into a pRSET vector (Invitrogen) and expressed in 1 L of *E. coli*. Using a nickel chelating resin, 200 $\mu$g were gel purified and injected into rabbits

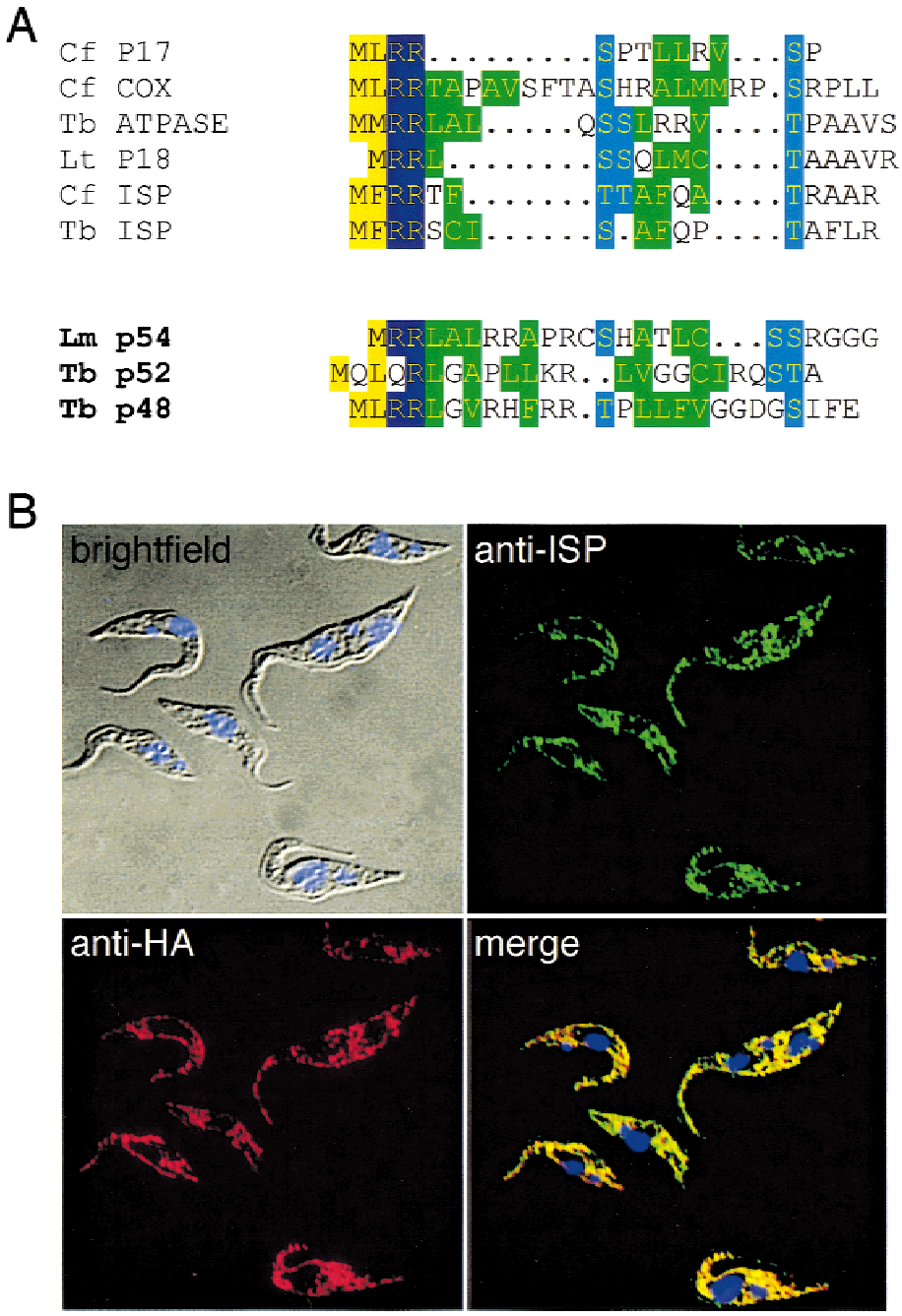


**FIGURE 4.** Mitochondrial localization of HA-tagged p48 in *T. brucei*. **A**: N-terminal sequences of Tb p48 and Tb p52 kinetoplastid RNA ligases are similar to kinetoplastid mitochondrial import targeting signals. The hydrophobic N-terminus is labeled yellow and the one or two charged residues that typically follow are shown in blue. Green represents patches of hydrophobic residues and blue indicates threonine and serine residues that flank a hydrophobic patch. The species abbreviations are as follows: Cf: *C. fasciculata*; Lt: *L. tarentola*; Tb: *T. brucei*; and Lm: *L. major*. ISP, p17, p18, ATPase, and COX correspond to known nuclear-encoded mitochondrial proteins (Bringaud et al., 1995; Priest & Hajduk, 1995, 1996; Torri & Englund, 1995; Xu et al., 1996; Chi et al., 1998). **B**: Subcellular localization of HA48 by immunofluorescence staining of procyclic trypanosomes. Brightfield: phase contrast embossed image; anti-HA: localization of the HA-tagged p48 transgene (red = AlexaFluor 594); anti-ISP: localization of the mitochondrial-localized iron sulfur protein (green = AlexaFluor 488); and merge: transposed images of the anti-ISP and anti-HA images (yellow = shared localization). The nuclear and kinetoplast DNA is stained with DAPI (blue).

(Cocalico). During the making of the polyclonal antibodies, the initial test bleed antibodies recognized a 48-kDa, and to a lesser degree, a 52-kDa protein that sediments in both 19S and 35–40S editing complexes (data not shown). However, over the course of the antigen boosts, the antibodies became specific for the 50-kDa protein, and did not recognize the 52-kDa protein. Therefore, all experiments were performed using the final boost antibodies.

### Immunoprecipitations

Glycerol gradient fractions containing Complex I were used for immunoprecipitations. For the immunoprecipitations (Fig. 3), 10 $\mu$L of the glycerol gradient fraction was preadenylated, and then added to 20 $\mu$L of a 50% slurry of protein A sepharose equilibrated in TTBSE (20 mM Tris, pH 7.8, 500 mM NaCl, 0.3% NP-40, and 20 mM EDTA). One milliliter of the designated antibody was added per immunoprecipitation reaction. This slurry was then shaken lightly on a vortex apparatus at RT. After 1 h, the beads were washed three times in a high volume of TTBSE (500 $\mu$L per wash), and proteins in the immunocomplex were eluted by boiling in 30 $\mu$L of reducing SDS sample buffer. For analysis, approximately 30 $\mu$L of each fraction (flow through, washes, and elution) was loaded onto the SDS-PAGE gel.

### Homologous recombination

The *T. brucei* 48-kDa open reading frame was amplified using a 3′ primer that contained the His or HA epitope sequence spaced three glycines from the C-terminal p48 sequence. Both the 5′ and 3′ primers contained restriction sites for cloning into the *Eco*RI site in the pXS2 vector. The final construct, pXS2p48His, was verified by sequencing and digested at a unique BSTXI site located in the middle of the tubulin intergenic sequence. The linearized pXS2p48His plasmid was electroporated into procyclic cells at a concentration of 1 $\mu$g/mL as described. G418 was added 18 h after transfection (final concentration, 50 $\mu$g/mL). Fourteen days after transfection, cells were spun down and resuspended in fresh medium containing 25 $\mu$g/mL G418.

### Immunofluorescence

Slide smears were prepared from procyclic cells in logarithmic growth, air dried, and fixed with 100% methanol at −20 °C for 7 min. Slides were washed three times in phosphate-buffered saline (PBS) and then incubated with primary antibody (rat anti-HA diluted 1:200 [Boehringer Mannheim], and either rabbit anti-50-kDa ligase diluted 1:400 [Cocalico] or mouse anti-iron sulfur protein diluted 1:800) in a humid chamber for 1 h. Slides were then washed three times in PBS and incubated with 1:1,000 dilution of AlexaFluor 594 goat anti-rat IgG and either AlexaFluor 488 goat anti-rabbit IgG or AlexaFluor 488 goat anti-mouse IgG (Molecular Probes) for 1 h. Slides were incubated with 4,6 diamino-2-phenylindole (DAPI, Sigma) at 0.4 mg/mL for 5 min and then washed three times in PBS, mounted in Fluoromount-G (Southern Biotechnology Associates, Inc.), and images captured on an Olympus digital fluorescence microscope at 150× magnification.

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