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RNA editing in the free-living bodonid *Bodo saltans*

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ABSTRACT

In parasitic kinetoplastid protozoa, mitochondrial (mt) mRNAs are post-transcriptionally edited by insertion and deletion of uridylate residues, the information being provided by guide (g) RNAs. In order to further explore the role and evolutionary history of this process, we searched for editing in mt RNAs of the free-living bodonid *Bodo saltans*. We found extensive editing in the transcript for NADH dehydrogenase (ND) subunit 5, which is unedited in trypanosomatids. In contrast, *B.saltans* cytochrome *c* oxidase (cox) subunit 2 and maxicircle unidentified reading frame (MURF) 2 RNAs display limited editing in the same regions as their trypanosomatid counterparts. A putative intramolecular cox2 gRNA and the gene for gMURF2-I directing the insertion of only one U in the 5′ editing domain of MURF2 RNA, are conserved in *B.saltans*. This lends (further) evolutionary support to the proposed role of these sequences as gRNAs. Phylogenetic analysis showed that *B.saltans* is more closely related to trypanosomatids than the cryptobiids *Trypanoplasma borreli* and *Cryptobia helicis*, in line with the trypanosomatid-like cox2 and MURF2 RNA editing patterns. Nevertheless, other features like the apparent absence of a catenated mtDNA network, are shared with bodonid and cryptobiid species. ND5 RNA editing may represent yet another example of editing 'on the way out' during kinetoplastid evolution, but in view of the fact that cox2 RNA is unedited in *T.borreli* and *C.helicis*, we infer that the editing of this RNA may have arisen relatively recently. Our results provide the first examples of RNA editing in a free-living kinetoplastid, indicating that there is no direct link between U-insertion/deletion editing and a parasitic lifestyle.

INTRODUCTION

RNA editing in kinetoplastid protozoa involves the addition and deletion of uridylate residues at numerous sites of mitochondrial (mt) mRNAs, via consecutive enzymatic cleavage/ligation reactions under the direction of guide (g) RNAs (reviewed in 1–4). So far, this type of RNA editing has been found in a large number of species belonging to the suborder Trypanosomatina (1,5) and in *Trypanoplasma borreli*, which is a cryptobiid belonging to the Bodonina suborder (6,7; for trypanosome taxonomy, see 8). This suggested that RNA editing was present in the common ancestor to these species, possibly existing >500 million years ago, which implied that RNA editing is an ancient process (6,9–11). Trypanosomatid mtDNA is characterized by the presence of an intricate network of maxicircles and minicircles which vary in size according to the species (reviewed in 12,13). The maxicircles contain the genes encoding pre-edited and unedited mRNAs and a few gRNA genes, whereas minicircles encode the large majority of the gRNAs, number and location of the gRNA genes depending on the species (reviewed in 1,2,14–16). It has been suggested that the function of the network is to reduce the risk of losing minicircles during mtDNA replication (17). Nevertheless, during prolonged periods of laboratory cultivation of *Leishmania tarentolae*, minicircles encoding gRNAs involved in the editing of apparently non-essential mRNAs are rapidly lost (18).

How and why kinetoplastid RNA editing has evolved remains enigmatic. On the one hand, RNA nucleotide insertion/deletion processes could be a relic of truly ancient, possibly prebiotic times (11,19,20). Alternatively, its origin could be more recent, having evolved e.g. as a mechanism to compensate for genomic mutations (21,22). The role of RNA editing in present-day kinetoplastids, if any, is also unclear. In principle, it could allow the production of multiple protein isoforms from one gene via differential editing of a pre-edited RNA (23,24) and/or provide an extra level of regulation of gene expression (25), but it is unknown whether RNA editing indeed functions this way. In addition, RNA editing seems to speed up the rate of evolution of mitochondrial proteins (26). Whether RNA editing provides a selective advantage also remains to be established (for a more extensive discussion, see 2 and references therein), but in view of the fact that all kinetoplastid species in which U-insertion/deletion editing has been found to date have a parasitic lifestyle, it has been envisaged that RNA editing is an adaptation to parasitism (20).

Members of the suborder Bodonina have largely been neglected in the analysis of the RNA editing process, in spite of the fact that they represent early branches of kinetoplastid evolution and are fascinating study objects for those interested in exploring the evolutionary history of RNA editing. Therefore, we searched for

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editing in the free-living bodonid *Bodo saltans* (and other bodonid species) and found extensive editing in NADH dehydrogenase (ND) subunit 5 RNA and limited editing in cytochrome *c* oxidase (cox) subunit 2 and maxicircle unidentified reading frame (MURF) 2 RNAs. Phylogenetic analysis of cox2 sequences showed that *B.saltans* indeed diverged early from the kinetoplastid lineage, albeit later than the cryptobiids *T.borreli* and *Cryptobia helicis*. In accordance with its intermediary position in the tree, the *B.saltans* mt genetic system displays trypanosomatid-like characteristics, but clear differences are also found.

The present data show for the first time that RNA editing also occurs in non-parasitic kinetoplastid flagellates.

MATERIALS AND METHODS

Cell culture and nucleic acid isolation

Crithidia fasciculata (Steinert strain, see 27) was grown as described (28). *Crithidia fasciculata* total DNA or RNA was isolated using the hot-phenol extraction method described by Borst and Fase-Fowler (29) and mtDNA was isolated as described (28); for RNA isolations, DNA was removed by digestion with DNase (5 µg/ml), according to Tullis and Rubin (30).

Bodo saltans (strain K1) was isolated by Doris Springmann (Limnology Institute, University of Constance) from the water of Lake Constance and was provided by Karel Simek (Institute of Hydrobiology, Czech Academy of Sciences, Ceské Budejovice). It was grown at 16°C in poor medium (90 mg bacto peptone and 10 mg yeast extract in 1 l tap water) with an optimal density of feeder bacteria (*Alcaligenes xylosoxidans denitrificans*) with mild shaking. After a cell density had been reached of ~10^7 cells/ml, the culture was spun down and the pellet was resuspended in 200 ml of fresh medium and left overnight at 4°C in a column to allow sedimentation of the feeder bacteria. Remaining bacteria were removed by differential centrifugation. Cells were washed repeatedly with NET-50 (50 mM EDTA, 100 mM NaCl, 10 mM Tris–HCl, pH 8.0) and lysed by the addition of 3% (w/v) N-laurylsarcosine (Fluka) and 1 mg/ml pronase E (Merck), for 1 h at 4°C. The lysate was extracted with phenol–chloroform and total DNA was precipitated with ethanol, air dried and resuspended in 10 mM Tris–HCl, pH 8.0, 0.1 mM EDTA. During the isolation procedures, vortexing and shearing were avoided. Total RNA was extracted without corrosive organic compounds with a Rapid RNA purification kit (Amresco), and mRNA was isolated with oligo (dT)-cellulose beads using a Messenger RNA isolation kit (Stratagene), according to the manufacturer's instructions.

Bodo uncinatus (strain ATCC 30904) was isolated from a freshwater pool. It was provided by Jaroslav Kulda (Department of Parasitology, Faculty of Sciences, Charles University, Prague) and was cultivated at 20°C, using the same protocol as for *B.saltans*. Cells of *C.helicis* were obtained from the receptaculum seminis following its dissection from sacrificed garden snails (*Helix pomatia*). After opening the wall of the receptaculum, flagellates were washed out by micromanipulation from the space between the receptaculum wall and spermatophorous matrix. *Trypanosoma avium* (strain A1412) was isolated from the bone marrow of a raven (*Corvus frugilegus*) by Jan Kucera (Institute of Veterinary Pharmacology, Jílové u Prahy) and cultured as described elsewhere (31). Total DNA from these flagellates was isolated as from *B.saltans*. *Endotrypanum monterogei* (strain M5725) was isolated from the blood of a twotoed sloth (*Choleopus didactylus*) by Ralph Lainson (Chagas Institute, Belem, Brasil). Total DNA from these organisms was provided by Dmitri Maslov and Agda Simpson (University of California, Los Angeles).

Electrophoresis, blotting, hybridisation and PCR

Standard agarose gel electrophoresis of DNA or RNA, Southern and northern blotting and hybridisation procedures were essentially as described (32–34). Analysis of DNA by the Contour-clamped Homogeneous Electric Fields technique (CHEF) was performed as described by Chu *et al.* (35). In a representative experiment, 5 µg of DNA was electrophoresed at 180 V with pulse times of 5 s for 24 h at 4°C in a 1% (w/v) agarose gel in 0.09 M Tris–borate, 0.2 mM EDTA, pH 8.3. In all electrophoresis experiments, kinetoplastid DNA was added at 37°C in low-melting agarose, which was allowed to solidify before the electrophoresis was started. Gels obtained from CHEF or normal gel electrophoresis were blotted following treatment of the gel with 0.25 M HCl for 20 min to facilitate the blotting of large DNA molecules.

For PCR amplification of DNA fragments, the following protocol was used: 1 ng of recombinant DNA or 100 ng of total *B.saltans* DNA was incubated with 40 pmol of oligonucleotides for 5 min at 95°C, after which it was amplified in 30 cycles of 1 min at 95°C, 1 min 30 s at 50°C, followed by 2 min at 74°C. The annealing temperature used in a particular experiment depended on the length and GC-content of the oligonucleotides in question: 4°C per G or C plus 2°C per A or T, minus 5°C. For these reactions, 1 U of Taq polymerase and buffers were used according to the manufacturer's (Promega) instructions. For amplification of RNA segments (RT-PCR), the PCR protocol was preceded by cDNA synthesis: 0.1 µg of *B.saltans* mRNA was denatured for 2 min at 70°C, then immediately put on ice and added to the RT-mix containing 300 ng of the downstream primer, 10 U reverse transcriptase (Promega), 10 U RNAsin (Promega) and Promega's RT buffer in a total volume of 30 µl. The mixture was incubated for 1 h at 42°C, followed by 5 min at 95°C; 5 µl of this reaction was used for PCR essentially as described above.

Cloning and sequencing of a 4.0 kb *B.saltans* mtDNA fragment

A fragment of 294 bp derived from the region containing the frameshift site of the cox2 gene, was synthesized by PCR with *B.saltans* DNA, utilising two degenerate oligonucleotides derived from universally conserved regions (G567 and G568, sequences available upon request). This fragment hybridised to a 2.1 kb fragment on a Southern blot, obtained upon digestion of *B.saltans* DNA with *Alu*I. The fragment was isolated using a DNA extraction kit (Qiagen) and cloned into the *Hin*cII site of pBluescript KS(+). Positive clones were picked and used for sequence analysis (see below). The insert was also used to probe recombinant plasmid libraries prepared from *B.saltans* total DNA and a clone containing a 4040 bp *Hae*III fragment was obtained. The region present in the 4040 bp clone was sequenced in its entirety using numerous oligonucleotides (sequences available upon request), following the procedure of Sanger *et al.* (36). The sequence has been deposited in GenBank under accession number AF041263.

Cloning and sequencing of a cox2 segment from various kinetoplastids

The oligonucleotides G567 and G568 that were used to amplify the 294 bp cox2 mt DNA fragment from *B.saltans* were also used to amplify the corresponding cox2 segment from *B.uncinatus*, *E.monterogei*, *T.avium* and *C.helicis*. The fragments were cloned in pT7blue vector (Novagen) and sequenced. The sequences have been deposited in GenBank under accession numbers AF011394–97 for *B.uncinatus*, *C.helicis*, *T.avium* and *E.monterogei*, respectively.

Sequence determination of cDNA

The sequences of cox2, MURF2 and ND5 cDNA sections were determined following RT-PCR mediated cloning of cDNA fragments with the oligonucleotides indicated in Figures 5 and 6 (sequences available upon request). PCR-fragments were cloned in the pGEM-T vector (Promega). The cDNA sequences have been deposited in GenBank under accession numbers AF041331 (cox2), AF041332 (MURF2) and AF041330 (ND5).

Microscopy

Immobilized *B.saltans* cells were visualised under the light microscope by Nomarski interference contrast. For transmission electron microscopy (EM), *B.saltans* cells were fixed overnight in 2% (w/v) glutaraldehyde at 4°C, post-fixed in 2% (w/v) osmium tetroxide in 0.2 M cacodylate buffer pH 7.5 for 1 h at room temperature, and embedded in Epon-Araldite resin as described (37). *Bodo saltans* and *C.fasciculata* DNA was spread for electron microscopy as described (6,38), using bacteriophage PM2 DNA (Boehringer) as an internal size marker. In some experiments, the DNA was extracted immediately before use from ~2 × 10^6 cells, embedded in blocks of 0.5% (w/v) low-melting agarose, by the lysis procedure described in Sambrook *et al.* (32).

Phylogenetic analysis

(c)DNA cox2 sequences from *L.tarentolae* (39), *C.fasciculata* (40), *T.brucei* (41,42), *T.borreli* (6), *B.saltans*, *B.uncinatus*, *E.monterogei*, *T.avium* and *C.helicis* (this paper) were aligned. To utilize all the informative positions, the (c)DNA rather than the amino acid sequences were analysed using the PHYLIP package (43), which is available at the WorldWide Web site http://evolution.genetics.washington.edu/phylip.html . The maximum parsimony tree was obtained by branch-and-bound algorithm (Dnapenny program), which performs an exhaustive search for all maximum parsimony trees. Once the tree was obtained, the statistical support for each branch was determined by bootstrap analysis. On the basis of the original matrix, 500 random sets of data were generated (Seqboot program) and analysed for maximum parsimony solution (Dnapars program). Finally, the consensus tree and bootstrap values were obtained by the Consense program.

RESULTS

Bodo saltans mtDNA organisation

Bodo saltans possesses two flagellae, which is the morphological hallmark of Bodonina species (Fig. 1A). Osmium-fixed- and Epon-Araldite-embedded cells, in which the ultrastructure is optimally preserved, were analysed by transmission electron microscopy. This approach clearly identified the *B.saltans* kinetoplast DNA (kDNA) disc in the anterior part of the mitochondrion close to the flagellar kinetosomes (Fig. 1B), kinetoplast structure and localisation resembling that found in trypanosomatids (44). We next isolated *B.saltans* DNA and analysed it by electron microscopy, using experimental procedures in which vortexing and other shearing of the DNA was avoided. In numerous experiments, no evidence could be found for the presence of a kDNA network (data not shown), even with DNA extracted from a small number of cells embedded and lysed in agarose involving a minimal number of manipulations (see Materials and Methods). At higher magnifications, *B.saltans*

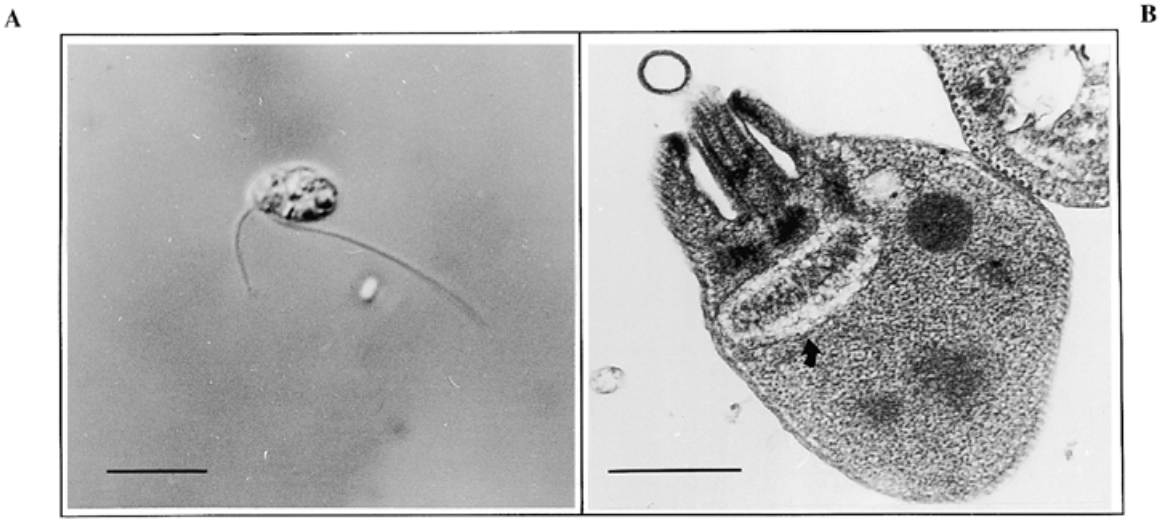

Figure 1. Light microscope- and Transmission EM pictures of *B.saltans*. (**A**) Light microscope picture (magnification 1800-fold, the size bar representing 10 µm). (**B**) Transmission EM picture (magnification 25 000-fold, the size bar representing 1 µm). The arrow points at the kinetoplast in which the kDNA disc is clearly visible. For details, see Materials and Methods.

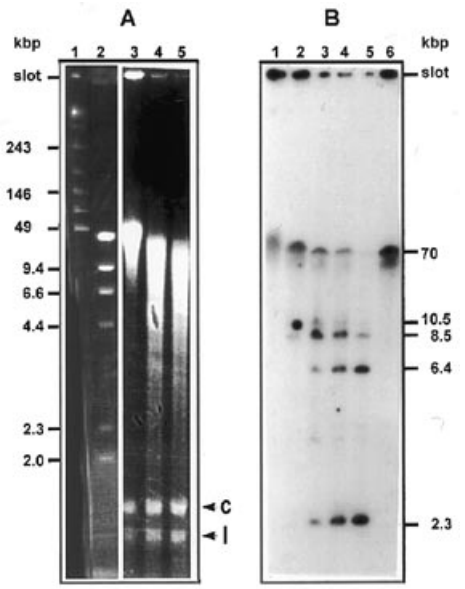

Figure 2. Electrophoresis of *B.saltans* DNA. (**A**) Ethidium bromide stain of a CHEF gel (see Materials and Methods). Lane 1, ladder of λ-DNA multimers; lane 2, λ-DNA cut with *Hin*dIII; lane 3, 5 µg *B.saltans* DNA, unrestricted; lane 4, 5 µg *B.saltans* DNA cut with *Bam*HI (25 U for 1 h); lane 5, 5 µg *B.saltans* DNA cut with *Eco*RI (25 U for 1 h). The position at which circular and linear forms of 1.4 kb minicircle-like molecules were found is marked with c and l, respectively. (**B**) Southern blot hybridisation of a CHEF gel of 5 µg of *B.saltans* DNA. Lane 1, unrestricted DNA; lanes 2–5, DNA cut with *Eco*RI (10 U) for 15, 30, 45 and 60 min, respectively; lane 6, DNA cut with *Bam*HI (25 U for 1 h). The probe was a cloned *B.saltans* maxicircle fragment (coordinates, 537–2628, see Fig. 3). The sizes of the hybridising bands have been indicated, as calculated from the position of marker DNAs [not shown, see (A)].

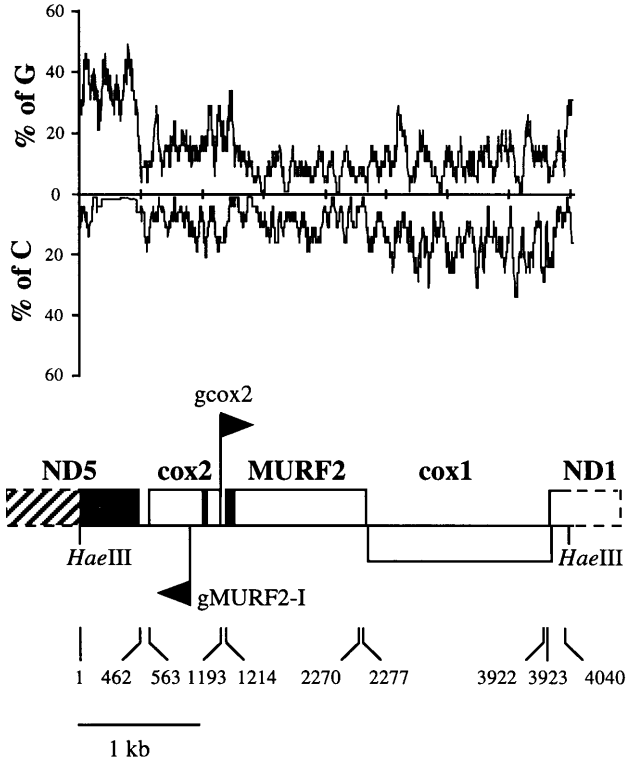

Figure 3. Gene content of a 4.0 kb maxicircle DNA fragment of *B.saltans*. The upper part of the figure contains a graphic representation of the GC-content of the fragment (window size: 30 nt). In the lower part, the coordinates of the genes found and the position of the *Hae*III sites used in the cloning procedures have been indicated; for a given gene the A of the (inferred) translational initiation codon is nt # 1, the last nucleotide of the stopcodon (A or G) is the last nucleotide of the gene. The black areas of the ND5, MURF2 and cox2 genes are cryptic, coding sequences being created by editing of the corresponding RNA segments, the striping representing the uncertain editing status of the 5′ moiety of ND5 RNA. The position of two gRNA genes encoding gcox2 (coordinates: 1191–1210) and gMURF2-I (coordinates: 990–952), respectively, has been indicated by flags. The direction of transcription is from left to right for the genes above the line and from right to left for the genes below. Abbreviations: cox, cytochrome *c* oxidase; ND, NADH dehydrogenase; MURF, maxicircle unidentified reading frame.

DNA appeared to contain a number of small circular DNAs of identical size, 1.40 ± 0.14 kb (n = 49), as determined from a comparison of their contour length with that of bacteriophage PM2 DNA. In addition, catenanes composed of dimers and trimers were also observed, albeit infrequently. We speculate that these circular DNAs represent the *B.saltans* equivalent of minicircles (see below and Discussion).

The characteristics of *B. saltans* mtDNA were further analysed by the Contour-clamped Homogeneous Electric Fields (CHEF) electrophoresis technique. Figure 2A shows an ethidium bromide stained gel. With uncut DNA, a prominent band was visible in the lower molecular mass region of the gel (lane 3), which, following excision and analysis by EM, turned out to predominantly consist of the 1.4 kb circles described above (c in Fig. 2A). A faster migrating, less prominent band was analysed in a similar fashion and appeared to consist largely of linear 1.4 kb molecules, most likely representing the linear form of the 1.4 kb circles (l in Fig. 2A). When CHEF gels were blotted and probed with a cloned mt maxicircle fragment (see below), a broad hybridising band was found with an apparent size of ~80 kb (Fig. 2B, lane 1). A sharper band migrating at 70 kb was observed following treatment of the DNA with a number of restriction enzymes (lanes 2–6 and data not shown). Under the conditions used, the 70 kb band was the first band to arise with *Eco*RI and the only one obtained with *Hin*dIII, *Bam*HI and *Xho*I. These results strongly suggest that the 70 kb band represents the linearized form of the *B.saltans* maxicircle, the band migrating at 80 kb being derived from the circular form. Approximately 50% of the hybridisation signal in lane 1 was found at the position of the slot, most likely due to trapping of the large circular molecules during the solidification of the low-melting agarose used to apply the DNA sample.

Sequence analysis and gene content of a 4.0 kb *B.saltans* maxicircle fragment

A 4040 bp fragment, obtained by digestion with *Hae*III of total *B.saltans* DNA, was cloned and sequenced as described in Materials and Methods. Comparison with database sequences resulted in the identification of genes encoding cox1, cox2, MURF 2 and a 115 bp N-terminal segment of ND1, with a high degree of identity to the corresponding genes from trypanosomatids and *T.borreli* (Fig. 3; Table 1). The presence of these genes clearly shows the mitochondrial origin of the sequenced DNA fragment. The cox2 and MURF2 genes have anomalies at the same position as their trypanosomatid counterparts: the cox2 gene is frameshifted (albeit that the *B.saltans* frameshift is +1 instead of –1), and the MURF2

reading frame appears to lack a proper N-terminus. This strongly suggested that, as in trypanosomatids, the corresponding RNAs are edited at these positions, which was confirmed by sequence analysis of the corresponding transcripts (see below). Upstream of the cox2 gene, we found a region without obvious similarity to known sequences. This region has a relatively high GC-content (35 versus 15% for the remainder of the fragment, see Fig. 3), which is reminiscent of the cryptic genes in kinetoplastid mtDNAs whose transcripts are extensively edited (15,45). As outlined below, RNAs derived from this fragment are indeed extensively edited, resulting in a sequence with 63% identity to ND5 gene sequences from trypanosomatids, in which ND5 RNA is not edited.

Editing of cox2 and MURF2 RNAs and conservation of cox2 and MURF2-I gRNA genes

Bodo saltans cox2 cDNA segments that encompass the frameshift region were cloned and sequenced. In four out of twelve cDNA clones, we found the insertion of two single Us at two sites (Fig. 4A), resulting in a continuous reading frame (Fig. 4B). As expected from the DNA sequences, the insertion pattern is different from that observed in trypanosomatids since two of the four Us that are inserted in trypanosomatid cox2 RNA are already encoded by the gene in *B.saltans*. Interestingly, a putative guiding sequence for frameshift region editing, found immediately downstream of the translational stopcodon in all trypanosomatid cox2 genes analysed to date (34,46,47), is also found at that position in *B.saltans* cox2, its sequence being changed in accordance with the altered editing pattern (Fig. 4A, see Fig. 3).

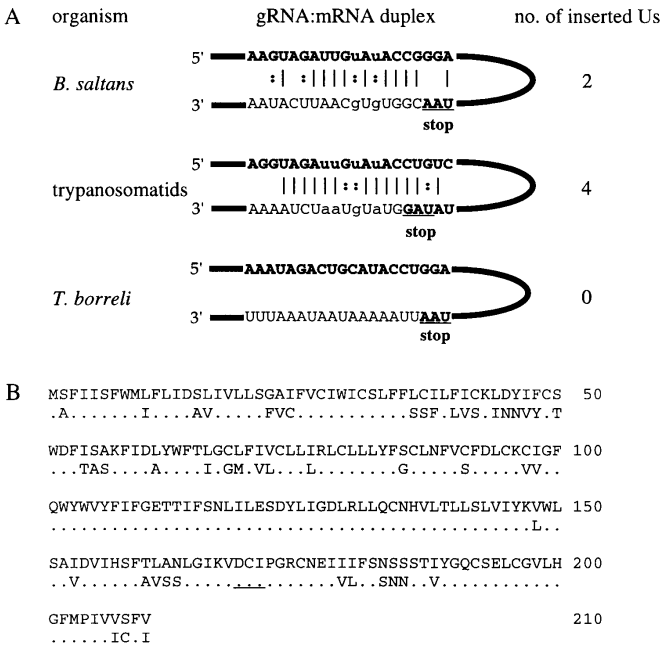

Figure 4. *Bodo saltans* cox2 RNA editing. (**A**) The sequence of cox2 RNA and conservation of an internal cox2 gRNA in various species. The figure shows the potential of the 3′ UTR to basepair with the editing region of cox2 mRNA, with the number of inserted U residues given on the right. Bold lettering is used for nucleotides that are in the protein coding part of the sequence, lowercase Us indicate inserted U residues; stop codons are underlined. Conventional Watson–Crick basepairs are indicated with vertical bars; non-Watson–Crick basepairs with :. (**B**) Comparison of the inferred amino acid sequence of *B.saltans* cox2 protein (upper lines) and *C.fasciculata* cox2 protein (lower lines). Identical amino acids are indicated with dots. Amino acids derived from edited RNA sections are underlined.

Table 1. Comparison of inferred mt protein sequences

	B.saltans	*Crithidia*[a]	*T.brucei*	*T.borreli*	*P.tetraurelia*	*S.cerevisiae*
ND5/cox2						
B.saltans	100	76.7	74.8	57.1	29.1	28.1
Crithidia[a]	48.3	100	80.4	54.3	25.7	31.2
T.brucei	42.9	71.8	100	56.2	26.3	31.2
T.borreli				100	28.1	28.1
P.tetraurelia					100	27.0
S.cerevisiae						100
MURF2/cox1						
B.saltans	100	74.9	74.9	70.8	28.3	41.3
Crithidia[a]	39.6	100	83.2	68.0	27.1	44.9
T.brucei	42.5	70.6	100	67.4	26.1	39.0
T.borreli				100	26.2	40.8
P.tetraurelia					100	30.2
S.cerevisiae						100

Percentages of amino acid identity are indicated, calculated with the GCG Bestfit and Gap program. Indicated below the diagonals are ND5 and MURF2 amino acid identities, cox2 and cox1 identities are indicated above the diagonal.
Abbreviations: *P.tetraurelia, Paramecium tetraurelia; S.cerevisiae, Saccharomyces cerevisiae*.
[a]For cox1, ND5 and MURF2 *C.oncopelti* has been used, for cox2 *C.fasciculata*.

We analysed MURF2 cDNAs in a similar fashion and we isolated a number of edited cDNAs (Fig. 5A). The frequency of editing of MURF2 RNA appears to be low: when a downstream unedited oligonucleotide was used in the RT-PCR procedure to amplify the editing region, only one out of five cDNAs showed the insertion of just one U (see Fig. 5A1). Only with the aid of a downstream oligonucleotide containing the extra nucleotide could we isolate the edited cDNAs shown in Figure 5A2. Seven out of the fifteen cDNAs analysed, had an identical editing pattern (21 Us inserted and 1 U deleted at 9 sites), containing two 5′ in-frame AUG codons that can be aligned with putative translational initiation codons in fully edited *T.brucei* MURF2 mRNAs, with the restriction that the *B.saltans* N-terminal MURF2 sequence is 1 aa shorter (Fig. 5B). We assume these cDNAs to be derived from the fully edited *B.saltans* MURF2 mRNA. A low frequency of complete editing was also observed in the 5′ region of apocytochrome *b* and cox1 RNAs of *T.borreli* (6). Apparently, for some bodonid RNAs low relative amounts of mature transcripts suffice to meet the demand for the protein product in question.

A putative MURF2 gRNA coding sequence was found on the non-coding strand of the cox2 gene at the position at which it is also found in trypanosomatids (34,46) (Fig. 3). This putative MURF2-I gRNA directs the insertion of only one U at editing site 1 of the MURF2 5′ editing region (Fig. 5C).

Bodo saltans ND5 RNA is extensively edited

In order to determine if the GC-rich sequences upstream of the cox2 gene encode an extensively edited transcript, we hybridised a Northern blot with probes derived from this region. Indeed, the blot showed the characteristic smearing caused by the abundant presence of partially edited RNAs with a varying degree of editing at their 3′-ends (data not shown; 15,45). Using the strategy outlined in Figure 6A, we cloned and sequenced a number of the corresponding cDNAs. Many edited RNAs were found with a varying degree of editing in the 3′ region, edited segments being separated from unedited sequences by incompletely edited junction sequences (data not shown; 2,15,45). The protein sequence inferred from the fully edited consensus sequence (Fig. 6A) showed ~45% identity to the 213 aa C-terminal part of trypanosomatid ND5 genomic sequences (Fig. 6B; Table 1). We have not extended the analysis further, therefore we do not know whether the 5′ 64% of the ND5 RNA, encoded by the section of the gene to the left of the *Hae*III restriction site (Fig. 3), is also extensively edited. Nevertheless, we conclude that, in contrast to trypanosomatids, in *B.saltans* a substantial portion of the ND5 gene is cryptic, protein-coding mRNA sequences being generated by extensive RNA editing.

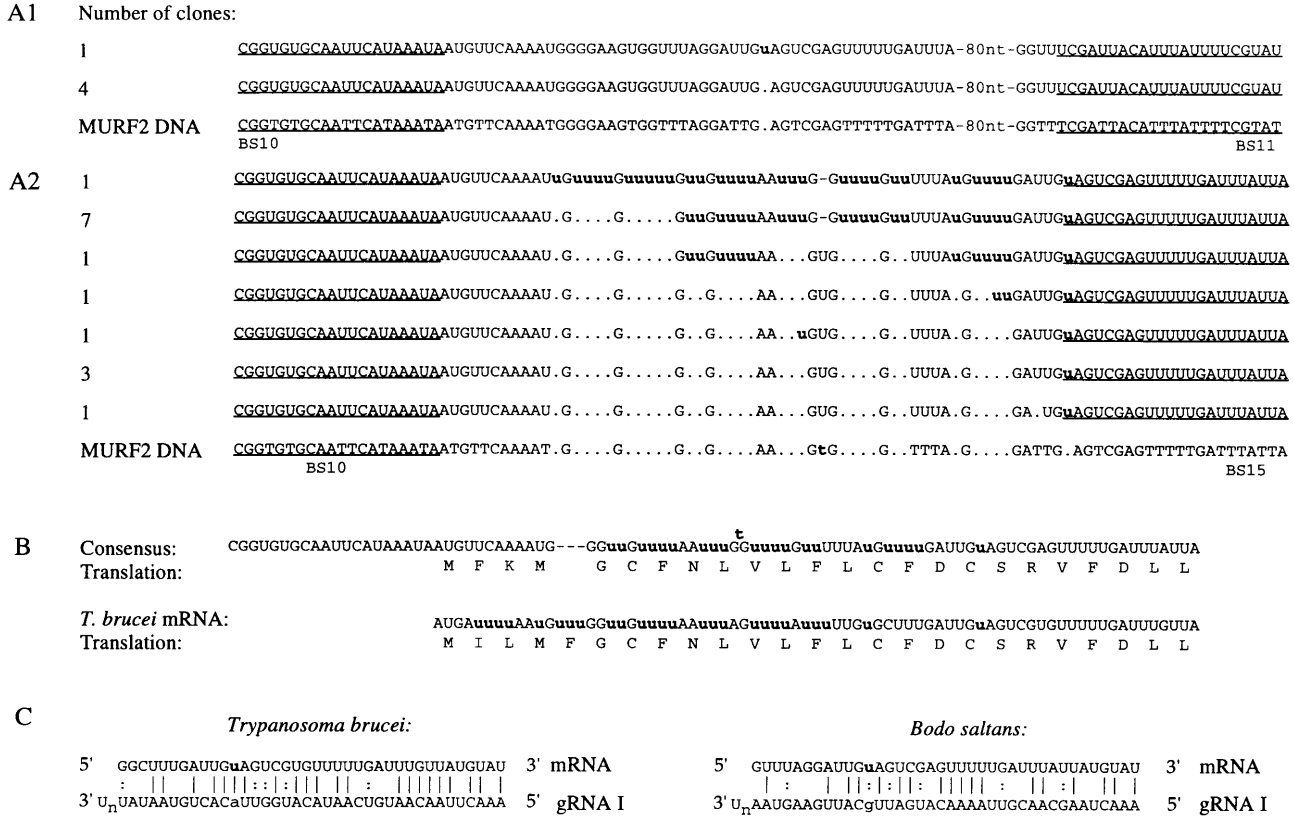

Figure 5. *Bodo saltans* MURF2 RNA editing. (**A1**) The sequence of five cDNA clones obtained by RT-PCR using unedited oligonucleotides (underlined in the figure). (**A2**) Sequences of cDNAs obtained with a downstream 'edited' oligonucleotide (underlined). In (A1) and (A2), lowercase Us indicate inserted U residues, whereas lowercase Ts indicate deletions. (**B**) The inferred amino acid sequence of the predominant cDNA (seven out of fifteen clones) has been given in a comparison with *T.brucei* MURF2 (48). (**C**) MURF2-I gRNA:mRNA duplexes from *T.brucei* (33) and *B.saltans*.

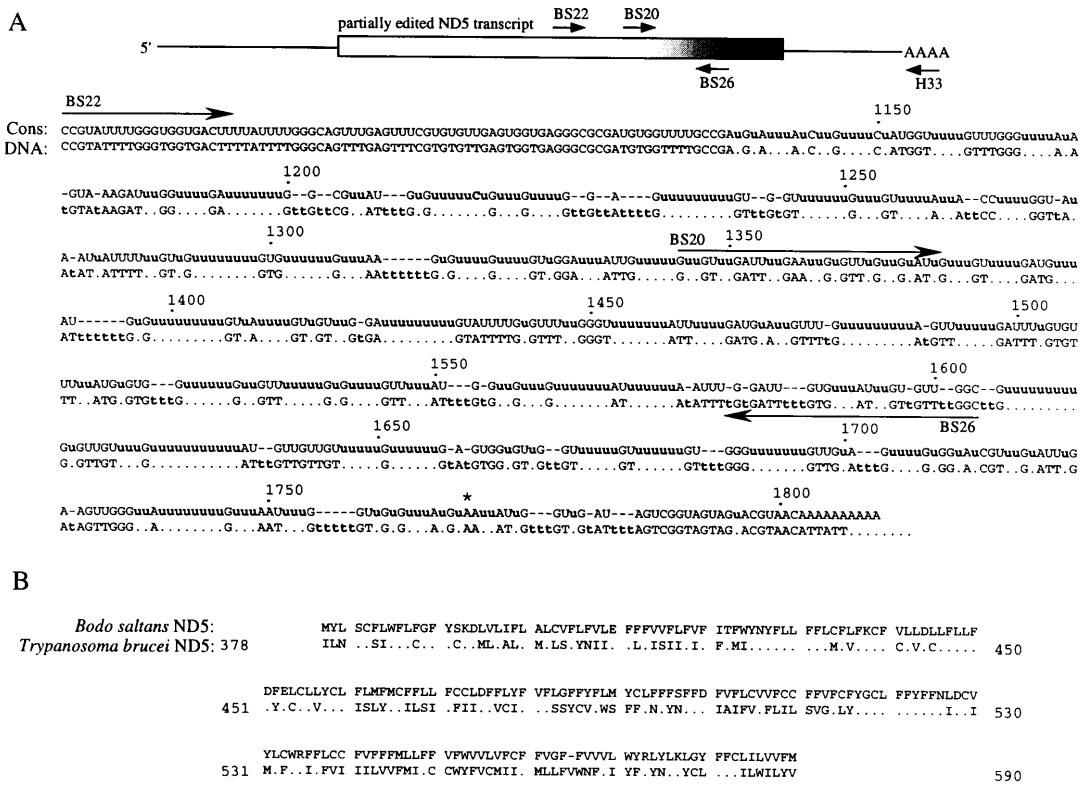

Figure 6. *Bodo saltans* ND5 RNA editing. (**A**) A comparison of the genomic sequence with the consensus edited ND5 RNA sequence, as assembled from sequence analysis of 20 different cDNA clones obtained by RT-PCR using the two-strep strategy outlined in the cartoon. In a first round of RT-PCR, cDNA clones were isolated with the aid of oligo dT as a downstream primer and two upstream unedited primers (BS20, 22 indicated by arrows above the line). In the second round, an 'edited' primer was used as downstream primer (BS26, underlined). Lowercase Us in the RNA sequence represent inserted U residues, whereas lowercase Ts in the DNA sequence indicate deletions; the asteriks marks the position of the translational stopcodon. In (**B**), the inferred amino acid sequence of 639 3′ base pair of edited *B.saltans* ND5 cDNA has been given in a comparison with the corresponding part of *T.brucei* ND5 (aa 378–590, ref. 41), identical amino acids being indicated by dots.

Phylogenetic analysis

In order to determine the phylogenetic relation of *B.saltans* to the trypanosomatids and *T.borreli*, a tree was constructed using multiple alignments of the sequence of a 296 bp internal cox2 fragment from a number of kinetoplastid species. A very similar tree could be constructed using sequences of nuclear small and large subunit rRNA genes (Lukes *et al.*, unpublished data; 7) and other mt protein sequences (6). The trees clearly show that the two cryptobiids *C.helicis* and *T.borreli* constitute the earliest offshots within Kinetoplastida, while *Bodo* spp. branch off more recently.

DISCUSSION

The mt genetic system of *B.saltans* has trypanosomatid-like and bodonid-like features

The results presented in this paper show that cox2, MURF2 and ND5 pre-mRNAs, encoded by the 70 kb maxicircle of the free-living bodonid *B.saltans* are edited by U-insertion/deletion, to our knowledge this species being the first representative of the family Bodonidae analysed in such detail. Our phylogenetic data show *B.saltans* to be more closely related to the trypanosomatids than the cryptobiids *T.borreli* and *C.helicis*, which is reflected in some of the characteristics of the *B.saltans* mt genetic system. The editing of cox2 and MURF2 RNAs (Figs 4 and 5) is found in the same region as that of their trypanosomatid counterparts, although subtle differences in editing patterns exist. In addition, as in trypanosomatids, cox1 and the 5′ part of ND1 RNAs are unedited, as judged from the alignment of these genes with their trypanosomatid counterparts (data not shown). Finally, the juxtaposition of the cox2, MURF2 and cox1 genes found in trypanosomatids is conserved in *B.saltans*, including the position of two putative gRNA genes, gcox2 and gMURF2-I (Fig. 3). We take the conservation of the gRNA genes as strong evolutionary evidence for the supposed role of their products. The exact reason for the conservation of gMURF2-I, which directs the insertion of one U only, is unclear, but it could be speculated that this gRNA plays a key role in regulating the amount of fully edited translatable mRNA. Other features of the *B.saltans* editing process are also conserved, such as the occurrence of junction sequences separating 3′ edited from 5′ non-edited RNA segments, suggesting a 3′ to 5′ overall polarity. It is to be expected, therefore, that the editing process in bodonids and in trypanosomatids is mechanistically very similar.

Clear differences between the *B.saltans* and trypanosomatid mt genetic systems are also found. The position of the *B.saltans* ND1 and ND5 genes is different from that in trypanosomatids and, more importantly, the ND5 gene is cryptic and transcribed into an

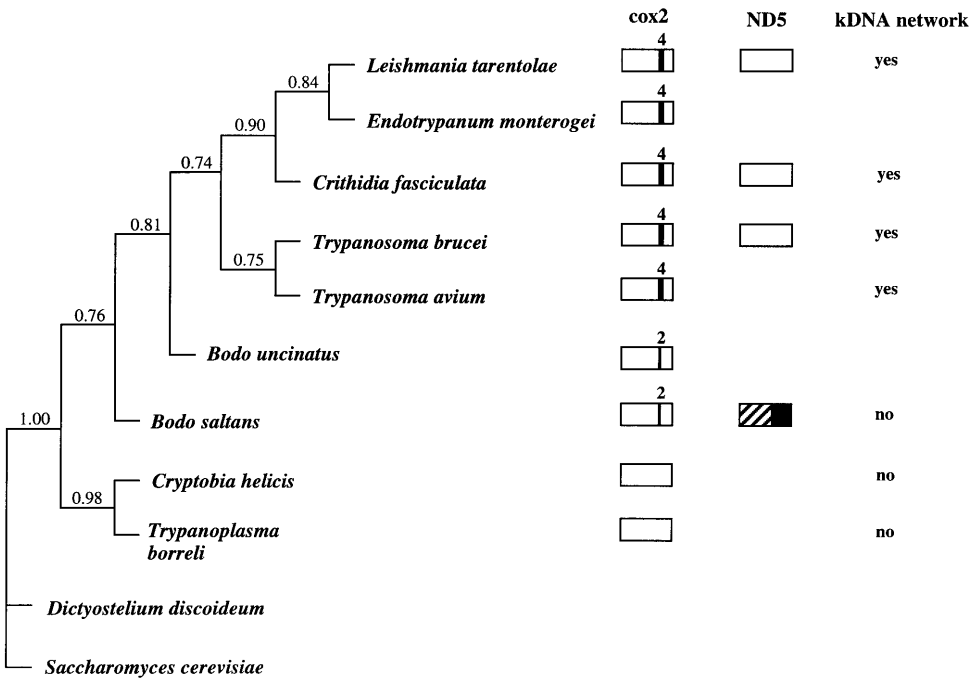

Figure 7. Phylogeny of kinetoplastid cox2 and ND5 RNA editing. Kinetoplastid phylogeny was inferred from the sequence of a 296 bp cox2 cDNA fragment (see Materials and Methods). Evolutionary distances are represented solely by the horizontal components of the tree. Bootstrap values ≥50% are indicated as percentages of 500 resamplings. The occurrence of editing in cox2 and ND5 RNA and the presence or absence of a kDNA network of the kinetoplastid species in which this has been investigated is indicated (see text for references). So far, U insertion/deletion RNA editing and mtDNA networks have not been found outside the kinetoplastid lineage. Edited RNA regions are indicated in black, the striping representing the uncertain editing status of the 5′ moiety of *B.saltans* ND5 RNA; the number of inserted Us in cox2 RNAs is also indicated.

extensively edited RNA (Fig. 6). This illustrates once more that there are no particular features in the sequence of a given gene and/or its transcript, that are incompatible *per se* with (extensive) RNA editing, and it leads to the prediction that, provided enough species are analysed, editing will be found in most if not all kinetoplastid mt mRNAs. What the driving force could be for the creation of specific patterns of edited and unedited RNAs in a given species remains to be elucidated (see below).

Another aspect in which *B.saltans* seems to be different from trypanosomatids, but similar to other bodonid and cryptobiid species, is the apparent absence of a large catenated mtDNA network. It is difficult to exclude the existence in *B.saltans* of an unusually fragile kDNA network, but the experiments were performed under conditions that allowed easy visualisation in total DNA of the kDNA network of the trypanosomatid *C.fasciculata* and very large chromosome-derived DNA molecules in *B.saltans* (data not shown), suggesting that our DNA preparations consist of intact, undegraded DNA. Surprisingly, unlike the other species that have no network (5,6,49,50, Lukes *et al.*, submitted), we found abundant minicircle-like molecules in *B.saltans*, with small catenanes occurring at a low frequency. We are currently in the process of further characterising these molecules and our preliminary analyses revealed the presence of sequences that are similar to conserved sequence elements present in trypanosomatid minicircles (51). If the 1.4 kb circles are indeed the *B.saltans* equivalent of minicircles and contain gRNA genes, one would be tempted to conclude that a large catenated network *per se* is not required for the ordered segregation of minicircles during replication to avoid the loss of essential gRNA genes, reinitiating the debate about the possible function of the network in trypanosomatids (17). In addition, even in the absence of a large network the *B.saltans* kDNA appears to form a disc (Fig. 1B), representing an area of highly concentrated DNA (52). The *B.saltans* kDNA disc is very similar in structure and localisation to that of trypanosomatids (44), but clearly distinct from the more dispersed kDNA arrangement observed in other bodonids and cryptobiids (pan-kDNA; 53,54).

The evolution of RNA editing

The placement of *B.saltans* and *B.uncinatus* in the tree of Figure 7 implies that our data do not provide any further information on the possible age and origin of the U-insertion/deletion processes. They do, however, shed some light on a number of interesting points. First, in our evolutionary trees parasitic kinetoplastids constitute two groups of species (*C.helicis* and *T.borreli* on the one hand and the trypanosomatids on the other), interspersed with the free-living *B.saltans* and *B.uncinatus*. Similar results have been obtained with trees constructed with the nuclear small and large subunit ribosomal RNA gene sequences (Lukes *et al.*, unpublished data). These results, although based on a limited dataset, are in disagreement with the notion that free-living members of the order should be evolutionary predecessors of the parasites. Therefore, it appears that monogenetic parasitism arose independently on separate occasions during evolution of kinetoplastids, although a less parsimonious explanation considering reacquistion of the free-living life style by the two bodonids studied, cannot be excluded until more data become available.

However, similar considerations have led to the proposal that the transition from monogenetic to digenetic parasitism also occurred independently at multiple occasions (9). If one assumes that free-living bodonid-like organisms were indeed the ancestors of the present-day kinetoplastids (20 and references therein), it is attractive to conclude that RNA editing arose prior to parasitism. Although it cannot be excluded that RNA editing provides some selective advantage to parasites, parasitism has clearly not served as a driving force for its evolution.

Next, our results suggest that the editing of different RNAs may be at a different evolutionary stage. On the one hand, RNAs such as ND5 mRNA could be considered to be yet another example of an RNA in which editing is 'on the way out', as judged from the disappearance of its editing in more recently evolved species (Fig. 7), presumably by retrotransposition of an edited cDNA into the genome (11,55 for a more extensive discussion). As stated before (6), it would be difficult to explain the existence of a small internal editing domain as found in cox2 RNAs as a result of such retrotransposition processes. The phylogeny of cox2 RNA editing (Fig. 7) suggests that cox2 editing arose relatively recently, displaying a very modest increase in complexity in trypanosomatids (four insertions at three sites instead of two insertions in two sites in bodonids). It could be considered, therefore, to be an RNA in which editing is 'on the way in', maybe to compensate for genomic (frameshift) mutations, along the lines of the model of Covello and Gray (21). In fact, this may be the way editing of other RNAs originated. If so, it can be anticipated that editing patterns that would be in line with this hypothesis are present in other 'primitive' kinetoplastids, making the continued analysis of these organisms highly worthwhile.

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