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Is the *Trypanosoma brucei* REL1 RNA Ligase Specific for U-deletion RNA Editing, and Is the REL2 RNA Ligase Specific for U-insertion Editing?*

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It was shown previously that the REL1 mitochondrial RNA ligase in *Trypanosoma brucei* was a vital gene and disruption affected RNA editing *in vivo*, whereas the *REL2* RNA ligase gene could be down-regulated with no effect on cell growth or on RNA editing. We performed down-regulation of REL1 in procyclic *T. brucei* (midgut insect forms) by RNA interference and found a 40–50% inhibition of Cyb editing, which has only U-insertions, as well as a similar inhibition of ND7 editing, which has both U-insertions and U-deletions. In addition, both U-insertion and U-deletion *in vitro* pre-cleaved editing were inhibited to similar extents. We also found little if any effect of REL1 down-regulation on the sedimentation coefficient or abundance of the RNA ligase-containing L-complex (Aphasizhev, R., Aphasizheva, I., Nelson, R. E., Gao, G., Simpson, A. M., Kang, X., Falick, A. M., Sbicego, S., and Simpson, L. (2003) *EMBO J.* 22, 913–924), suggesting that the inhibition of both insertion and deletion editing was not due to a disruption of the L-complex. Together with the evidence that down-regulation of REL2 has no effect on cell growth or on RNA editing *in vivo* or *in vitro*, these data suggest that the REL1 RNA ligase may be active *in vivo* in both U-insertion and U-deletion editing. The *in vivo* biological role of REL2 remains obscure.

Uridine (U)[1]-insertion/deletion RNA editing occurs in the mitochondrion of kinetoplastid protozoa (1, 2). The mechanism in all cases but one involves the annealing of a trans-acting guide RNA to the pre-edited mRNA just downstream of the initial editing site, specific cleavage of the mRNA at the unpaired editing site, deletion of unpaired uridines or addition of uridines to the 3′ end of the 5′ cleavage fragment, which can form base pairs with guiding A or G nucleotides in the guide RNA, and ligation of the 5′ and 3′ cleavage fragments (3–8). Two mitochondrial RNA ligases have been identified in *Trypanosoma brucei* (9–11). Conditional disruption of both alleles of the REL1 ligase in bloodstream *T. brucei* was lethal and affected *in vivo* RNA editing (12). Conditional expression of a mitochondrial targeted *REL1* transgene with a K86R mutation at the AMP-binding site in procyclic *T. brucei* also produced a growth phenotype and disrupted RNA editing (13). Significant effects on *ND7* and *MURF2* editing were reported, both of which involve both U-insertions and U-deletions, but no detectable effects on *Cyb* and *COII* editing were reported, both of which involve only U-insertions (13). Full round *in vitro* U-deletion editing using mitochondrial extract from cells expressing the dominant negative *REL1* transgene was inhibited ~50%, whereas U-insertion editing was apparently unaffected (13). It was also reported that more ATP was required for *in vitro* U-deletion editing than for U-insertion editing, and that this correlated with the levels of ATP required for adenylation of REL1 and REL2, respectively. In addition, we showed that overexpression of tandem affinity purification-tagged REL1 in *Leishmania tarentolae* causes the appearance of a minor L-subcomplex containing REL1, LC-3, LC6a, and LC-4, which is the homologue of MP63 in *T. brucei* (14),[2] and others have found another L-subcomplex consisting of REL2, RET2, and MP81.[3]

These observations led to the model that REL1 mediates ligation at U-deletion editing sites and REL2 mediates ligation at U-insertion sites (13, 15). The fact that down-regulation of expression of the REL2 RNA ligase by conditional RNAi in procyclic or bloodstream *T. brucei* showed no phenotype, either in terms of cell growth or editing (16), was interpreted as a substitution of the REL2 role by REL1, although the reverse does not occur.

In this paper, we have re-analyzed the effects of RNAi down-regulation of REL1 and REL2 in terms of this hypothesis and we conclude that the situation may be more complex than originally envisioned.

EXPERIMENTAL PROCEDURES

Trypanosome Culture and RNAi—To construct the pREL1-H2H vector for inducible RNAi, two 495-bp PCR fragments from the 5′ end of the *T. brucei REL1* coding region were inserted in a head-to-head configuration adjacent to a green fluorescent protein stuffer fragment under the control of a tetracycline-regulatable procyclic acidic repetitive protein promoter (17). The vector was transfected into procyclic *T. brucei* strain 29–13 cells (18), and drug-resistant cell clones were selected by plating on agarose (19). Cells were cultured in SDM-79 medium, and RNAi was induced with 1 μg/ml tetracycline. Cells were maintained in log phase growth by daily dilution. The pREL2-H2H vector was constructed similarly with a 456-nucleotide fragment (nucleotides 69–524) of *REL2* in a head to head configuration.

Oligonucleotides used for PCR of REL1 and REL2 (added restriction sites are in boldface) are as follows: for *REL1*, 5076, 5′-**AAG CTT** ATG CAA CTC CAA AGG TTG GG-3′ (1st forward primer with *Hin*dIII site); 5077, 5′-**TCT AGA** ATA CTT GGC ACC AAA CAG TT-3′ (1st reverse

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[1] The abbreviations used are: U, uridine; RNAi, RNA interference; MOPS, 4-morpholinepropanesulfonic acid; ND, NADH dehydrogenase; MURF, maxicirels unidentified reading frame.

[2] R. Aphasizhev and L. Simpson, unpublished results.

[3] K. Stuart, A. Panigrahi, and A. Schnaufer, personal communication.

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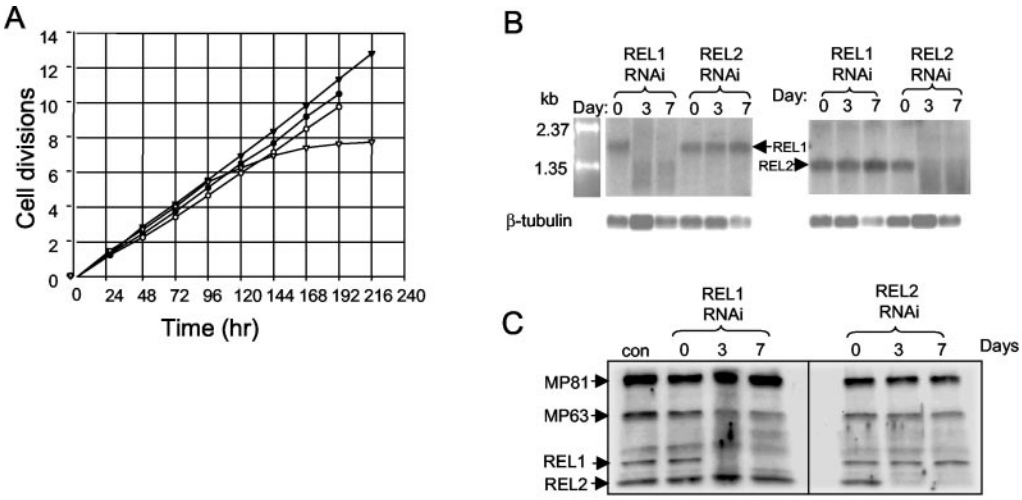

FIG. 1. *A*, effect of tetracycline induction of RNAi of REL1 and REL2 expression on growth of procyclic *T. brucei.* The cells were maintained in log phase by daily dilutions. The cumulative number of cell divisions is plotted *versus* the time after the addition of tetracycline (*tet*). *Open triangles*, REL1 RNAi + tet; *filled triangles*, REL1 RNAi no tet; *open circles*, REL2 RNAi + tet; *filled circles*, REL2 RNAi no tet. *B*, specific degradation of REL1 mRNA by induction of REL1 RNAi and specific degradation of REL2 mRNA by induction of REL2 RNAi. Total cell RNA was isolated from cells 0, 3, and 7 days after addition of tetracycline and fractionated by electrophoresis. The gels were blotted, and the blots were hybridized with a labeled REL1 probe (*left panel*) or a labeled REL2 probe (*right panel*). Hybridization of β-tubulin mRNA was used as a loading control. *C*, specific down-regulation of REL1 protein expression by REL1 RNAi and REL2 protein expression by REL2 RNAi. Mitochondrial extract from 29–13 cells and from the transfected cells 0, 3, or 5 days after the addition of tetracycline was fractionated by SDS acrylamide gel electrophoresis. Western analysis was performed using antibodies against the MP81, MP63, REL1, and REL2 proteins. *Left*, REL1 RNAi. *Right*, REL2 RNAi.

primer with *Xba*I site); 5078, 5′-**GGA TCC** ATG CAA CTC CAA AGG TTG GG-3′ (2nd forward primer with *Bam*HI site); and 5079, 5′-**GGA TCC** ATA CTT GGC ACC AAA CAG TT-3′ (2nd reverse primer with *Bam*HI site); for *REL2*, 5093, 5′-**AAG CTT** CAT TTT TGA GCG CTA CAC AG-3′ (1st forward primer with *Hin*dIII site); 5094, 5′-GC**T CTA GA**A TGT CGA ATG CGT AAA AGT G-3′ (1st reverse primer with *Xba*I site); 5095, 5′-**GGA TCC** CAT TTT TGA GCG CTA CAC AGA-3′ (2nd forward primer with *Bam*HI site); and 5096, 5′-G**GG ATC C**AT GTC GAA TGC GTA AAA GTG-3′ (2nd reverse primer with *Bam*HI site).

Protein Expression and Western Blotting—The full-length *REL1* and *REL2* genes were cloned into the pMAL c2x vector (New England Biolabs). The plasmids were transformed into *Escherichia coli* BL21 DE3 (Stratagene). Expression was induced with 0.3 mM isopropyl-1-thio-β-D-galactopyranoside at 37 °C for 3 h. The recombinant REL1 and REL2 proteins fused with the maltose-binding protein were purified by binding to amylose resin (New England Biolabs) and elution with 10 mM maltose. The recombinant proteins were further purified by SDS-PAGE gel electrophoresis. Polyclonal antibodies against REL1 and REL2 were prepared by Covance Research Products Inc. Monoclonal antibodies against the *T. brucei* MP81, MP63, and MP42 proteins were kind gifts from Ken Stuart. Western blotting was performed using the Super-Signal West Pico chemiluminescent system (Pierce).

RNA Analysis—Total RNA was purified by the acid guanidium isothiocyanate method (20). Primer extension was performed as described previously (21). *Cyb* (oligonucleotide 3812) and *Murf2* (oligonucleotide 3807) mRNAs were analyzed by run-off extensions. *COI* (oligonucleotide 3808), *COII* (oligonucleotide 3809), *ND7* (oligonucleotide 4282), *A6* (oligonucleotide 3882), and calmodulin (oligonucleotide 3813) mRNAs were analyzed by poisoned primer extension using ddGTP to chain-terminate the extensions at the first C residue after 26 editing sites for *A6*, the entire editing domain for *COII*, and 9 editing sites for *ND7* (22). For normalization, oligonucleotides 3813 and 3808 for the cytosolic calmodulin mRNA and the never-edited *COI* mRNA were extended in the same reaction. The following oligonucleotides were used: 3807, 5′-CAACCTGACATTAAAAGAC-3′; 3808, 5′-GTAATGAGTACGTTGTAA-AACTG-3′; 3809, 5′-ATTTCATTACACCTACCAGG-3′; 3812, 5′-GTTC-TAATACATAACAAATCAAAAACACG-3′; 3813, 5′-GTTGATCGGCCA-TCGTAAATCAAGTGGATG-3′; 3882, 5′-ATAAACTAGAATAAGATAT-TGAGG-3′; and 4282, 5′-CTTTTCTGTACCACGATGC-3′.

Northern Analysis—Total RNA (30 μg) was fractionated on a 1.5% agarose-formaldehyde gel in 20 mM MOPS, 5 mM sodium acetate, and 1 mM EDTA, pH 7.0, and the gel was blotted onto a Zeta-probe membrane (Bio-Rad). The filter was hybridized with full-length *REL1* or *REL2* PCR-amplified DNA labeled with [α-^{32}P]ATP using the Prime-It II random primer labeling kit (Stratagene).

Extract Preparation, Glycerol Gradient Sedimentation, and Native Gel Electrophoresis—Purified mitochondria (25 mg protein/ml) were lysed with 0.5% Nonidet P-40 in 10 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, and 60 mM KCl. The clarified extract (300 μl) was centrifuged on a 10–30% glycerol gradient in the SW41 rotor (Beckman) for 20 h at 30,000 rpm, and 150.75-ml fractions were collected from the top using the Isco density gradient fractionator. Aliquots (10 μl) of each fraction were mixed with 0.5 μl (10 μCi/μl) of [α-^{32}P]ATP for 30 min at 27 °C. The reaction was mixed with SDS loading buffer for denaturing gradient gel analysis, and the gels were blotted onto nitrocellulose membranes (Protran) for Western analysis. For both adenylation (23) and Western blot analysis, an aliquot from fraction 9 of an identical gradient of mitochondrial extract from untransfected 29–13 cells was used as an internal loading control in each gel. Sedimentation values were calculated using aldolase (9 S), thyroglobulin (19 S) and *E. coli* ribosome 30 S subunits.

RNA Substrates and in Vitro Editing—The following RNA substrates were chemically synthesized (Oligos Etc. and Xeragon) and gel-purified: 5′ fragment, 5′-GCACUACACGAUAAAUAUAAAAAG-3′; 5′-UU fragment, 5′-GCACUACACGAUAAAUAUAAAAAGUU-3′; 3′ fragment, 5′-AACAUUAUGCUUCUUCGddC-3′; AG brRNA, 5′-AAGAAGCAUAAU-GUUAGCUUUUUAUAUUUAUCGUGUAGUCddG-3′; and 0 brRNA, 5′-AAGAAGCAUAAUGUUCUUUUUAUAUUUAUCGUGUAGUCdd-G-3′.

RNAs were 5′-phosphorylated with T4 polynucleotide kinase (Invitrogen) and [γ-^{32}P]ATP. Complementary RNAs were annealed by heating and slow-cooling. For *in vitro* editing assays, the L-complex fractions (8–10) were pooled and concentrated to 300 μl. The concentrated gradient fractions were stored at −20 °C in 50% glycerol, 1 mg/ml bovine serum albumin, and 1 mM dithiothreitol. The fractions were fractionated by electrophoresis on 8–16% denaturing gradient gels, and the gels were stained with Coomassie Blue (Sigma) to monitor the protein concentrations. The *in vitro* editing reactions were performed at 27 °C for 2 h in 20 μl of 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mM ATP. 2 mM UTP was added for the insertion reaction. The products were separated in a sequencing gel and detected by autoradiography.

RESULTS

Down-regulation of REL1 Ligase Expression Inhibits Both U-insertion and U-deletion Editing in Vivo—Down-regulation of REL1 by conditional RNAi produced a growth inhibition by day 6 of induction (Fig. 1*A*), which was preceded by a selective degradation of *REL1* mRNA and down-regulation of REL1 protein expression by day 3 (Fig. 1*B*). A correlated down-regulation of the MP63 L-complex protein was also observed (Fig. 1*C*). The relative abundances of edited and pre-edited

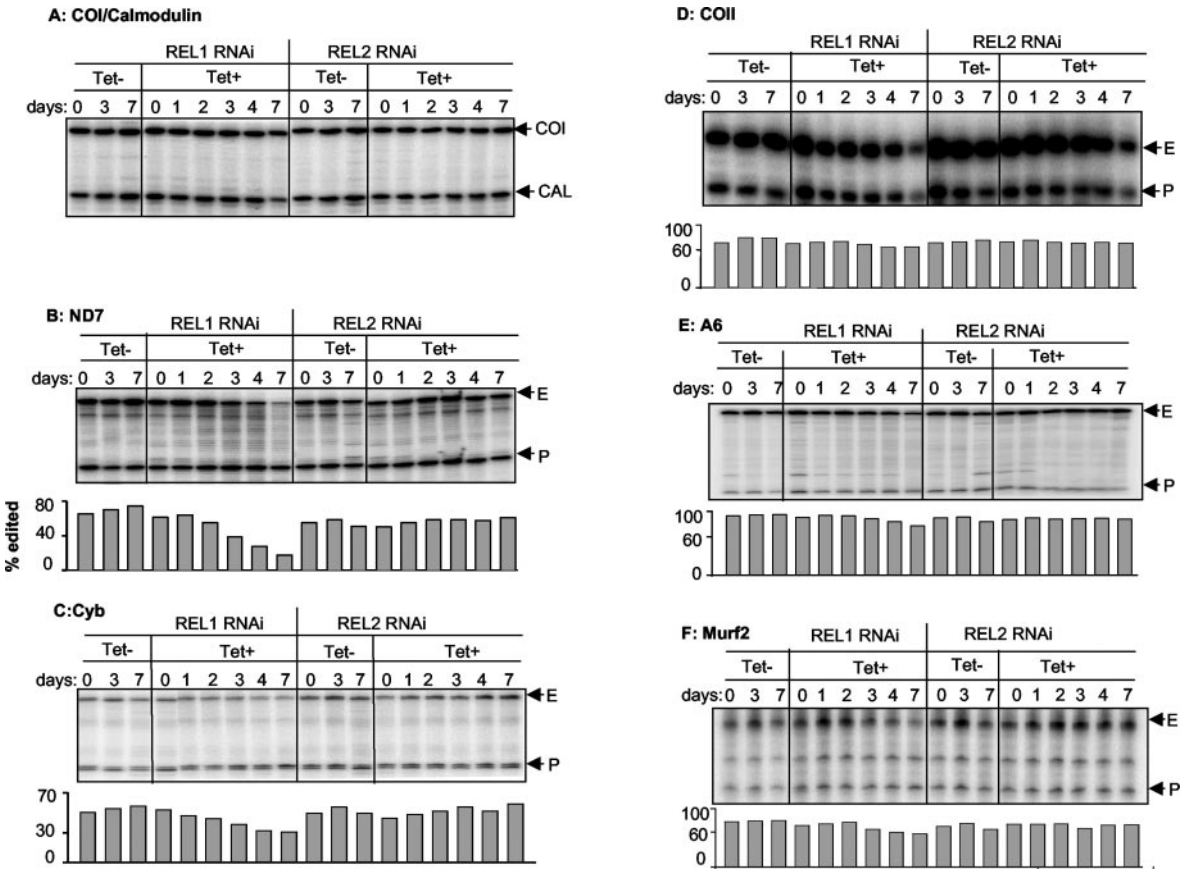

FIG. 2. **Effect of REL1 and REL2 RNAi on RNA editing *in vivo*.** The abundance of pre-edited and edited mRNAs was assayed using primers downstream of the editing domains. *A*, primer extensions of never-edited *COI* mRNA and cytosolic calmodulin mRNA were used as loading controls. *B*, *ND7* editing. *C*, *Cyb* editing. *D*, *COII* editing. *E*, *A6* editing. *F*, *MURF2* editing. Quantitation of the autoradiographs are shown below each figure. The ordinate represents the percent of editing (edited band/the edited plus pre-edited bands × 100). The fully edited band is indicated by *E*, and the pre-edited band is indicated by *P*.

mRNAs were examined for five genes by primer extension analysis (Fig. 2). Editing of *Cyb* and *ND7* mRNAs decreased 40–70% after 7 days of RNAi induction, and editing of *COII*, *MURF2*, and *A6* mRNAs decreased slightly. There was no change in the abundance of pre-edited RNAs or the never-edited *COI* mRNA, suggesting a specific effect on editing.

As reported previously (16), down-regulation of REL2 by RNAi produced no growth phenotype (Fig. 1*A*) or editing phenotype (Fig. 2), although there was a selective decrease of *REL2* mRNA by day 3 (Fig. 1*B*) and REL2 protein by day 3 (Fig. 1*C*).

Down Regulation of REL1 Affects Both U-insertion and U-deletion Pre-cleaved in Vitro Editing—Mitochondrial extracts from cells induced for REL1 RNAi for 0, 3, or 5 days were tested for pre-cleaved *in vitro* editing activity (Fig. 3). U-insertion editing and U-deletion editing were inhibited to similar extents. There was no effect on the addition or deletion of 3′-terminal uridines to or from the 5′-mRNA cleavage fragment. In the case of REL2 RNAi, there were no detectable changes in the extent of U-insertion or U-deletion pre-cleaved *in vitro* editing (Fig. 3).

Down Regulation of REL1 Has No Effect on the S Value or Abundance of the L-Complex—One possible explanation for the inhibition of both U-insertion and U-deletion *in vitro* editing by down-regulation of *REL1* would be that there was a decrease in stability of the L-complex. In fact, it was previously reported that knocking out of one *REL1* allele in *T. brucei* procyclics produced a partial breakdown of the L-complex (13). To test this possibility, mitochondrial extract from cells induced for REL1 RNAi for 0, 3, or 5 days was fractionated in a glycerol gradient and each fraction was incubated with [α-^{32}P]ATP to label the REL1 and REL2 RNA ligases, which represent markers for the L-complex. It should be noted that the REL1 ligase was labeled to a greater extent than the REL2 ligase because of the latter being pre-charged with AMP (11). The S value of the L-complex did not change as the REL1 protein decreased in abundance (Fig. 4*A*). Western analysis of each fraction using antibodies against MP81, MP63, and REL1 was also performed (Fig. 4*A*). Despite the loss of detectable REL1 protein, the L-complex was unaffected in terms of location in the gradient and relative abundance. Down-regulation of REL2 likewise had no effect on the S value or relative abundance of the L-complex (Fig. 4*B*).

DISCUSSION

The presence of two mitochondrial RNA ligases, both of which are components of the L-complex, has raised an interesting question. Several lines of evidence have led to the model that REL1 is involved in U-deletion editing and REL2 is involved in U-insertion editing (13, 15, 24). The complete absence of any phenotype with REL2 down-regulation has been explained by the suggestion that REL1 can substitute for REL2 in the REL2 down-regulated cells and mediate both types of editing (13, 15). The lethality of the REL1 knock-out has been explained by assuming that REL2 is not capable of substituting for REL1 in U-deletion editing.

The results presented in this paper raise some doubts about this model. We showed that down-regulation of REL1 expression affected the *in vivo* editing of different mRNAs to different extents, but the inhibitory effect was apparently correlated

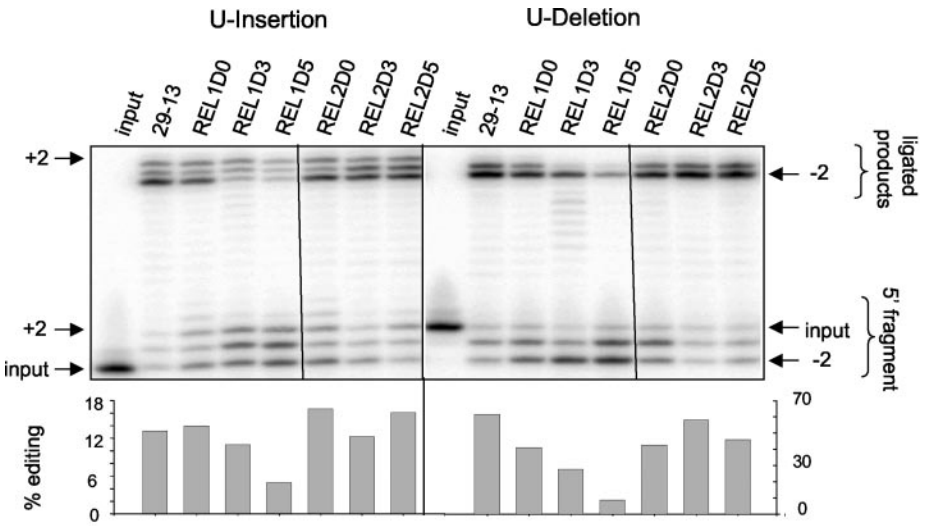

FIG. 3. **Effect of REL1 RNAi and REL2 RNAi on pre-cleaved *in vitro* editing.** Both +2U-guided U-insertion and −2U-guided U-deletion editing were assayed. Mitochondrial extract was isolated from untransfected 29–13 cells and from transfected cells 0, 3, and 5 days after the addition of tetracycline. The extracts were fractionated in glycerol gradients and fractions 8–10 (of 15 total fractions) containing the peak of the L-complex and were pooled and concentrated. The concentrated fractions were used for *in vitro* editing assays. *Input lane*, no enzyme. The relative amounts of the fully edited species were determined by PhosphorImager analysis, and the quantitation is plotted beneath the figures. The ordinate represents the percent of editing (mature edited band/input band × 100).

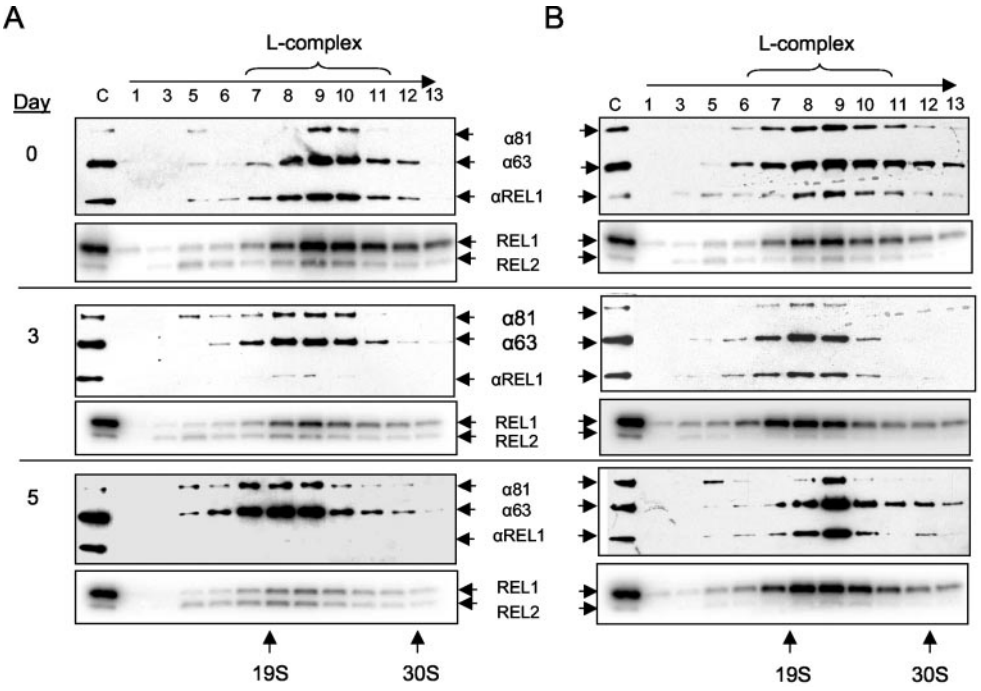

FIG. 4. **Effect of REL1 RNAi and REL2 RNAi on S value and relative abundance of L-complex.** The direction of sedimentation is indicated by an *arrow*. *A*, REL1 RNAi. Mitochondrial extracts from transfected cells 0, 3, and 5 days after addition of tetracycline were fractionated in glycerol gradients and aliquots (10 μl) of each fraction electrophoresed in an SDS acrylamide gel, which was blotted for Western analysis. α81, α63, and αREL1 represent antibodies against MP81, MP63, and REL1, respectively. The *lower panel* in each case is an autoradiograph of an identical gel of 10-μl aliquots of each fraction labeled with [α-^{32}P]ATP. The adenylated REL1 and REL2 bands are seen. The location of the L-complex is indicated by *brackets*. *Lane C*, fraction 9 from gradient of mitochondrial extract from 29–13 control cells. *B*, REL2 RNAi. See *A* for details.

with the percentage of editing of the specific mRNA rather than with the presence of U-insertions alone or U-insertions and U-deletions. For example, the editing of *Cyb* mRNA, which only involves U-insertions, was inhibited to a similar extent as the editing of *ND7* mRNA, which involves both U-insertions and U-deletions. The editing of *A6*, *MURF2*, and *COII* mRNAs, the former two involving both insertions and deletions and the latter involving only insertions, was inhibited to a lesser extent, both decreasing ~20% by day 7. The magnitude of the effect on editing appeared to be inversely correlated with the normal extent of editing, in that *A6*, *MURF2*, and *COII* editing is normally very efficient (>90% edited RNA), whereas *Cyb* and *ND7* editing is less efficient (~70% edited RNA). The reason for this variation in the extent of inhibition of editing with different mRNAs is not known. Furthermore, we showed that pre-cleaved U-insertion and U-deletion *in vitro* editing were inhibited to similar extents. These results differ from those reported previously both in terms of the effect on *Cyb* mRNA editing *in vivo* and full-round *in vitro* editing (13), the reasons for which are not clear but may be related to the different modes of gene knockdown and to the different *in vitro* assays. We eliminated the possibility that RNAi down-regulation of REL1 affected the overall stability of the L-complex and thereby also affected U-insertion editing by showing that there was no large change in S value or abundance of the L-complex in the REL1 RNAi-induced cells. We conclude that the simplest interpretation is that REL1 performs ligations for both U-insertion and U-deletion editing and that REL2 is less active or even inactive *in vivo*, at least under these physiological conditions. We realize that the simplest interpretation is not always valid and that,

for example, down-regulation of REL1 may affect the functional interaction of REL2 with other L-complex proteins and thereby also affect U-insertion editing, but this would have to be established. It is clear from both the apparently functional suborganization of proteins in the L-complex (14) and from the striking ATP titration correlations (15) that REL1 and REL2 probably have differing biological roles, but the precise nature of these roles is yet unclear. We speculate that REL2 may be active under different physiological or developmental conditions. The precise roles of these two RNA ligases await reconstitution of activities with recombinant proteins.

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