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Disruption of a Gene Encoding a Novel Mitochondrial DEAD-Box Protein in *Trypanosoma brucei* Affects Edited mRNAs

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The majority of mitochondrial pre-mRNAs in kinetoplastid protozoa such as *Trypanosoma*, *Leishmania*, and *Crithidia* are substrates of a posttranscriptional processing reaction referred to as RNA editing. The process results in the insertion and, to a lesser extent, deletion of uridylates, thereby completing the informational content of the mRNAs. The specificity of the RNA editing reaction is provided by guide RNAs (gRNAs), which serve as templates for the editing apparatus. In addition, the process relies on mitochondrial proteins, presumably acting within a high-molecular-mass ribonucleoprotein complex. Although several enzymatic activities have been implicated in the editing process, no protein has been identified to date. Here we report the identification of a novel mitochondrial DEAD-box protein, which we termed mHel61p. Disruption of the *mHEL61* alleles in insect-stage *Trypanosoma brucei* cells resulted in a reduced growth rate phenotype. On a molecular level, the null mutant showed significantly reduced amounts of edited mRNAs, whereas never-edited and nuclear mRNAs were unaffected. Reexpression of mHel61p in the knockout cell line restored the ability to efficiently synthesize edited mRNAs. The results suggest an involvement of mHel61p in the control of the abundance of edited mRNAs and thus reveal a novel function for DEAD-box proteins.

RNA editing of mitochondrial pre-mRNAs in kinetoplastid protozoa is a unique form of posttranscriptional gene transcript maturation. The process is characterized by the site-specific insertion and deletion of exclusively uridylate residues, thereby creating functional mRNA molecules for translation (reviewed in reference 19). RNA editing relies on small, metabolically stable RNA molecules known as guide RNAs (gRNAs), which act as templates in the process (6, 44, 45). gRNAs probably initiate the processing reaction by the formation of a short intermolecular duplex structure with the pre-mRNA, located proximal to the sequence domain to be edited. Bordering this helical segment, unpaired gRNA nucleotides then specify via base pairing the U insertion event with free UTP as the substrate (6, 29). Non-base-paired uridylates in the pre-mRNA are deleted. The reaction seems to occur within a high-molecular-mass complex (9, 39), involving a cascade of enzymatic activities (15) such as gRNA-directed endonuclease, terminal uridylyl transferase, terminal uridylyl nuclease, and RNA ligase (12; reviewed in reference 19). Although both pre-mRNAs and gRNAs have been identified in stable complexes with mitochondrial proteins (4; reviewed in references 18 and 30), no polypeptide has been shown to date to directly participate in the editing process.

By analogy to other biochemical processes that are catalyzed by macromolecular ribonucleoprotein complexes, it is likely that RNA editing is a highly dynamic process. The participating RNA and protein molecules probably have to undergo a defined series of conformational changes not only during the assembly of the functional particle but also as the reaction proceeds and terminates. Some mRNAs are edited throughout their entire length, which requires the sequential base pairing of the pre-mRNA molecule with several gRNAs (31, 35). To allow the ordered interplay of successive gRNAs, which often partially overlap, the participation of an RNA chaperone function in the form of an RNA helicase has been postulated (36). In fact, such an activity could be demonstrated in the mitochondrion of *Trypanosoma brucei*, displaying all characteristic features of RNA-unwinding enzymes (9, 36). The majority of RNA helicases belong to the superfamily of DEAD-box (DEAH/DExH) proteins, a name coined due to the presence of a conserved DEAD (Asp-Glu-Ala-Asp) amino acid motif within their primary sequence. Prototypical members of the family share additional highly conserved motifs and promote the unwinding of RNA duplex regions in a nucleoside triphosphate (NTP)-dependent manner. DEAD-box proteins play important roles in a variety of cellular processes requiring RNA-RNA and RNA-protein interactions, including nuclear and mitochondrial pre-mRNA splicing, rRNA processing, RNA degradation and stabilization, translation initiation, and ribosome assembly (reviewed in references 16 and 42). Hitherto, two mitochondrial members of this protein family have been described: Suv3p (48), a DExH-box protein from *Saccharomyces cerevisiae* which is involved in the degradation of excised group I intron RNAs (33), and the yeast DEAD-box protein pMSS116, which affects splicing of group I and group II introns in vivo and in vitro (38, 46).

In this study, we describe the identification and first characterization of a novel mitochondrial DEAD-box protein, mHEL61p, from the parasitic organism *T. brucei*. We report the generation of an *mHEL61* null mutant and demonstrate a reduction in the abundance of exclusively edited mRNAs in vivo due to the lack of mHEL61p. We further demonstrate that the low abundance of edited mRNAs in the mutant cell line can be rescued by ectopically reexpressing mHel61p. Finally, we show that mitochondrial lysates from mHel61p-minus cells per se are capable of performing RNA editing in vitro, indi-

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cating that the observed in vivo effect is the result of a post-catalytic involvement of mHel61p.

MATERIALS AND METHODS

Trypanosome cell growth, preparation of extracts, and subcellular fractionation. The procyclic life cycle stage of the *T. brucei* serodeme IsTaR 1 (49) was grown at 27°C in SDM-79 medium supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (5). Bloodstream trypanosomes (stock 427, variant MITat 1.2) (10) were grown in HMI-9 medium by the method of Hirumi and Hirumi (24). Whole-cell lysates were prepared as described previously (32), and nuclear and cytosolic extracts were isolated by standard procedures (11, 34). Mitochondrial vesicles were isolated by isopycnic centrifugation in preformed linear 15 to 35% (vol/vol) Percoll gradients (20).

Isolation of nucleic acids and Southern and Northern analysis. Genomic DNA was prepared as described previously (32). Poly(A)$^+$ mRNA was purified from whole-cell RNA preparations (7) with paramagnetic oligo(dT)$_{25}$ polystyrene beads. Digestion of DNA with restriction enzymes, agarose gel electrophoresis, and transfer of nucleic acids onto nylon membranes followed standard protocols. Hybridizations were performed with digoxigenin-11-dUTP-labelled probes obtained by PCR from relevant plasmids. Hybridized fragments were detected with an anti-digoxigenin alkaline phosphatase-conjugated antibody in conjunction with a digoxigenin luminescence detection kit (Boehringer).

DNA oligonucleotides. Oligodeoxynucleotides were synthesized by automated solid-support chemistry with *O*-cyanoethyl-*N*,*N*-diisopropylphosphoramidites. The following oligodeoxynucleotides were used: 105CPL-5′ (GTCGTC GACTTTTCTCTAGCTAGCATACGGCACATAAG), 105CPL-3′ (TCTTCTA GAAAGATTTATTTCAACATTTTTCCATCGTG), ACTIN-3′ (GGAAAAT CATAAGCACT), αTUB-5′ (CCGTGGCATATGGCAAG), αTUB-3′ (GGG GGTCGCACTTTGTC), COI-RT (GTAATGAGTACGTTGTAAAACTG), COII-RT (ATTTCATTACACCTACCAGG), Cyb-RT (CAACCTGACATTA AAAGAC), HEL105.3′R (GTCGTCGACCAATGGTTGCGATGTTCTCG), HYGRO-5′ (ACCGCAAGGAATCGGTC), HYGRO-3′ (TCTGCGGGCGA TTTGTG), NEO-5′ (CTGCCGAGAAAGTATCC), and UAP (GGCCA CGCGTCGACTAGTAC).

Glycerol gradients. Fractionation of cleared mitochondrial lysates was performed by ultracentrifugation in linear 10 to 30% (vol/vol) glycerol gradients as described by Pollard et al. (39). Apparent sedimentation coefficients for the various fractions were determined with the following markers: bovine serum albumin (4.3S), thyroglobulin (19.3S), *Escherichia coli* 30S ribosomal subunits, *E. coli* 50S ribosomal subunits, and *E. coli* 70S ribosomes.

Cloning of *mHEL61*. PCRs were performed with genomic DNA from *T. brucei* IsTaR 1 (49). The 5′-terminal end of the *mHEL61* gene had been identified previously during the 5′ rapid amplification of cDNA ends (RACE) of *HEL64* (37). The 3′ terminus was amplified by 3′ RACE with the *mHEL61*-specific primer HEL105.3′R and the universal amplification primer UAP (Gibco BRL), whose sequence had been introduced during cDNA synthesis. PCR products were ligated into plasmid pCR-Script SK (+) (Stratagene) and transformed into *E. coli* XL1-Blue MRF′ Kan cells. The 5′ RACE and 3′ RACE products were sequenced, and the sequence information was used for PCR amplification of the full-length *mHEL61* gene from *T. brucei* genomic DNA with primers 105CPL-5′ and 105CPL-3′. The 1,995-bp product was cloned into pCR-Script SK (+), resulting in plasmid pHEL105. The nucleotide sequences of both strands of the insert were determined by automated sequencing.

Production and purification of anti-mHEL61p antibodies and immunoblotting. Anti-mHel61p antibodies were produced against a truncated version of mHel61p (mHel61.TR), encompassing amino acids 2 to 247 of the unprocessed protein and containing six histidines at the N terminus. mHel61.TR was affinity purified from *E. coli* extracts by Ni chelate chromatography and used to immunize rabbits. Affinity purification of the antibodies was performed as described previously (40). Protein extracts were separated on sodium dodecyl sulfate-containing 10% (wt/vol) polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked with phosphate-buffered saline containing 5% (wt/vol) bovine serum albumin and probed with the antibodies. Detection was performed with an enhanced chemiluminescence detection system (Amersham).

Gene disruption experiments. Constructs used for the disruption of the *mHEL61* gene are based on plasmid pHEL105 containing the entire *mHEL61* open reading frame (ORF). For construction of plasmid p105KO-NEO, plasmid pHD102 (provided by C. Clayton, Heidelberg, Germany) was cut with *Nsi*I and *Eco*47III. The fragment containing the complete transcription unit for the *neo* gene, including the PARP promoter and *actin* 5′ and 3′ untranslated regions, was ligated into pHEL105 which had been cut with *Nsi*I and *Hpa*I, thereby removing 143 nucleotides from the coding region of *mHEL61*. Similarly, the *Nsi*I-*Eco*47III fragment of pHD132 (51) was integrated into *Nsi*I- and *Hpa*I-treated pHEL105, resulting in plasmid p105KO-HYGRO. *T. brucei* insect-stage cells were transfected essentially as described previously (2). The constructs were digested with *Eco*RV and *Not*I. This treatment left 35- and 10-bp vector sequences at the 5′ and 3′ ends of the fragments used for the recombinant integration. Both constructs were used at 50 μg per transfection. Selection was started after overnight incubation with 25 μg of G418 (*neo* transfectants) and hygromycin B (*hyg* transfectants) per ml. After 48 h, the concentrations of both drugs were further increased to 50 μg/ml. For double gene replacement, the G418-resistant cell line was transfected with the p105KO-HYGRO fragment. Outgrowth of the transfected cells was seen after 10 to 12 days.

RNA helicase and RNA editing assays. The RNA helicase assay was performed as described by Missel and Göringer (36). As a substrate, gRNA gA6-14 hybridized to its cognate domain of the edited ATPase 6 mRNA was used. The uridylate deletion RNA-editing assay was carried out as described previously (44) except that a cleared mitochondrial lysate was used instead of a 20S glycerol gradient fraction.

Primer extension analysis. 5′ ^{32}P-labelled oligodeoxynucleotide (0.1 pmol) was mixed with 50 μg of steady-state RNA, heated to 65°C for 60 min, and annealed at room temperature for 30 min in 0.1 M Tris-HCl (pH 8.3)–0.15 M KCl–1 mM disodium EDTA. Primer extension was performed in 50 mM Tris-HCl (pH 8.3)–50 mM KCl–10 mM $MgCl_2$–5.5 mM dithiothreitol, 150 μM each dATP, dCTP, and dTTP, 75 μM ddGTP, and 10 U of avian myeloblastosis virus reverse transcriptase (Stratagene) at 42°C for 45 min. RNA was removed by RNase A digestion, and the reaction products were precipitated with ethanol and fractionated on a 7 M urea-containing 10% (wt/vol) polyacrylamide gel. The gels were analyzed by PhosphorImager analysis or autoradiography followed by densitometry (Howtek Scanmaster 3, pdi software version 2.4).

Ectopic reexpression of mHel61p. To reexpress mHEL61p, we used pHD415 (3): this vector permits integration of cloned DNA into the multicopy tubulin locus, and posttranscriptional processing signals for the cloned DNA are provided by aldolase sequences 5′ and 3′ of the cloned DNA. A downstream *phleo* cassette permits selection of transfectants. Integration into the β-tubulin locus is achieved by linearizing the construct within the β-tubulin sequence. To clone the *mHEL61* ORF and flanking sequences into pHD415, pHEL105 and pHD415 were cut with *Not*I and *Bam*HI, respectively, and the ends were processed with T4 DNA polymerase. Both linearized plasmids were then cut with *Hin*dIII. The doubly cut pHD415 and the 2.04-kb fragment from pHEL105 were recovered and ligated to yield pHD415-mHEL61. A unique *Not*I site within the β-tubulin sequence (from pHD415) was used to linearize the construct for integration.

Nucleotide sequence accession number. The sequence of *mHEL61* has been submitted to GenBank under accession no. U86382.

RESULTS

Cloning of *mHEL61*. Mitochondrial extracts of *T. brucei* have been shown to exhibit an NTP-dependent unwinding activity of double-stranded (ds) RNA substrates (9, 36) which is characteristic for RNA helicases of the DEAD-box protein family (16, 42). We set out to clone potential RNA helicase genes from *T. brucei* in a nested PCR with degenerate oligodeoxynucleotides directed against conserved DEAD-box protein motifs (37, 42). Here we report the amplification of a DNA fragment containing a continuous ORF encoding a protein of 546 amino acids with a calculated molecular mass of 60.7 kDa. The protein sequence contains all the amino acid motifs typical for the DEAD-box protein family of putative RNA helicases and was named mHel61p. The protein-coding region is flanked by a 5′ untranslated region of 69 nucleotides (including the spliced leader sequence) and a 3′ untranslated region of 308 nucleotides. In contrast to most other trypanosomatid mRNAs (26), it contains a consensus AAUAAA polyadenylation signal, located at position −10 from the polyadenylation site. The DNA sequence and the deduced amino acid sequence of mHel61p are shown in Fig. 1a. Aside from the conserved DEAD-box motifs, no significant homology to other RNA helicases or other proteins in the various databases was found (Fig. 1b). Therefore, mHel61p can be considered a new member of the DEAD-box protein family.

A Southern and Northern blot analysis revealed that mHel61p is encoded by a single-copy gene expressed as a 2.3-kb transcript in both the insect and bloodstream life cycle stages of trypanosomes (Fig. 2a and b). Thus, expression of mHel61p does not seem to be regulated between the two major life cycle stages of trypanosomes. This result was confirmed in a Western blot experiment. Equal amounts of whole-cell lysates from procyclic and bloodstream trypanosomes were analyzed with a polyclonal antiserum raised against a truncated N-terminal polypeptide of mHel61p (amino acids 2 to 247). In both life cycle stages, similar signal intensities with the expected size of approximately 60 kDa were identified (Fig. 2c).

a

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TTTCTCTAGCTAGCATACGGCACATAAGCATGAGGGCCCTGCGTTGTGTTAGGAGGGGTGTTTATCGCCAGTCAGTACGACTCTGTTATTTTATGTCGCTGGAGTGTTCACTACGTCCAA  120
                          M  R  A  L  R  C  V  R  R  G  V  Y  R  Q  S  V  R  L  C  Y  F  M  S  L  E  C  S  L  R  P  I   31

TCACAGGTGCCTCCGCACGATTACTGTGCCGATTTACTGCTCCGACTTGGCCCAGAAAAAATGAGGTGGAGGAAGGGGATGTGGGGACGACAGAGTCCACTTCGCAAGATGTGCGGTCAA  240
  T  G  A  S  A  R  L  L  C  R  F  T  A  P  T  W  P  R  K  N  E  V  E  E  G  D  V  G  T  T  E  S  T  S  Q  D  V  R  S  I   71

TATTTCGTAGTAACATCGAAACACACTCCGAAGTATCGCTCGAATCCTCAGCAAGTGCAGCTTTGCCACCCAAAACTGCGGCGTTGGATGTGTCTGTTCTCGACAGTAAGGGTAACGCGG  360
  F  R  S  N  I  E  T  H  S  E  V  S  L  E  S  S  A  S  A  A  L  P  P  K  T  A  A  L  D  V  S  V  L  D  S  K  G  N  A  V  111

TCCCAGTGAATCCTGTTAAGCTCTTTTCTGATCTCGACAATCTACCTGATTGGCTGTCGAAGGGACTCCAGTCGTCCGGATTTTCATGTACCACCCCCATTCAAAGCTATACTATTCCAG  480
  P  V  N  P  V  K  L  F  S  D  L  D  N  L  P  D  W  L  S  K  G  L  Q  S  S  G  F  S  C  T  T  P  I  Q  S  Y  T  I  P  V  161

TCCTTGACGAGGGTCACGATATGATCGGCCTGGCTCCTACAGGTTCGGGGAAAACTGTAGCCTTCGCCGTTCCCGCCCTAAAAAAGTTTCAGTGGAGTCCGAACGGTTCACCAAGGATAG  600
  L  D  E  G  H  D  M  I  G  L  A  P  T  G  S  G  K  T  V  A  F  A  V  P  A  L  K  K  F  Q  W  S  P  N  G  S  P  R  I  V  191

TGGTTCTCGCACCAACAAGGGAGCTAGTTCAACAAACTGCGAAAGTGTTCCATCAGTTGAGTTCGGGGAAAGTTCGTGTGTGCGAACGGTACGGTGGTGCCCCACGTGAAGCGCAGGCCC  720
  V  L  A  P  T  R  E  L  V  Q  Q  T  A  K  V  F  H  Q  L  S  S  G  K  V  R  V  C  E  R  Y  G  G  A  P  R  E  A  Q  A  R  231

GTCGGTTGCACAATGGTTGCGATGTTCTCGTGGCGTGCCCAGGTCGGTTGAAGGACTTCTTGCAGAACGGCGACGTGATCTTTGATGAAGTGTCGTTTTTGGTCTTCGATGAGGCTGATC  840
  R  L  H  N  G  C  D  V  L  V  A  C  P  G  R  L  K  D  F  L  Q  N  G  D  V  I  F  D  E  V  S  F  L  V  F  D  E  A  D  R  271

GTCTACTGGACATGGGGTTCAAGGTCCAATTAGACGACATTCTGGGTTACTTTTCGAGCCACCGCCCTGCTCAAACGATGATGTGGTCCGCTACCTGGCCCCCAGTTGTGGAGCAACTGG  960
  L  L  D  M  G  F  K  V  Q  L  D  D  I  L  G  Y  F  S  S  H  R  P  A  Q  T  M  M  W  S  A  T  W  P  P  V  V  E  Q  L  A  311

CCCAAGAGTATCTCTCACAGAATCGCTACGTAATCCGGTCTGGAACCGCCGGAACCGGACTTCAGGTCAACGAGAATATTAAGCAGCACATTTTCTTTGCTGATGCCCCCGAAGAGCGCG 1080
  Q  E  Y  L  S  Q  N  R  Y  V  I  R  S  G  T  A  G  T  G  L  Q  V  N  E  N  I  K  Q  H  I  F  F  A  D  A  P  E  E  R  V  351

TGAAAACACTTGTTTCTCTCATTAAGGAGGGGAAAATTGACGAGAATACCGCTAAGATGATGATATTTGTCGAGCGTCAAACTGACACAGAGAATGCAGCCTACGCGCTGGCCCGTATGT 1200
  K  T  L  V  S  L  I  K  E  G  K  I  D  E  N  T  A  K  M  M  I  F  V  E  R  Q  T  D  T  E  N  A  A  Y  A  L  A  R  M  L  391

TGGGGATTCATAGCAGATGCATTGGAGTGGTGCACGGCGGGATGCAACAGCGGCAGAGGGATCATATAATGGGCATATTCAAGGAAGGACGGATTCGGATACTTGTAGCCACTGACGTGG 1320
  G  I  H  S  R  C  I  G  V  V  H  G  G  M  Q  Q  R  Q  R  D  H  I  M  G  I  F  K  E  G  R  I  R  I  L  V  A  T  D  V  A  431

CCTCTCGTGGACTTGACTTCCCGGATGTAACTTGCGTTGTTAACCTCATAGCCCCCAAGAACATTGACAGCTACTGCCACCGCATTGGTCGGACCGGAAGGGCTGGTCGCACCGGTGAGT 1440
  S  R  G  L  D  F  P  D  V  T  C  V  V  N  L  I  A  P  K  N  I  D  S  Y  C  H  R  I  G  R  T  G  R  A  G  R  T  G  E  S  471

CCTTTACCTTTATTGGGCGTTCGGATGGATCTCTGGCGAAGGACTTGATAAATTACCTGGAAAAATGTGGTATGGACGTCCCTCAGCGGTTAGTGGAGTTTGCTGAAGAGCACGCCCAAG 1560
  F  T  F  I  G  R  S  D  G  S  L  A  K  D  L  I  N  Y  L  E  K  C  G  M  D  V  P  Q  R  L  V  E  F  A  E  E  H  A  Q  G  511

GGGAGGAGATAAAGCGTATGAAACGACCGTGGCGCGGTAGGCAGCAGCGTGAGGGGAGGAGCCAGTCGCGGGGTGGTCGTGAGCCGGCACCTCGTGACAGTGGTTGGTAGTTCGTGCGCA 1680
  E  E  I  K  R  M  K  R  P  W  R  G  R  Q  Q  R  E  G  R  S  Q  S  R  G  G  R  E  P  A  P  R  D  S  G  W  *              546

GTTTTGTTGCTGTCAGGACTTGTGGTGACTGACATGTTTCTGTCATTTCTACTTTAGCACATTAGTTTCACATAGTGATCCTGGCATATGATGGACCTTCTTTCCTCGTCACTGGTGCGT 1800
GAACCCCTTTTCGGTGTGAGGCCGTACCTTTTGTTCGTTTTTTCACGTGTGTTATTTCCTAGAGCTTCACATCTGGTGATAGGCTCACGTGGCGTTGTAGTTAAATTCTTTAAGAAACCT 1920
GCGAGAGGTATTTCTAAAATAGTACAACACGATGGAAAAATGTTGAAATAAATCTT                                                                    1976
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b

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p68                                 MSGYSSDRDRGRDRGFGAPRFGGSRAGPLSGKKFGNPGEKLVK
RhlB
mHEL61p     MRALRCVRRGVYRQSVRLCYFMSLECSLRPITGASARLLCRFTAPTWPRKNEVEEGDVGT

p68         KKWNLDELPKFEKNFYQEHPDLARRTAQEVETYRRSKEITV.....RGHNCPKPVLNFYE
RhlB                                                      SKTHLTEQKFSD
mHEL61p     TESTSQDVRSIFRSNIETHSEVSLESSASAALPPKTAALDVSVLDSKGNAVPVNPVKLFS

p68         ..ANFPANVMDVIARQNFTEPTAIQAQGWPVALSGLDMVGVAQTGSGKTLSYLLPAIVHI
RhlB        ..FALHPKVVEALEKKGFHNCTPIQALALPLTLAGRDVAGQAQTGTGKTMAFLTSTFHYL
mHEL61p     DLDNLPDWLSKGLQSSGFSCTTPIQSYTIPVLDEGHDMIGLAPTGSGKTVAFAVPALKKF
                                                    A  G GKT
p68         NHQPFLE..RGDGPICLVLAPTRELAQQVQQVAAEYCRACRLKSTCIYGGAPKGPQIRDL
RhlB        LSHPAIADRKVNQPRALIMAPTRELAVQIHADAEPLAEATGLKLGLAYGGDGYDKQLKVL
mHEL61p     QWSP.......NGSPRIVVLAPTRELVQQTAKVFHQLSSG.KVRVCERYGGAPREAQARRL
                                 PTRELA                        GG
p68         ERGVEICIATPGRLIDFLECGKTNLRRTTYLVLDEADRMLDMGFEPQIRKIVDQIRP..D
RhlB        ESGVDILIGTTGRLIDYAKQNHINLGAIQVVVLDEADRMYDLGFIKDIRWLFRRMPPANQ
mHEL61p     HNGCDVLVACPGRLKDFLQNGDVIFDEVSFLVFDEADRLLDMGFKVQLDDILGYFSSHRP
                          TPGR                      DEAD
p68         RQTLMWSATWPKEVRQLAEDFLKDYIHINIGALELSANHNILQIV.DVCHDVEKDEKLIR
RhlB        RLNMLFSATLSYRVRELAFEQMNNAEYIEVEPEQKTGHRIKEELF.YPSN....EEKMRL
mHEL61p     AQTMMWSATWPPVVEQLAQEYLSQNRYVIRSGTAGTGLQVNENIKQHIFFADAPEERVKT
                   SAT
p68         LMEEIMSEKEN....KTIVFVETKRRCDE....LTRKMRRDGWPAMGIHGDKSQQERDWV
RhlB        LQTLIEEEWPD....RAIIFANTKHRCEE....IWGHLAADGHRVGLLTGDVAQKKRLRI
mHEL61p     LVSLIKEGKIDENTAKMMIFVERQTDTENAAYALARMLGIHSRCIGVVHGGMQQRQRDHI

p68         LNEFKHGKAPILIATDVASRGLDVEDVKFVINYDYPNSSEDYIHRIGRTARSTKTG....
RhlB        LDEFTRGDLDILVATDVAARGLHIPAVTHVFNYDLPDDCEDYVHRIGRTGRAGASGHSIS
mHEL61      MGIFKEGRIRILVATDVASRGLDFPDVTCVVNLIAPKNIDSYCHRIGRTGRAGRTGESFT
                                ARG D                   HRIGR
p68         ...TAYTFFTPNNIKQVS.........DLISVLREANQAIN...PKLLQLVEDRGSGRSRG
RhlB        LACEEYALNLPAIETYIG.........HSIPVSKYNPDALMTDLPKPLRLTRPRTGNGPRR
mHEL61p     FIGRSDGSLAKDLINYLEKCGMDVPQRLVEFAEEHAQGEEIKRMKRPWRGRQQREGRSQS

p68         RGGMKDDRRDRY&
RhlB        TGAPRNRRRSG*
mHEL61p     RGGREPAPRDSGW*
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FIG. 1. (a) cDNA and deduced protein sequence of the *mHEL61* gene. The DNA sequence starts with the spliced leader acceptor site and ends with the polyadenylation site. Conserved DEAD-box motifs (42) are in boldface italics and underlined. (b) Amino acid sequence comparison of *T. brucei* mHel61p with the human p68 RNA helicase (23, 25) and the *E. coli* RhlB protein (41). The alignment was performed with the PILEUP program of the GCG software package (Wisconsin package version 8.1; Genetics Computer Group [GCG], Madison, Wis.). Identical amino acids are in white on a black background, and gaps necessary for optimal alignment are shown as dots. The conserved DEAD-box motifs are given in white and italics on a grey background. & at the end of the p68 sequence, additional C-terminal amino acids, which are not shown.

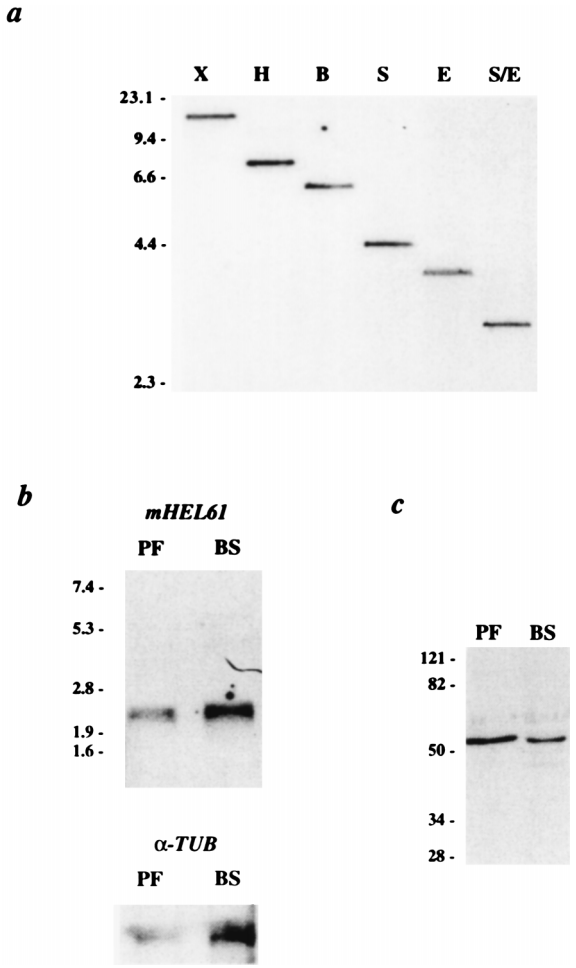

FIG. 2. Genome organization and expression of *mHEL61*. (a) Southern blot analysis of genomic DNA from *T. brucei* (5 μg per lane) with the following restriction enzymes: X, *Xba*I; H, *Hin*dIII; B, *Bam*HI; S, *Sal*I; E, *Eco*RI; S/E, double digest with *Sal*I and *Eco*RI. After blotting, hybridization was performed with a *mHEL61*-specific, digoxigenin-labelled probe. Size markers are given in kilobase pairs. (b) Northern blot analysis. Whole-cell RNA was isolated from procyclic-form (PF) and bloodstream (BS) trypanosomes and separated in a formaldehyde-agarose gel (20 μg per lane). After blotting onto a nylon membrane, hybridization was performed with an *mHEL61*-specific digoxigenin-labelled probe (top panel). For comparison, hybridization with a probe against the constitutively expressed α-tubulin (α-TUB) mRNA is shown (lower panel). RNA size markers are given in kilobases. (c) Western blot analysis. Whole-cell lysates of procyclic form (PF) and bloodstream (BS) trypanosomes were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (20 μg of protein per lane) and blotted onto a nitrocellulose membrane. Hybridization was performed with affinity purified anti-mHel61p antibody as outlined in Materials and Methods. Protein size markers are given in kilodaltons.

Mitochondrial localization of mHel61p. The deduced N terminus of mHel61p is rich in basic and hydroxylated amino acids, whereas negatively charged amino acids are absent. It can be folded into an amphiphilic helix (data not shown), thereby displaying characteristic traits of a mitochondrial targeting presequence (21). These features led us to examine the intracellular localization of the protein. Extracts from the trypanosome nucleus, the cytosol, and mitochondria were probed in an immunoblot analysis with an antibody directed against mHel61p. No traces of the protein were detected in the nuclear and cytoplasmic lysates. In contrast, mHel61p was clearly present within the mitochondrial extract, showing a strongly enriched signal in comparison to the whole-cell extract (Fig. 3a). Control experiments with antibodies directed against the mitochondrial matrix protein Hsp60 and the cytosolic translation initiation factor eIF-4A paralleled the results obtained with the anti-mHel61p antibody and verified the absence of cross-contamination of the different cell compartments (Fig. 3b). Thus, mHel61p is a nucleus-encoded protein, located within the mitochondrion of the trypanosome cell.

mHel61p and mitochondrial helicase activity. As mentioned above, *T. brucei* mitochondria contain an NTP-dependent activity capable of unwinding RNA duplexes with a preference for substrates mimicking RNA-editing intermediates (36). Due to its mitochondrial localization and its membership of the DEAD-box protein family, mHel61p was a likely candidate to exhibit this RNA helicase activity. To test this hypothesis, we analyzed mitochondrial extracts fractionated either by the stepwise addition of ammonium sulfate or by isokinetic centrifugation in linear glycerol gradients (39). All of the fractions were assayed in parallel for RNA helicase activity (36) and for the presence of mHel61p by immunoblotting. Neither of the separation methods led to a clear cofractionation of mHel61p with the RNA helicase activity. In the ammonium sulfate precipitation experiment, the unwinding activity was found almost exclusively in the 30 to 40% saturation fraction whereas mHel61p was detected in four of five fractions with the highest abundance in the 40 to 50% saturation fraction (data not shown). Similarly, in the glycerol gradient analysis, the RNA helicase activity was found to migrate in a broad peak, equivalent to apparent Svedberg values of ca. 13S to 30S. However, the bulk of mHel61p was identified in the first three fractions at the top of the gradient, and only small amounts of mHel61p were found to overlap with the peak of the unwinding activity (Fig. 4).

Gene disruption of *mHEL61*. Based on the ambiguity of this result, we decided to perform gene disruption experiments with the hope of gaining information on the biochemical function of mHel61p. The two wild-type alleles of *mHEL61* were sequentially disrupted with sequences conferring resistance to

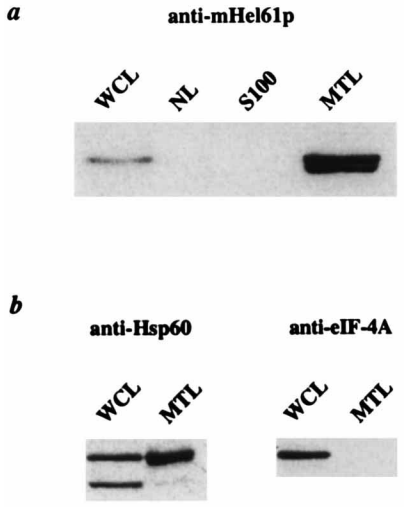

FIG. 3. mHel61p is located within the mitochondria of trypanosome cells. (a) Lysates from whole cells (WCL), nuclei (NL), a cytosolic S100 supernatant (S100), and mitochondria (MTL) were tested for the presence of the protein with an anti-mHel61p antibody. The double band in the MTL lane is probably due to an mHel61p degradation product. (b) Testing the mitochondrial lysate with antibodies against the mitochondrial Hsp60 protein and the cytosolic translation factor eIF-4A confirmed the absence of cytosolic contamination within the mitochondrial preparation.

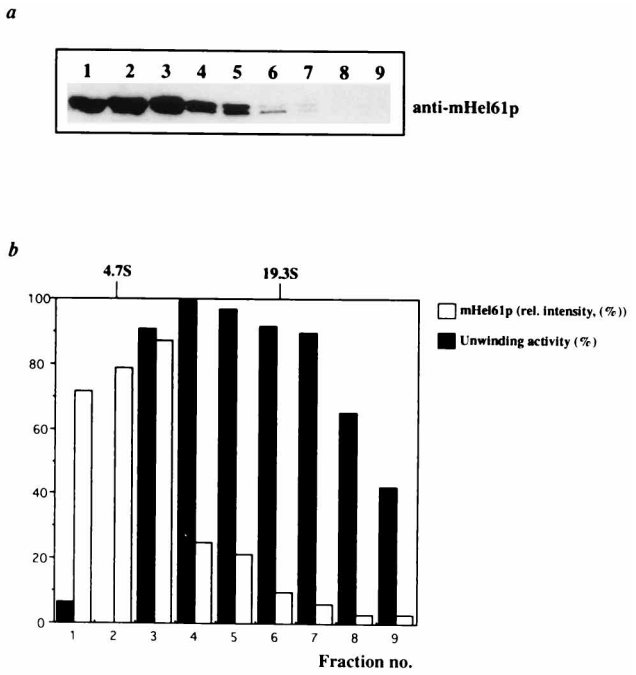

FIG. 4. Correlation of mHel61p with the mitochondrial RNA helicase activity. (a) Cleared mitochondrial lysates from insect-stage trypanosomes (1 mg) were separated by isokinetic centrifugation in linear 10 to 30% (vol/vol) glycerol gradients and fractionated into 24 fractions (0.5 ml each). One-tenth of each fraction was separated by SDS-PAGE, blotted onto nylon membranes, and probed for the presence of mHel61p with an affinity-purified antibody against the polypeptide. mHel61p was detected in fractions 1 to 7 (where 1 corresponds to the top of the gradient). (b) Open bars show a graphical representation of the distribution of mHel61p in fractions 1 to 9, expressed as relative signal intensities derived from a densitometer analysis of the fluorograph shown in panel *a*. Solid bars show the relative RNA helicase activities of the same gradient fractions tested as outlined in Materials and Methods. Apparent S values derived from marker proteins centrifuged in separate gradients are given at the top of the graph.

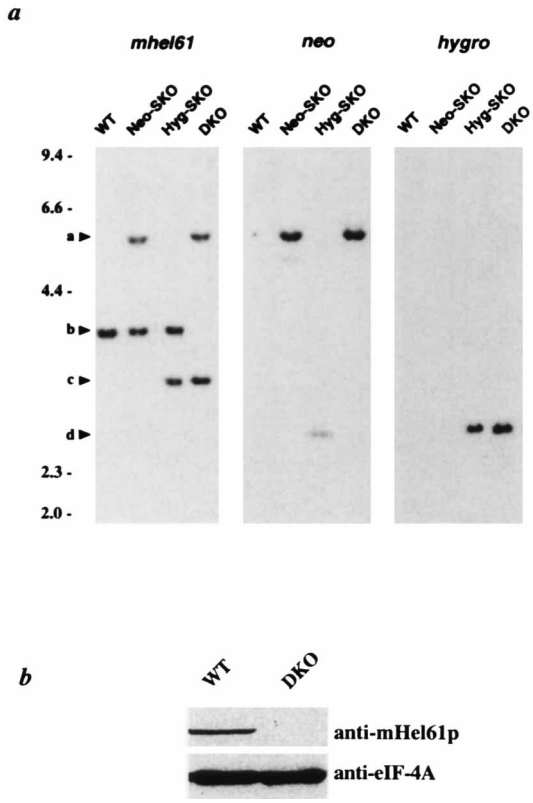

FIG. 5. Southern blot analysis of *mHEL61* transfectants. (a) *Eco*RI-digested genomic DNA samples (10 μg per lane) from wild-type (WT) trypanosomes, neomycin single-knockout transfectants (Neo-SKO), hygromycin single-knockout transfectants (Hyg-SKO), and the double-knockout cell line (DKO) were separated by agarose gel electrophoresis and blotted onto nylon membranes. Hybridization was performed with an *mHEL61*-specific probe (left panel, *mhel61*), a neomycin phosphotransferase-specific probe (center panel, *neo*), and a hygromycin phosphotransferase-specific probe (right panel, *hyg*). Bands labeled "a" contain the neomycin resistance gene, those labelled "b" contain the *mHEL61* gene, and those labelled "c" and "d" correspond to the hygromycin resistance gene. The existence of two different fragment sizes for the *hyg* gene is due to an internal *Eco*RI cleavage site within the *mHEL61/hyg* fragment used for the transfection experiment. The 5′ part of the resulting fragment is recognized by the *mHEL61*-specific probe, whereas the 3′ fragment is recognized by the hygromycin phosphotransferase-specific probe. Size markers are given in kilobase pairs. (b) *mHEL61* double-knockout cells do not express mHel61p protein. Whole-cell lysates (50 μg per lane) from wild-type trypanosomes and *mHEL61* double-knockout cells were separated by SDS-PAGE and tested with an antibody against mHel61p. No signal can be detected in the mHel61p-minus cell extract. However, both cell lines express equal quantities of the cytosolic DEAD-box protein eIF-4A.

the aminoglycoside antibiotics G418 (*neo*), a neomycin analog, and hygromycin B (*hyg*). In a first-round transfection experiment, we introduced the *neo* cassette into wild-type trypanosomes and selected for G418-resistant cells. This cell line was then transfected with the *hyg* construct and incubated in the presence of both antibiotics to select for the double-knockout event. DNA from the resultant cell line was analyzed by Southern blotting with *neo*-, *hyg*-, and *mHEL61*-specific probes to confirm the presence of both resistance marker genes and the disruption of *mHEL61* genes (Fig. 5a). To verify that the null mutant cells no longer synthesized mHel61p, whole-cell lysates of wild-type and double-knockout trypanosomes were analyzed by Western blotting. No signal for mHel61p could be detected in the null mutant strain (Fig. 5b). As a control, we tested whole-cell extracts from both strains with an antibody against the translation initiation factor eIF-4A, another DEAD-box protein. No differences in the abundance of eIF-4A were observed between the wild type and the mutant lacking mHel61p (Fig. 5b).

Since we were able to disrupt both *mHEL61* alleles, obviously the protein is not essential in insect stage trypanosomes. We also did not identify any gross morphological changes in the *mHEL61* null mutant cells. However, the mutant strain showed a significantly decreased growth rate compared to wild-type cells. Its doubling time increased to 14 ± 2 h compared to 8 ± 1 h for wild-type trypanosomes. The single-knockout cells had not shown any growth defect in the presence of G418, and a separately constructed single-knockout cell line with the *hyg* resistance gene as a marker also showed no growth difference from wild-type trypanosomes. Although we cannot rule out a synergistic effect due to the presence of both antibiotics, the reduced growth rate of the *mHEL61* double-knockout cells was suggestive of an effect on the trypanosomal metabolism and may be due to a decreased mitochondrial RNA helicase activity. To investigate this possibility, we isolated mitochondrial vesicles from the *mHEL61* double-knockout strain and compared the RNA-unwinding activity of mitochondrial lysates to that of lysates isolated from wild-type mitochondria. Surprisingly, no difference in the RNA-unwinding activity between the wild type and the null mutant could be detected (data not shown). Thus, the mitochondrial RNA helicase activity cannot be attributed to mHel61p.

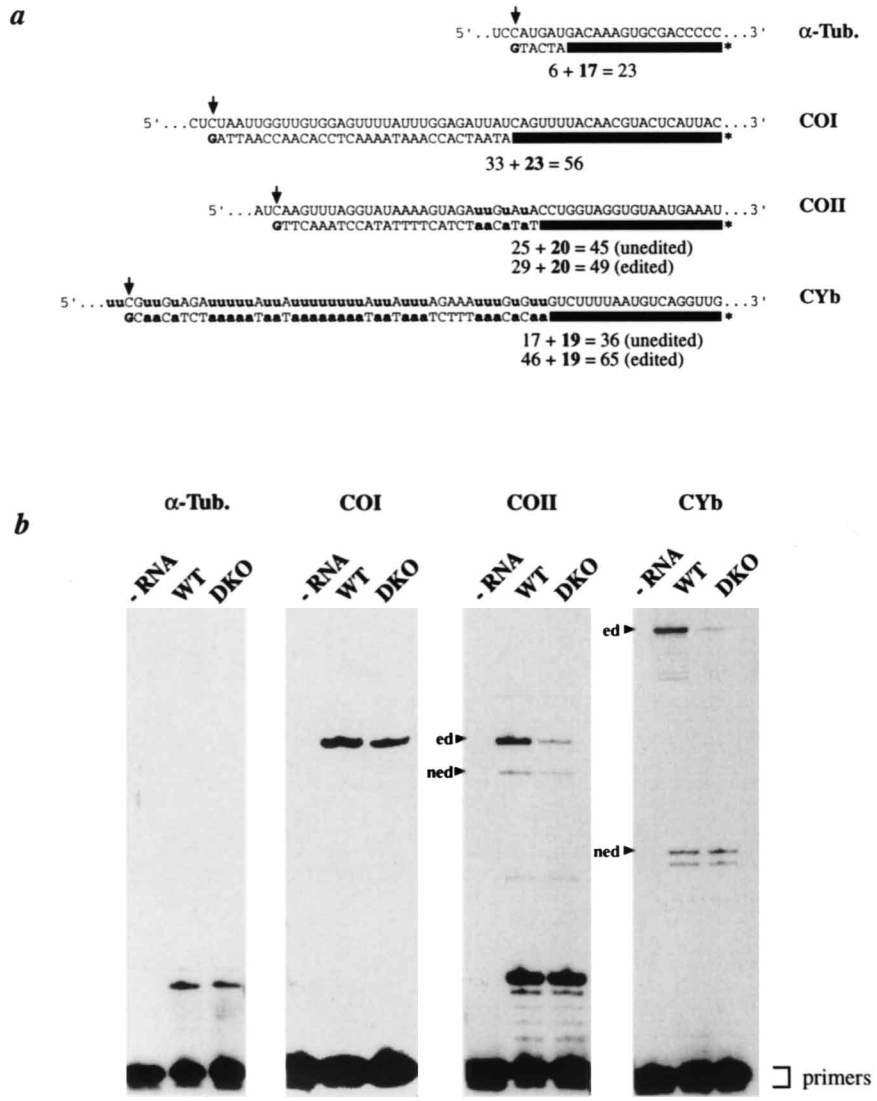

FIG. 6. Trypanosome cells lacking mHel61p show reduced levels of edited mRNAs. (a) Graphical representation of the relevant sequences of the four mRNAs tested by poisoned primer extension analysis. α-Tub., α-tubulin. Uridylate residues inserted as the result of RNA editing are shown in lowercase letters. Oligodeoxynucleotide primers are represented as black bars, and the asterisk represents the radioactive phosphate at the 5′ ends. The nucleotide lengths of the expected extension products are given below each diagram. Arrows indicate the primer extension stops at the first C residue in each mRNA. (b) Representative autoradiographs of the primer extension analysis for all four mRNAs. WT, wild-type RNA; DKO, RNA preparations from the *mHEL61* double-knockout cell line; −RNA sample with no RNA template added but otherwise treated identically throughout the experiment. Arrows point to extension products representing the edited (ed) and nonedited (ned) versions of the COII and CYb mRNAs. Experiments were performed with three independent steady-state RNA preparations from late-log-phase insect-stage trypanosomes.

RNA editing in mHel61p null mutants. As a consequence, we had to conclude that another mechanism must account for the observed growth rate phenotype of the mHel61p knockout strain. Insect-stage trypanosomes rely on the expression of mitochondrial gene products, which generally require RNA editing for their expression. Therefore, we decided to assay for RNA editing in vivo and to test whether RNA editing in the *mHEL61* null mutant strain was impaired. To detect and quantitate unedited and edited versions of the same mRNAs simultaneously, we performed a "poisoned" primer extension analysis (47). Steady-state RNA isolated from both wild-type and mHel61p-minus cells were compared. Two edited mRNAs, apocytochrome *b* (CYb) and cytochrome oxidase II (COII), a never-edited mRNA, cytochrome oxidase I (COI) (22), and a nuclear transcript, α-tubulin (27), were tested (Fig. 6a). COII mRNA is edited by the insertion of 4 uridylates (1), and CYb is processed by the addition of 34 uridylates (14). Primer molecules were designed to anneal immediately downstream of the first editing sites, and extensions were performed in the presence of ddGTP to stop cDNA synthesis at the first cytosine residue. A representative set of results is shown in Fig. 6b. For the nuclear transcript α-tubulin and the mitochondrial but never-edited COI RNA, no significant difference in the RNA abundance between wild-type and *mHEL61* null mutant cells was observed. The unedited forms of the COII and CYb transcripts were either present in similar amounts in both cell lines (CYb) or moderately reduced in the double-knockout strain (COII). However, the amounts of the edited versions of both RNAs were substantially decreased in cells lacking mHel61p. If the ratio of edited to unedited transcripts in wild-type cells

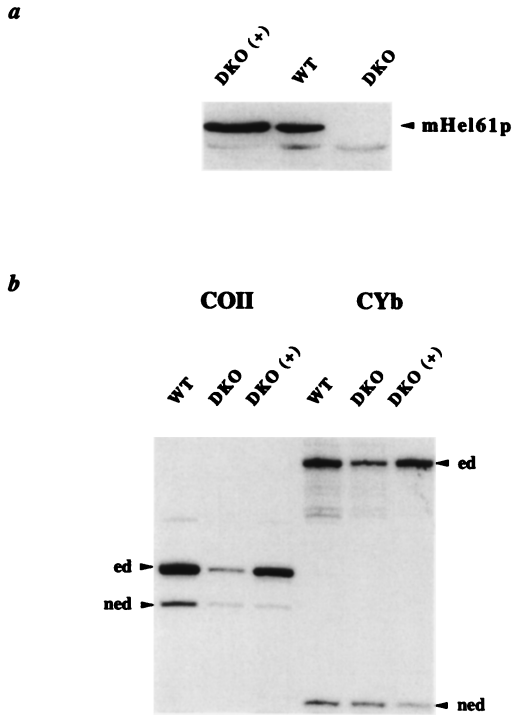

FIG. 7. Reexpression of mHel61p in the *mHEL61* double-knockout cell line rescues the phenotype of low abundance of edited mRNAs. (a) Western blot analysis of whole-cell extracts from wild-type trypanosome cells (WT), *mHEL61* double-knockout cells (DKO), and DKO trypanosomes reexpressing mHel61p [DKO (+)]. (b) Comparative primer extension analysis of COII and CYb mRNAs from wild-type (WT) cells, mHel61p-minus cells (DKO), and double-knockout cells reexpressing mHel61p [DKO (+)]. Annotations are as described in the legend to Fig. 6.

is represented as 100%, the ratios for the COII and CYb transcript are only 26 and 28%, respectively, in the double-knockout strain.

Ectopic reexpression of *mHEL61*. On the basis of these results, we tested whether the phenotype of the mHel61p deletion strain could be rescued by introducing a functional *mHEL61* gene back into the knockout cell line. We constructed a gene cassette to redirect a full-length *mHEL61* gene together with a phleomycin (*phleo*) resistance marker into the C-terminal domain of the β-tubulin gene. Transfectants were grown in the presence of all three antibiotics (G418, hygromycin B, and phleomycin), and the reexpression of mHel61p in the double-knockout [DKO (+)] cells was tested in a Western blot analysis (Fig. 7a). Clearly, the cells had regained the capacity to synthesize mHel61p. A densitometer analysis of the Western blot suggested that reexpressing cells made about 20 to 30% more protein than did wild-type trypanosomes. This could be due to multiple integration events into the multicopy tubulin locus or could simply reflect an enhanced transcription rate. An analysis of the growth rate of the mHel61p reexpressing cells demonstrated that they grew with a doubling time of 12 hours, which is faster than that of the parental double-knockout cells (14 h) but still slower than that of wild-type cells.

A primer extension analysis, to determine the quantities of edited and unedited CYb and COII mRNAs in the reexpressing cell line, confirmed the restoration of the editing efficiency (Fig. 7b). The phenotype of the *mHEL61* double-knockout cell line, with respect to the relative abundance of the two edited mRNAs, was completely reversed. For both mRNAs, the ratios of edited to unedited transcripts increased to values significantly above the wild-type value (COII, 185%; CYb, 170%), which is in line with the above data of the Western analysis.

In vitro RNA-editing activity of mitochondrial extracts from mHel61p-minus cells. The in vivo data clearly demonstrated an influence (either directly or indirectly) of mHel61p on the editing process. However, the data did not allow us to draw a conclusion about the step of the reaction at which the protein might function. To address this question, we decided to use an in vitro RNA-editing system which monitors the gRNA-dependent deletions of four uridylate residues from a synthetic mRNA, representing the unedited 3′ end of the *T. brucei* ATPase 6 mRNA (44, 45). The assay is able to identify, next to a single, specific editing product, the formation of a mRNA 3′-end cleavage intermediate and the formation of gRNA/mRNA chimeras, presumed dead-end products of the editing reaction. Interestingly, the comparison of the in vitro editing activity of mitochondrial lysates from mHel61p null mutant cells and lysates from wild-type cells revealed no difference (Fig. 8). Product formation, mRNA cleavage, and chimera formation were indistinguishable from the situation in wild-type mitochondrial extracts. Although the assay very probably reflects only a limited set of steps of the editing process (editing at a second site and annealing of gRNAs located farther upstream cannot be monitored), it was obvious that mitochondrial lysates from mHel61p-minus cells were editing competent. Thus, mHel61p must act at a postcatalytic reaction step.

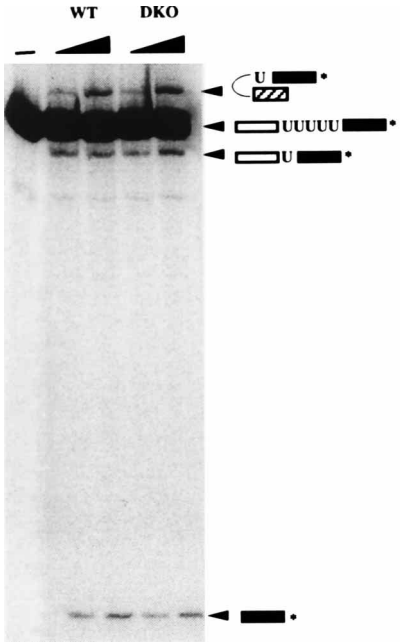

FIG. 8. Comparison of the in vitro RNA-editing activity in mitochondrial lysates from wild-type trypanosomes and mHel61p-minus cells. Reactions were performed, as outlined in Materials and Methods, with 10 and 30 μg of mitochondrial lysate. The reaction products were analyzed in a denaturing polyacrylamide gel and then subjected to autoradiography. WT, wild-type lysate; DKO, lysate isolated from the *mHel61* double-knockout cell line. The minus sign represents a sample of the radioactively labelled input mRNA treated identical throughout the experiment without the addition of mitochondrial lysate. Graphical representations of the different RNA molecules are given on the right next to the corresponding bands and represent (from top to bottom) gRNA/mRNA chimeric molecules, mRNA educt, edited mRNA product (minus four uridylate residues), and the 3′ endonucleolytic mRNA cleavage product. An asterisk indicates the radioactive phosphate residue at the 3′ ends of the different molecules.

DISCUSSION

Our results identify the novel mitochondrial DEAD-box protein mHel61p as being involved in the control of edited mRNAs in trypanosome mitochondria and thus reveal an undocumented property of DEAD-box proteins. Members of this protein family have been reported to contribute to a variety of biochemical processes (16, 42) but not to the RNA editing process. The absence of mHel61p caused a >70% reduction in the editing efficiency of the mRNAs studied, and *mHEL61* null mutant cells were shown to grow more slowly. The protein is encoded in the trypanosome nucleus and presumably is imported into the mitochondrial organelle via a cleavable import sequence.

mHel61p does not contribute to the mitochondrial RNA helicase activity in trypanosome mitochondria. The ds RNA-unwinding activity of mitochondrial lysates from mHel61p-minus cells was not different from that of lysates of wild-type mitochondria. Several explanations can account for this observation. First, mHel61p might not function as an unwinding enzyme, although it is clearly a member of the DEAD-box family of putative RNA helicases. Second, other RNA helicases might be present within the *T. brucei* mitochondrion which might mask the absence of activity in the *mHEL61* null mutant. The latter explanation includes the possibility that more than one helicase participates in the RNA editing process. Similar scenarios have been identified in other biochemical processes, most notably for the splicing reaction, which requires the participation of several DEAD-box proteins as auxiliary factors (8, 43). As a consequence, the different mitochondrial RNA helicases might provide, as well as their specific molecular functions, a basic level of a redundant and promiscuous unwinding activity, which in turn might explain the nonlethal phenotype of the *mHEL61* null mutant strain (13, 50).

mHel61p does not seem to be an integral component of the 20S RNA editing in vitro activity complex (9, 29, 44). The majority of the polypeptide was identified as a noncomplexed mitochondrial protein, and only small amounts of the protein were found in fractions with an apparent Svedberg value of 20S. Among several possible scenarios, this might suggest that mHel61p interacts only transiently with the editing machinery or that mHel61p functions independently of the editing apparatus. Support for this interpretation can be gained from the result that the RNA editing in vitro activity was not impaired in the mHel61p null mutant. No qualitative or quantitative difference was observed with respect to the formation of the correct editing product, the mRNA 3′-end cleavage intermediate, or the formation of gRNA/mRNA chimeras. This implies that the editing machinery can be properly assembled in the mutant cell line and functions indistinguishably from the apparatus in wild-type trypanosome mitochondria. The result also excludes a potential chaperone function to adjust the secondary structures of gRNA and pre-mRNA molecules before or during the editing reaction. Thus, we conclude that the involvement of mHel61p in the editing process is indirect, functioning at a postcatalytic event which is not monitored in the in vitro editing system. This could be a simple but specific stabilization of fully edited mRNAs, a feature of DEAD-box proteins which has been reported in other systems (28). The altered U-content of the edited mRNAs could potentially serve as the recognition signal for the binding of the protein. However, given the extreme extent of RNA editing in trypanosomes, it is hard to envisage that the addition of only four uridylate residues, as in the case of the COII mRNA, would be enough to distinguish between the edited and preedited versions of the molecule. Therefore, we favor the interpretation that mHel61p might act as an RNA helicase for the unwinding of the fully base-paired gRNA/mRNA duplex regions which presumably form after the completion of all editing events specified by one gRNA. Failure to disrupt these helical domains might trigger the degradation of the paired RNA structures and, as a consequence, result in reduced amounts of edited mRNAs. This would suggest a synchronization of the editing reaction with processes controlling mRNA stability or RNA turnover. This is a testable assumption; however, it requires as a first step the experimental verification of RNA helicase activity of mHel61p.

We have no satisfactory explanation why the growth rate of mHel61p-reexpressing trypanosomes did not achieve a wild-type doubling time. The most obvious explanation might lie in the somewhat increased expression level of the protein in the reexpressing cell line. This would suggest a precise regulation of the intramitochondrial mHel61p concentration. Increased amounts of the polypeptide might not be fully tolerated, because of the promiscuity of RNA helicases and thus the interference with other biochemical reactions that require RNA unwinding or stabilization of RNA helices.

Finally, we want to point out that mHel61p might act as a heteromultimeric protein. The primary sequence of the polypeptide lacks ds RNA binding motifs, a feature of DEAD-box proteins that function in a complex with other polypeptides (17).

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