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Fibrillarin-associated Box C/D Small Nucleolar RNAs in *Trypanosoma brucei*

SEQUENCE CONSERVATION AND IMPLICATIONS FOR 2′-*O*-RIBOSE METHYLATION OF rRNA*

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David A. Dunbar‡, Steven Wormsley§, Todd M. Lowe¶, and Susan J. Baserga‡‖**

From the Departments of ‡Therapeutic Radiology, §Cell Biology, and ‖Genetics, Yale School of Medicine, New Haven, Connecticut 06520-8040 and the ¶Department of Genetics, Washington University School of Medicine, St. Louis, Missouri 63110

We report the identification of 17 box C/D fibrillarin-associated small nucleolar RNAs (snoRNAs) from the ancient eukaryote, *Trypanosoma brucei*. To systematically isolate and characterize these snoRNAs, the *T. brucei* cDNA for the box C/D snoRNA common protein, fibrillarin, was cloned and polyclonal antibodies to the recombinant fibrillarin protein were generated in rabbits. Immunoprecipitations from *T. brucei* extracts with the anti-fibrillarin antibodies indicated that this trypanosomatid has at least 30 fibrillarin-associated snoRNAs. We have sequenced seventeen of them and designated them TBR for *T. brucei* RNA 1–17. All of them bear conserved box C, D, C′, and D′ elements, a hallmark of fibrillarin-associated snoRNAs in eukaryotes. Fourteen of them are novel *T. brucei* snoRNAs. Fifteen bear potential guide regions to mature rRNAs suggesting that they are involved in 2′-*O*-ribose methylation. Indeed, eight ribose methylations have been mapped in the rRNA at sites predicted by the snoRNA sequences. Comparative genomics indicates that six of the seventeen are the first trypanosome homologs of known yeast and vertebrate methylation guide snoRNAs. Our results indicate that *T. brucei* has many fibrillarin-associated box C/D snoRNAs with roles in 2′-*O*-ribose methylation of rRNA and that the mechanism for targeting the nucleotide to be methylated at the fifth nucleotide upstream of box D or D′ originated in early eukaryotes.

In all eukaryotes the rRNA genes are transcribed in the nucleolus as large 35–45 S precursor transcripts. In yeast and metazoans the rRNA precursor is processed into the mature 18 S, 5.8 S, and 28 S (25 S in yeast) rRNAs of the ribosome (1, 2). In trypanosomes, however, the large subunit rRNA (28 S) is further processed into six rRNAs, called 28 Sα, 28 Sβ, sr1, sr2, sr4, and sr6 (3–5). These processing steps are not peculiar to trypanosomes because *Euglena gracilis*, which shares a common ancestor with trypanosomatids, also contains a multiply fragmented 28 S rRNA (6, 7). It has been hypothesized that the origin of contiguous high molecular weight rRNAs started from an ancient ribosome that consisted of primarily fragmented rRNAs (4, 8, 9).

Small nucleolar RNAs (snoRNAs)[1] are required for both processing of the pre-rRNA precursor and in the extensive nucleotide modification (2′-*O*-ribose methylation and pseudouridine formation) that occurs on the rRNAs (reviewed in Refs. 2, 10, 11–22). There are two major classes of snoRNAs that are named for specific conserved nucleotide sequences: box C/D and box H/ACA snoRNAs (23). In yeast, the box C/D snoRNAs are characterized by their association with the nucleolar proteins fibrillarin, Nop5/Nop58, and Nop56 (and perhaps other proteins, 24, 25–29)[2] in small nucleolar ribonucleoproteins. Of the 41 box C/D snoRNAs predicted to function in 2′-*O*-ribose methylation of rRNA in *Saccharomyces cerevisiae*, 37 have been experimentally confirmed (30, 31). Only four other known box C/D snoRNAs in yeast perform other functions (U3) or have no assigned function (snR190, snR4, and snR45). Many of the vertebrate box C/D snoRNAs are also required for site-specific rRNA 2′-*O*-ribose methylation (31–35). However, the vertebrate U3, U22, and U8 box C/D snoRNAs are required for pre-rRNA cleavage events (36–40). The U14 snoRNA is unusual in that it is the only box C/D snoRNA shown so far to function in both 18 S rRNA maturation and 18 S rRNA nucleotide modification (35, 41–43).

The box C/D snoRNAs involved in nucleotide modification, also called methylation guide RNAs, are characterized by the presence of conserved box C and D sequences near their 5′- and 3′-ends, respectively. The methylation guide RNAs also often bear internal box D′- and box C′-sequences (44, 45). Most methylation guide RNAs have a single region of complementarity to mature rRNA regions upstream from their box D or D′ sequences; however, some of them contain two complementary sequences and are referred to as double methylation guide RNAs. Results from several laboratories suggest that the fifth nucleotide upstream of the box D or D′ sequence, within the complementary sequence, specifies the ribose of the nucleotide to be methylated in the target rRNA (31, 32, 35). Investigation of functional constraints on the guide RNA-rRNA duplex indicates that both the length and composition of the complementary sequence influence the extent of the methylation reaction (46).

Our current understanding of the trypanosomatid snoRNAs involved in ribosome biogenesis is just beginning to emerge.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF168719-AF168736.

** To whom correspondence should be addressed: Yale School of Medicine, P. O. Box 208040, 333 Cedar St., New Haven, CT 06520-8040. Tel.: 203-785-4618; Fax: 203-785-6309; E-mail: susan.baserga@yale.edu.

[1] The abbreviations used are: snoRNA, small nucleolar RNA; GAR, glycine and arginine rich; TBR, *T. brucei* RNA 1–17; PCR, polymerase chain reaction; nt, nucleotide(s); TMG, trimethylguanosine.

[2] D. Lafontaine and T. Tollervey, personal communication.

Previous studies revealed the existence of a U3 homolog in *Trypanosoma brucei* (47–50). The first methylation guide box C/D snoRNA in trypanosomes was described by Levitan *et al.* (51) who determined that it has the potential to guide methylation of a 5.8 S rRNA nucleotide. Two additional box C/D snoRNAs from *Leishmania tarentolae, Trypanosoma cruzi,* and *T. brucei* were identified by Roberts *et al.* (52). However, it is clear from the work of Hartshorne and Agabian (48) that *T. brucei* has many more fibrillarin-associated snoRNAs. The identity of the other fibrillarin-associated snoRNAs in *T. brucei* remains unknown.

A comparison of the box C/D snoRNA sequences from such widely divergent species as trypanosomes, yeast, and vertebrates is likely to yield important information about the evolution of ribosome biogenesis, particularly with reference to pre-rRNA cleavage and 2′-*O*-ribose methylation. We reasoned that the box C/D snoRNAs in *T. brucei* could be isolated and sequenced by enrichment via immunoprecipitation with anti-fibrillarin antibodies; however, antibodies specific to the *T. brucei* fibrillarin are not available and antibodies that cross-react are scarce. To identify the box C/D snoRNAs in trypanosomes in a systematic way, we cloned the *T. brucei* fibrillarin cDNA and determined that it bears both the glycine- and arginine-rich (GAR) and methyltransferase domains present in fibrillarins characterized in other eukaryotes. We expressed the *T. brucei* fibrillarin in *Escherichia coli* and purified it and then used the recombinant protein to generate polyclonal antibodies in rabbits. Immunoprecipitation experiments on *T. brucei* extracts using the anti-fibrillarin antibodies indicated that trypanosomes contain at least thirty fibrillarin-associated snoRNAs. Seventeen of them were sequenced and designated TBR for *T. brucei* RNA 1–17. Their sequences and mapping of the methylation sites in the rRNA revealed that, like in yeast and metazoans, many have the potential to be guide RNAs for 2′-*O*-ribose methylation of rRNA, suggesting that the box C/D snoRNAs existed in early eukaryotes. Interestingly, six of them appear to be homologs of methylation guide snoRNAs found in yeast and vertebrates. This implies that the mechanism of specifying the methylation site in rRNA has been conserved from an ancient eukaryote.

EXPERIMENTAL PROCEDURES

Growth of T. brucei—The procyclic form of *Trypanosoma brucei rhodesiense* YTaT1.1 strain obtained from Elisabetta Ullu (Yale University School of Medicine) was used in this study. Cells were grown at 28 °C in SM medium supplemented with 20% fetal calf serum (53).

Cloning of the T. brucei Fibrillarin Gene—The fibrillarin protein from a related trypanosomatid, *Leishmania major*, has been cloned and sequenced (54). We used this sequence to search the EST data base for related *T. brucei* sequences and identified a candidate partial clone in *T. brucei rhodesiense* (accession number W00261). A cDNA clone representing this expressed sequence tag was made in the following way. Reverse transcription on total *T. brucei* RNA was performed with Tbfib.1 (5′-GGACAAAAATGCGAGGTGGG). A double-stranded product was synthesized using the single-stranded cDNA as template in the polymerase chain reaction (PCR) with Tbfib.1 and Tbfib.2 (CTACTTCTACACGCTTTCCCG) as primers. The PCR product was cloned into the TA cloning vector, PCRII (Invitrogen). A radiolabeled probe for library screening was synthesized by performing PCR in the presence of [^{32}P]dCTP with Tbfib.1 and Tbfib.2 as primers. This fibrillarin fragment was used to screen a *T. brucei rhodesiense* cDNA library (from strain YTAT1.1) cloned into Lambda Zap (Stratagene). The library was kindly provided by Elisabetta Ullu, Yale School of Medicine. Library filters were hybridized to the labeled probe in 50% formamide (Hybrisol I, Oncor) at 42 °C. Filters were washed once in 2× SSC 0.05% SDS at room temperature for 1 h and twice in 1× SSC 0.1% SDS at 65 °C for 1 h each wash. Inserts from phage giving positive signals were plaque purified, and plasmids recovered according to the manufacturer's instructions (yields an insert in a Bluescript plasmid). Automated DNA sequencing of one positive clone was carried out on an Applied Biosystems 373 Stretch sequencer by primer walking of both strands.

Expression and Purification of the T. brucei Fibrillarin Protein in E. coli—The polymerase chain reaction was used to amplify the full-length fibrillarin cDNA with the appropriate restriction sites for cloning in frame into the *E. coli* expression vector pET28a (Novagen). This vector places a 6X histidine tag on the amino terminus of the protein. The 5′-oligonucleotide contains the first 23 nt of the fibrillarin sequence and also a 5′-*Bam*HI site (Tbfib.3, 5′-CCGCGGATCCATGCGAGGTGGGTTTGGACG). The 3′-oligonucleotide is complementary to the last 15 nucleotides and has an *Ava*I site (Tbfib.4, 5′-GTCAGGCTCGAGACATTATTATTGTTGTACTGC). These oligonucleotides were used in the polymerase chain reaction with the fibrillarin plasmid as a template with these cycling steps: 94, 55, and 72 °C (15 s each, 20 cycles). The product was purified by ethanol precipitation, digested with *Bam*HI and *Ava*I, and the resulting band was gel-purified. This fragment was ligated into *Bam*HI, and *Xho*I cut pET28a.

The fibrillarin cDNA cloned into pET28a was transformed into BL21 (DE3) cells for expression and protein purification. Partial solubility of the fibrillarin protein was obtained when the cells were induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside and grown overnight at 30 °C. The resulting soluble protein was purified by metal chelation chromatography and eluted from the column with 1 M imidazole. The fibrillarin protein, insoluble upon elution, was dialyzed overnight into phosphate-buffered saline prior to injection into rabbits. Injections were performed by Immunization Services at the Yale School of Medicine. The sera were checked for reactivity with fibrillarin expressed in *E. coli* by Western blot analysis.

Immunoprecipitations and RNA Analysis—For anti-fibrillarin immunoprecipitation experiments, 3 mg of protein A-Sepharose CL-4B (Amersham Pharmacia Biotech) was mixed with 50 μl of either rabbit anti-fibrillarin or preimmune serum in 0.5 ml of NET-2 (40 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Nonidet P-40) overnight at 4 °C on a rotating nutator. The bound antibodies were washed four times with cold NET-2 and used as described below. For anti-trimethylguanosine (TMG) experiments, 15 μl of anti-TMG antibody and 15 μl of rabbit anti-mouse IgG were mixed with protein A-Sepharose and then incubated and washed in NET-2 as described above.

T. brucei cells were grown to a density of 2–3 × 10^7 cells/ml. Cells from a 50-ml culture were pelleted and resuspended in 10 ml of wash buffer (20 mM Tris-HCl pH, 7.5, 100 mM NaCl, 3 mM $MgCl_2$). Cells were repelleted and resuspended in 0.6 ml of either NET-2, NET-5, NET-6, or NET-7 containing protease inhibitors (2 μg of aprotinin/ml, 1 μg of leupeptin/ml, and 1 μg of pepstatin A/ml). NET-5, 6, and 7 are the same as NET-2 except that they contain either 500, 600, or 700 mM NaCl, respectively. Cells were lysed by vigorous vortexing with one-half volume of glass beads (0.45–0.5-mm diameter) for five minutes. The lysate was cleared by centrifugation at 13,000 × *g* for 10 min at 4 °C. The cleared lysate containing either 150, 500, 600, or 700 mM NaCl was added to the protein A-Sepharose beads treated with either rabbit anti-fibrillarin serum, preimmune serum, or anti-TMG antibodies and incubated for 1 h at 4 °C on a rotating nutator. The beads were washed six times with either NET-2, -5, -6, or -7 buffer. RNA was recovered by adding 300 μl of NET-2 to beads and extraction with phenol/chloroform/isoamyl alcohol and ethanol precipitation. Immunoprecipitated RNAs were labeled with 5′-[^{32}P]cytidine 3′,5′-bisphosphate and T4 RNA ligase according to Ref. 55 and separated on a 10% sequencing gel.

Sequencing and Cloning of Trypanosome snoRNAs—For direct RNA sequencing, immunoprecipitations and 3′-end labeling of snoRNAs were performed as described above. Individual labeled snoRNAs were gel-isolated and eluted in 0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA (pH 8.0), 0.1% SDS overnight at room temperature on a rotating nutator. Approximately 30 nt of the 3′-end sequence of labeled RNAs was obtained by enzymatic sequencing using RNases T1, U2, PhyM, and *Bacillus cereus* (Amersham Pharmacia Biotech).

Full-length cDNAs were obtained using Superscript II reverse transcriptase (Life Technologies, Inc.) with ^{32}P-labeled deoxyoligomer primers complementary to the 3′-ends of snoRNA sequences (see Table I). RNA was isolated from an anti-fibrillarin immunoprecipitation performed on 50 ml of *T. brucei* cells and used for individual primer extension reactions to generate full-length cDNAs. These cDNAs were gel-eluted and poly(A)-tailed in 25 μl of buffer containing 0.1 M potassium cacodylate (pH 7.2), 20 mM $CoCl_2$, 0.2 mM dithiothreitol, 0.2 mM dATP, and 15 units of terminal deoxynucleotidyl transferase (Life Technologies, Inc.) for 1 h at 37 °C. The poly(A)-tailed cDNAs were extracted with phenol/chloroform/isoamyl alcohol and ethanol-precipitated. The poly(A)-tailed cDNAs were amplified using a (dT) oligomer and the 3′-oligomer by PCR with these cycling steps: 94, 55, and 72 °C (30 s each, 30 cycles). The PCR products were cloned using the CLONTECH TA-cloning kit and sequenced using an Applied Biosystems 373 Stretch sequencer.

TABLE I
Oligonucleotides used to clone the T. brucei snoRNAs

The 5′-oligos were used to identify snoRNA 3′-ends, and the 3′-oligos were used to identify snoRNA 5′-ends. See text for descriptions of both the 5′- and 3′-snoRNA cloning strategies.

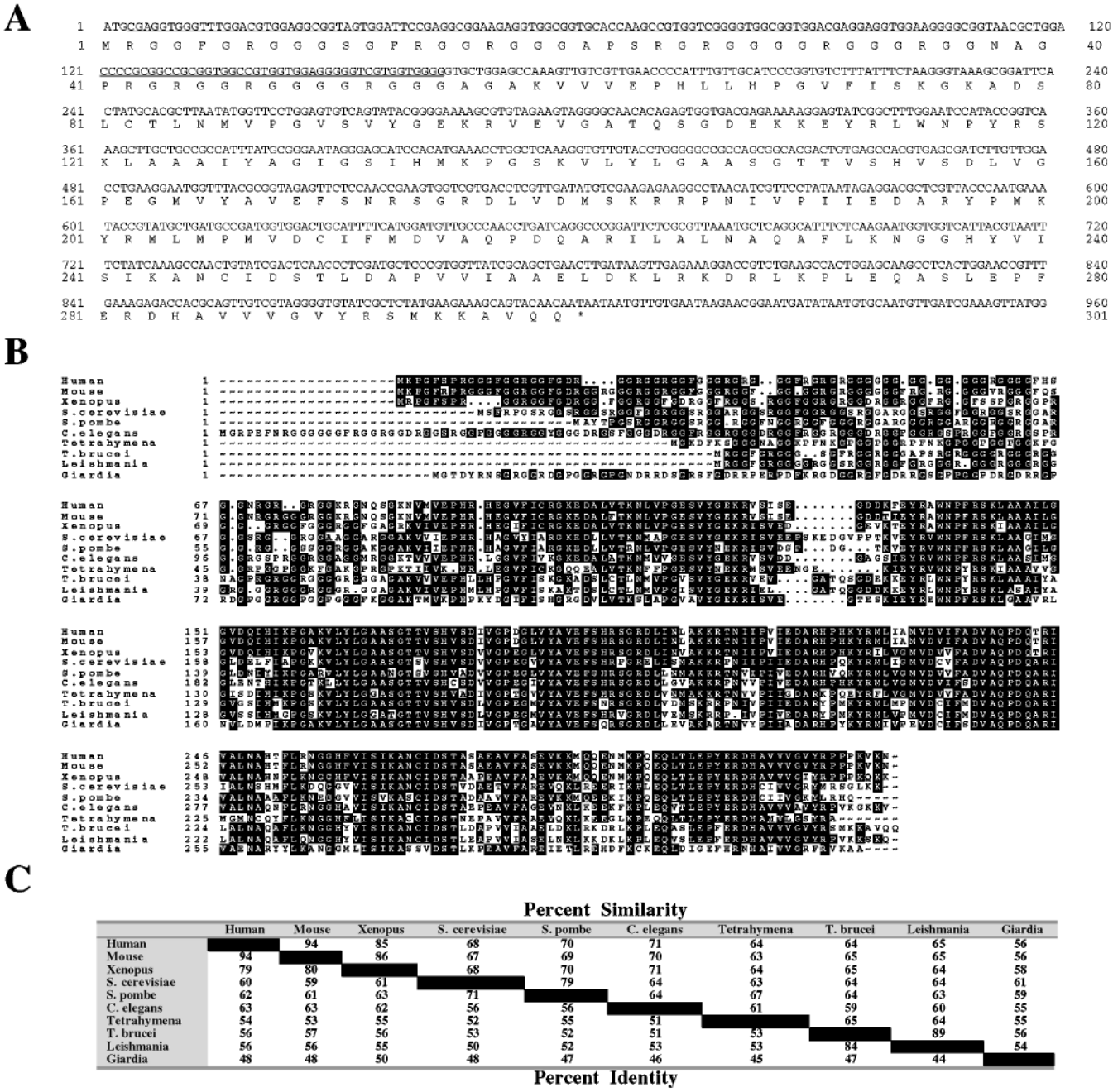
TBR1 5'	5'GCCCATGACGATAAACCACTT3'
TBR1 3'	5'CGCCTCGGATGTAGAA3'
TBR2 5'	5'CGCGTGATGAGGTGCAGAAGG3'
TBR2 3'	5'CATCAGATATTGTTCACCCAT3'
TBR3 5'	5'AACGTGACGAAAAACCTTTCA3'
TBR3 3'	TCAGAATGCCGTCTGTGATCG3'
TBR4 5'	5'CGGTTGATTAGCAGTGCGTCTTCCACCTAA3'
TBR4 3'	5'TCTCATTTACTAAATTTGATATA3'
TBR5 5'	5'GAAGTGATTGACACCTAGGCC3'
TBR5 3'	5'ATCATCAGGACATGAAATGGT3'
TBR6 5'	5'CGCGTGATGACATACAAAGTT3'
TBR6 3'	5'TTCAGATGGTGTTCTTGT3'
TBR7 5'	5'GAATGATGACTGACAAAACAT3'
TBR7 3'	5'TCATCAGGGGCAACAATTTTTC3'
TBR8 5'	5'GTGACGATGTACAATATGTTC3'
TBR8 3'	5'TTCAGTGCAAGTCTGTGTTATGGTGTC3'
TBR9 5'	5'CGCTGATGAAGTTGATATGGT3'
TBR9 3'	5'CTCAGAAGATGTGCTGTTGTGCGTC3'
TBR10 5'	5'CCAATGATGTTGTTATTTAAT3'
TBR10 3'	5'CACTTCAGTGTTTCCTCTC3'
TBR11 5'	5'TGATGATTATGATACGATGCCTGGTCAACA3'
TBR11 3'	5'TCTCATTTACTAAATTTGATATA3'
TBR12 5'	5'CTGCATGATGTGCTCAACTGGAATTAC3'
TBR12 3'	5'GTCAGTGATACGAGTAACAGCGGAG3'
TBR13 5'	5'CAAATGATGCTAACAATCGAGGCATTT3'
TBR13 3'	5'CATCAGATATTGTTCACCCAT3'
TBR14 5'	5'CACATGATGTCATTTCGTATTCTG3'
TBR14 3'	5'TCAGATGCCGGTAGTCAT3'
TBR15 5'	5'CCCCTTGATGATTGTGGCAACTCTC3'
TBR15 3'	5'TCAGAGATCTTGGTTGG3'
TBR16 5'	5'AGCATGATGATCATACGTGCAATT3'
TBR16 3'	5'TCGGAGCTGGTTTGTT3'
TBR17 5'	5'CCGCGACAAGGTCAGCCTGAGGGCACACCT3'
TBR17 3'	5'GGTCATGTATAATGCATGGTTTAGTGTGCTTA3'

The sequences of the 3′-ends were verified independently. Adenosine nucleotides were added to the 3′-ends of total snoRNAs isolated from an anti-fibrillarin immunoprecipitation experiment with 5 units of poly(A) polymerase (Amersham Pharmacia Biotech) in 50 μl containing 40 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 2.5 mM $MnCl_2$, 250 mM NaCl, 250 μM ATP, 50 μg/ml bovine serum albumin for 30 min at 37 °C. The 3′-poly(A)-tailed RNAs were extracted with phenol/chloroform/isoamyl alcohol and ethanol-precipitated. Full-length cDNAs complementary to the poly(A)-tailed RNAs were made with a (dT) oligomer and Superscript II reverse transcriptase. Aliquots of the primer extension reaction were then used to amplify individual snoRNAs by PCR using a (dT) oligomer and an oligomer containing the 5′-sequence specific for a particular snoRNA using the cycling conditions described above (Table I). The PCR products were cloned and sequenced as described above.

Northern Blot Analysis of snoRNAs—To isolate *T. brucei* total RNA, a 50-ml culture of cells grown to a density of 2–3 $\times$ 10^7 cells/ml was pelleted and resuspended in wash buffer. Cells were repelleted and resuspended in 250 μl of solution D (4 M guanidinium thiocyanate, 26.4 mM sodium acetate, pH 7.0, 0.5% sarcosyl, 0.72% β-mercaptoethanol). The lysed cells were extracted with an equal volume of acid phenol and 50 μl of chloroform and precipitated with three volumes of ethanol. RNA from an anti-TMG cap immunoprecipitation and an anti-fibrillarin immunoprecipitation were resolved with total RNA on a 10% sequencing gel and transferred to a Zeta-probe membrane.

Plasmids containing the TBR17 snoRNA clone and a plasmid containing the *T. brucei* U4 small RNA were used in PCR reactions with clone-specific primers and [α-^{32}P]dCTP (3000 Ci/mmol) to make labeled probes. Blots were hybridized to 1 $\times$ 10^7 cpm of labeled probes in 5$\times$ saline/sodium phosphate/EDTA, 10$\times$ Denhardt's solution, 7% SDS at 65 °C. Blots were washed twice in 3$\times$ SSC 0.1% SDS at room temperature for 15 min each wash and once in 3$\times$ SSC 0.1% SDS at 65 °C for 10 min and exposed to x-ray film.

Mapping of 2′-O-Ribose Methylations by Primer Extension—The ribose methylation mapping protocol was modified from Ref. 56. Oligonucleotides complementary to rRNA 3′ to sites of predicted 2′-0-ribose methylation were synthesized. *T. brucei* total RNA (7 μg) was annealed to 15 ng of the ^{32}P-labeled oligonucleotides for 10 min at 70 °C. Primer extension reactions were carried out in 20 μl of a buffer containing 50 mM Tris-HCl, pH 8.5, 8 mM $MgCl_2$, 30 mM KCl, and 1 mM dithiothreitol in the presence of 0.5 units/μl avian myeloblastosis virus reverse transcriptase (Roche Molecular Biochemicals) with either 1 mM dNTPs/μl or 0.004 mM dNTPs/μl for 1 h at 42 °C. The extension products were resolved on an 8% sequencing gel next to an RNA sequencing ladder. For generation of RNA sequencing ladders, 5 μg of *T. brucei* total RNA



	Human	Mouse	Xenopus	S. cerevisiae	S. pombe	C. elegans	Tetrahymena	T. brucei	Leishmania	Giardia
Human		94	85	68	70	71	64	64	65	56
Mouse	94		86	67	69	70	63	65	65	56
Xenopus	79	80		68	70	71	64	65	64	58
S. cerevisiae	60	59	61		79	64	63	64	64	61
S. pombe	62	61	63	71		64	67	64	63	59
C. elegans	63	63	62	56	56		61	59	60	55
Tetrahymena	54	53	55	52	55	51		65	64	55
T. brucei	56	57	56	53	52	51	53		89	56
Leishmania	56	56	55	50	52	53	53	84		54
Giardia	48	48	50	48	47	46	45	47	44	

FIG. 1. *A,* nucleotide and amino acid sequence of the *T. brucei* fibrillarin protein. The *underlined* sequence indicates the GAR domain. *B,* conservation of fibrillarin proteins among 10 different eukaryotic organisms. The *black shaded* residues indicate sequence identity and the *gray shaded* residues indicate sequence similarity. The sequences were aligned using Pileup (GCG) and homologous amino acids were shaded using the Boxshade server. Accession numbers are: human (X56597), mouse (Z22593), *Xenopus laevis* (M28874), *S. cerevisiae* (J05230), *Schizosaccharomyces pombe* (X69930), *Caenorhabditis elegans* (Z78413), *Tetrahymena thermophila* (X77962), *T. brucei* (AF168719), *L. major* (L26252), and *G. lamblia* (L28115). *C,* identity and similarity among 10 eukaryotic fibrillarins. The programs BoxShade and Bestfit (GCG) were used.

was hybridized to 15 ng of labeled oligonucleotide for 10 min at 70 °C. Dideoxy RNA sequencing was performed in a 20-μl reaction using 10 units/μl Superscript II reverse transcriptase, 1 mM dNTPs/μl and 1 mM of the appropriate ddNTP at 42 °C for 1 h.

RESULTS

Cloning and Expression of T. brucei Fibrillarin—A radiolabeled short fragment (600 nt) of the putative *T. brucei* fibrillarin gene was used to screen a *T. brucei* cDNA library to obtain the full-length fibrillarin clone. The nucleotide and amino acid sequence are shown in Fig. 1*A*. The *T. brucei* fibrillarin is 300 amino acids long and has a predicted molecular mass of 31.7 kDa. The *T. brucei* fibrillarin is therefore among the shortest of the fibrillarins, the shortest being the *Tetrahymena* fibrillarin at 292 amino acids (57). Like most of the other fibrillarins, the *T. brucei* fibrillarin has a GAR domain at its amino terminus (58) and a potential *S*-adenosylmethionine binding/methyltransferase domain (amino acids 141–157; 59, 60). Comparison of the *T. brucei* fibrillarin to the nine other known eukaryotic fibrillarins (Fig. 1, *B* and *C*) indicates a high degree of conservation. It is most identical to the fibrillarin of a related trypanosomatid, *L. major*, with 84% sequence identity; it is least similar to the *Giardia lamblia* fibrillarin with 47% identity. Fig. 1*B* indicates that the highest sequence conservation among fibrillarins occurs carboxyl-terminal to the GAR domain.

Previous studies indicate that the human fibrillarin can re-

store growth to a *S. cerevisiae* strain with a null fibrillarin allele (Δnop1), though the strain is temperature-sensitive (61). The human and *S. cerevisiae* fibrillarins are 60% identical/68% similar. We assessed whether the *T. brucei* fibrillarin, which is 53% identical/64% similar to the budding yeast fibrillarin, could restore growth at the nonpermissive temperature to strains with five temperature-sensitive fibrillarin alleles (nop1.2, nop1.3, nop1.4, nop1.5, and nop1.7; 62). The *T. brucei* fibrillarin cDNA was cloned into the yeast expression vector, p415GPD (63), and transformed into the temperature-sensitive strains. Subsequent restreaking of the colonies and growth at both 22 and 37 °C indicated that the *T. brucei* fibrillarin does not complement these temperature-sensitive fibrillarin alleles (data not shown). This suggests that the *T. brucei* fibrillarin is too dissimilar to function in *S. cerevisiae*. It is also possible that the *T. brucei* fibrillarin cannot attain the correct cell compartment in yeast because its nuclear import/nucleolar targeting signals are different.

The *T. brucei* fibrillarin was cloned into pET28a for expression in *E. coli* as a histidine-tagged fusion protein. When the cells were induced and grown at 37 °C, the fibrillarin protein is insoluble, and upon solubilization in 6 M guanidine, HCl does not bind to the metal chelation column. Growth at 30 °C allowed partial solubility, and the protein was purified under nondenaturing conditions. However, when fibrillarin was eluted from the metal chelation column it became insoluble and could be visualized in column fractions as a cloudy precipitate. Gel electrophoresis of this precipitate revealed a protein of the expected mobility (32 kDa). The cloudy precipitate was injected into rabbits for the production of antibodies.

Anti-fibrillarin Antibodies Immunoprecipitate the U3 and Many Other snoRNAs—Sera from two rabbits were tested for reactivity with the fibrillarin produced in *E. coli* by Western blots, and one was chosen for further study. Immunoprecipitations were performed on *T. brucei* whole cell extracts with anti-fibrillarin polyclonal antibodies and compared with immunoprecipitations performed with preimmune serum at different salt concentrations (0.5–0.7 M). RNAs from immunoprecipitations were labeled at their 3′-ends and resolved on a 10% polyacrylamide denaturing gel. Anti-fibrillarin antibodies immunoprecipitated at least thirty specific bands at all three sodium chloride concentrations (Fig. 2, *lanes 2*, *4*, and *6*). None of these RNAs were immunoprecipitated with preimmune serum, indicating that they result from co-immunoprecipitation with anti-fibrillarin antibodies (compare *lanes 1* and *2*, *3* and *4*, and *5* and *6*).

Several strategies were used to determine the snoRNA sequences. The full-length sequences of RNAs TBR1–10 (Fig. 2, *lanes 2*, *4*, and *6*) were determined by direct RNA sequencing and by using a 5′-end cloning strategy. Because some of the nucleotides obtained by direct enzymatic sequencing were ambiguous, the RNA 3′-end sequence was confirmed using a 3′-end cloning strategy. The sequences of TBR11, -13, -15, and -16 were obtained when attempting to clone other snoRNAs with deoxyoligonucleotides based on the RNA 3′-end sequence. Similarly, the 3′-end of TBR12 was obtained when attempting to verify the 3′-end sequence of a different snoRNA. The 5′-end sequence of the *T. brucei* homolog of the *L. collosoma* snoRNA-2 was obtained using the 5′-end cloning strategy with a labeled deoxyoligonucleotide complementary to the 3′-end of the *Leptomonas collosoma* snoRNA-2 (51). The 3′-end sequence of this snoRNA was verified using the 3′-end cloning strategy and named TBR14. The presence of each snoRNA in total RNA and in fibrillarin immunoprecipitates was verified by Northern blot analysis (data not shown).

Sequencing revealed that all sixteen snoRNAs have box C, D, C′, and D′ sequences, characteristic of snoRNAs associated with fibrillarin (Fig. 3). Box C and D sequences are located at the 5′- and 3′-ends of the RNAs, respectively, as are all but one (snR13) of the 2′-*O*-ribose methylation guide RNAs described previously in yeast and vertebrates. Fourteen of the snoRNAs that we have sequenced are newly identified in *T. brucei*. TBR5 and TBR7 were previously identified and proposed to be box C/D snoRNAs (52, 64) but had not been shown to be associated with the fibrillarin protein. Direct RNA sequencing suggested the presence of at least two TBR10 species, A and B, and revealed the previously identified U3 snoRNA and a U3 3′-end breakdown product that migrates just above 5 S rRNA (Fig. 2).

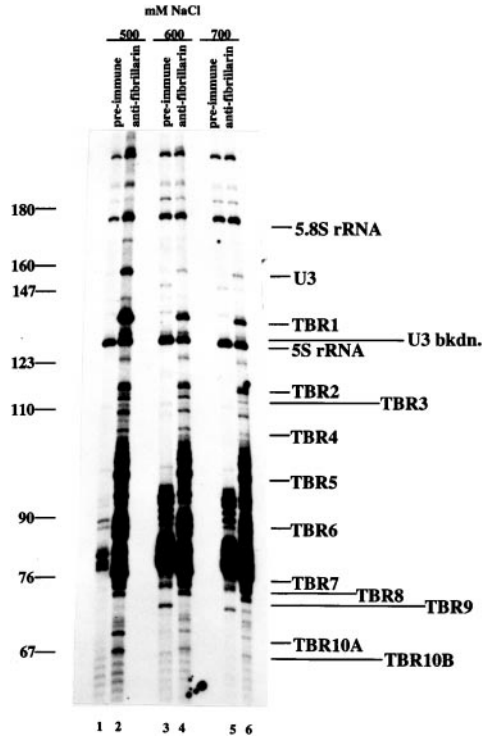


FIG. 2. **Anti-fibrillarin antibodies immunoprecipitate U3 and many other snoRNAs.** Immunoprecipitations were performed on *T. brucei* whole cell extracts with rabbit preimmune serum (*lanes 1, 3*, and *5*) or anti-fibrillarin rabbit serum (*lanes 2, 4*, and *6*) in the indicated salt concentrations. The RNAs isolated from the immunoprecipitations were labeled with 5′-[^{32}P]cytidine 3′,5′-bisphosphate and T4 RNA ligase and analyzed on a 10% denaturing polyacrylamide gel. The sizes of the labeled pBR322-*Msp*I markers are indicated.

Using a snoRNA search algorithm and model scoring program, the snoRNAs were tested for complementarity to the *T. brucei* rRNAs, U2, U3, U4, U6, spliced leader RNA, spliced leader associated RNA, and 7SL RNA. This program has been used to identify 22 novel methylation guide snoRNAs in yeast; the algorithm and scoring scheme are described elsewhere (30). Complementary sequences that gave the highest scores for each individual snoRNA are shown in Fig. 3. Because they have complementarity to rRNA upstream of box D or D′, 15 of them are potential methylation guide snoRNAs, 4 with sequence complementary to 18 S rRNA (TBR3, TBR7, TBR8, and TBR12), 1 with sequence complementary to both 18 S and 5.8 S rRNA (TBR14), and 8 with sequence complementary to 28 S rRNA. Of the large subunit rRNA methylation guide RNAs, two are complementary to 28 Sα (TBR6, TBR9) and eight are complementary to 28 Sβ (TBR1, TBR2, TBR4, TBR10, TBR11, TBR13, TBR15, and TBR16). Given confirmed methylation sites at the predicted targets, all the potential guide regions yielded scores that were in the same range as the previously characterized yeast snoRNA guide regions. None of the

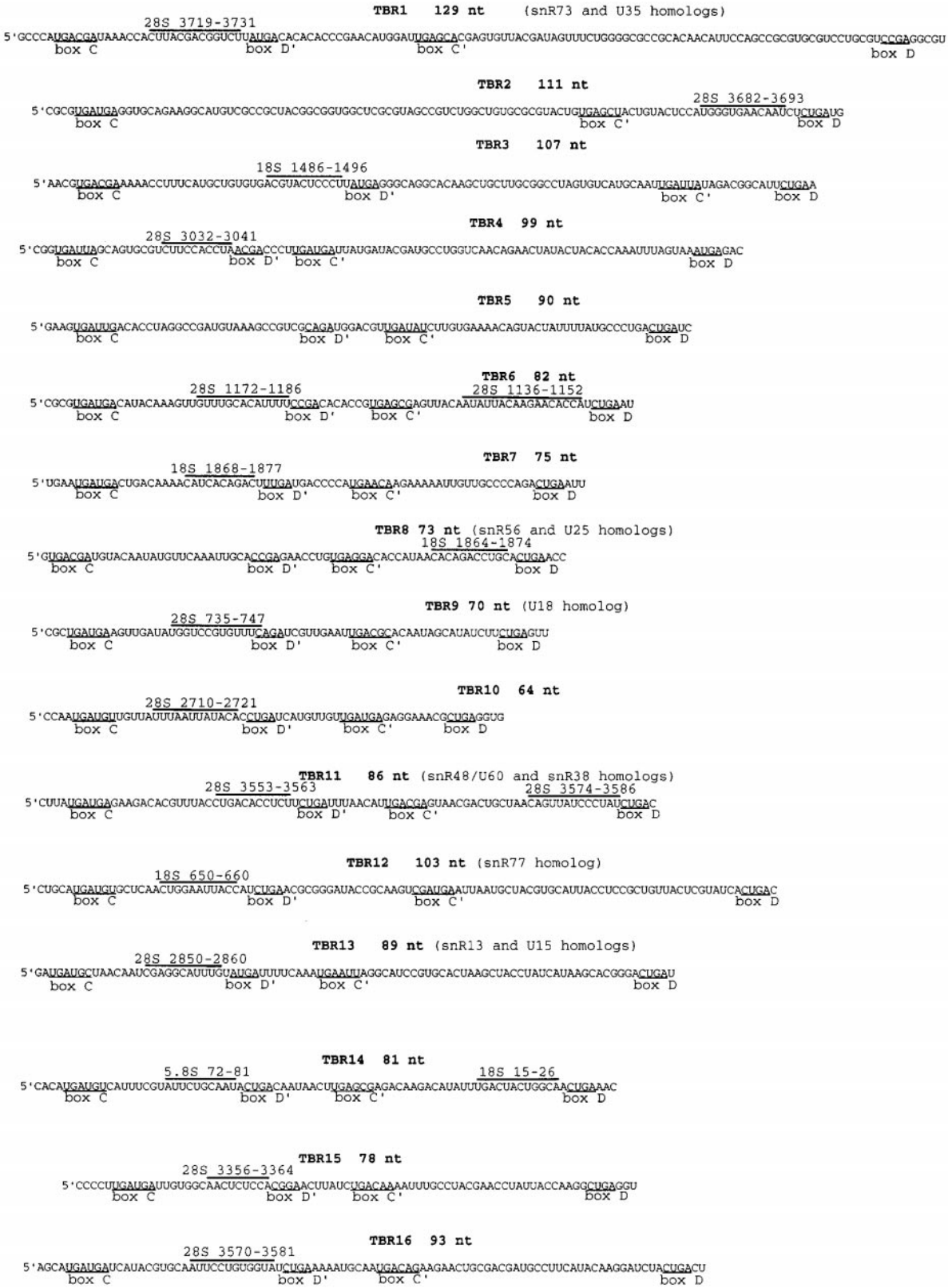


FIG. 3. **Full-length sequence of 16 *T. brucei* snoRNAs.** The conserved boxes C, D, C′, and D′ are *underlined*. The rRNA sequence complementarity is indicated, and the potential vertebrate and yeast snoRNA homologs are shown. The accession numbers for the snoRNAs are: TBR1 (AF168720), TBR2 (AF168729), TBR3 (AF168730), TBR4 (AF168731), TBR5 (AF168732), TBR6 (AF168733), TBR7 (AF168734), TBR8 (AF168735), TBR9 (AF168736), TBR10 (AF168721), TBR11 (AF168722), TBR12 (AF168723), TBR13 (AF168724), TBR14 (AF168725), TBR15 (AF168726), TBR16 (AF168727), and TBR17 (AF168728). The accession numbers for the *T. brucei* rRNA sequences are: X14553 for 28 S, M12676 for 18 S, and X02483 for 5.8 S.

snoRNAs had a significant degree of complementarity to RNAs other than rRNA. One, TBR5, does not have any significant complementarity to any known RNA.

We mapped some of the 2′-*O*-ribose methylation sites in trypanosome rRNA to demonstrate that they do indeed occur at sites targeted by the snoRNAs that we have identified (Fig. 4).

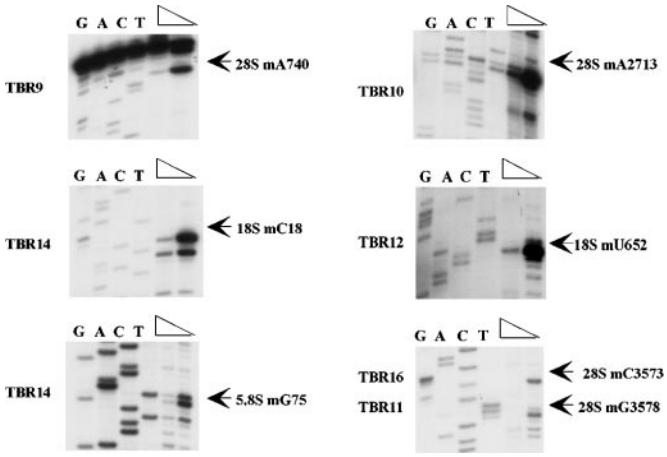


FIG. 4. **Mapping of predicted 2′-*O*-ribose methylation sites on *T. brucei* rRNA.** Oligonucleotides complementary to 18 S rRNA nt 40–61, 18 S rRNA nt 677–697, 5.8 S rRNA nt 97–117, 28 S nt 761–772, 28 S nt 2739–2758, and 28 S nt 3599–3619 were used to perform primer extensions on rRNA in the presence of decreasing dNTP concentrations (*triangles*). The methylated nucleotides are indicated by *arrows*.

Methylations occur at 28 S mA740 (target of TBR9), 28 S mA2713 (target of TBR10), 18 S mC18 (target of TBR14), 18 S mU652 (target of TBR12), 5.8 S mG75 (target of TBR14), 28 S mC3573 (target of TBR16), and 28 S mG3578 (target of TBR11). In all cases, the methylation site occurs complementary to the nucleotide in the snoRNA that is 5 nucleotides upstream of box D or D′, suggesting that trypanosomes snoRNAs conform to the "box D + 5 rule" as in other eukaryotes.

Several T. brucei snoRNAs Are Possible Functional Homologs to Yeast and Vertebrate snoRNAs—Trypanosomes are among the earliest diverged eukaryotes, yet our search program has identified several potential *T. brucei* functional homologs to yeast and vertebrate snoRNAs based on the presence of 5′- and 3′-box C, D, C′, and D′ sequences and rRNA complementary sequences (Table II). We define functional homologs as box C/D snoRNAs with similar (some are identical) rRNA complementary sequences that have the potential to methylate the ribose of the same nucleotide. These include functional homologs to the yeast snR73/vertebrate U35 (TBR1), yeast snR56/vertebrate U25 (TBR8), yeast/vertebrate U18 (TBR9), yeast snR38 (TBR11), yeast snR48/vertebrate U60 (TBR11), yeast snR77 (TBR12), and yeast snR13/vertebrate U15 (TBR13). Thus, trypanosomes are the earliest diverged eukaryote with snoRNAs homologous to yeast and humans. This implies conservation of the mechanism to target 2′-*O*-ribose rRNA methylation throughout evolution.

TBR17 Is a Box C/D Fibrillarin-associated snoRNA—Roberts *et al.* (52, 64) have previously identified a *T. brucei* snoRNA of 270 nt, which is somewhat larger in size than the other identified trypanosome snoRNAs. We will refer to this snoRNA as TBR17. We have investigated whether it is one of the box C/D fibrillarin-associated snoRNAs. First, as it appeared that Roberts *et al.* (52) had only a partial *T. brucei* TBR17 sequence, we cloned the full-length TBR17 snoRNA using the 5′- and 3′-poly(A)-tailing PCR-based technique. The full-length sequence of TBR17 was then used as a probe to examine whether it is associated with fibrillarin.

Immunoprecipitations were performed on trypanosome whole cell extracts using anti-TMG antibodies and anti-fibrillarin antibodies and compared with immunoprecipitations performed with preimmune serum. The Northern blot was hybridized with probes to U3, U4, and TBR17. As expected, the anti-TMG antibodies immunoprecipitate the U3 and U4 RNAs, because they both bear a TMG cap at their 5′-ends (Fig. 5*A*, *lane 2*). However, TBR17 is not immunoprecipitated with anti-TMG antibodies, indicating that this RNA does not contain a 5′-TMG cap (Fig. 5*A*, *lane 2*). This is consistent with the fact that the majority of the *T. brucei* box C/D snoRNAs do not possess a 5′-TMG cap (this work, data not shown, and Ref. 48). The anti-fibrillarin antibodies specifically immunoprecipitate both the U3 and TBR17 RNAs (Fig. 5*A*, compare *lanes 3* and *4*). These results demonstrate that like U3, TBR17 is a fibrillarin-associated RNA.

The TBR17 sequence that we obtained was 99% identical to the published sequence and contains both box C and D elements (52). However, the 3′-end of TBR17 that we obtained has seven additional nucleotides when compared with the published sequence, including a consensus box D element located two nucleotides from the 3′-end of the molecule (Fig. 5*B*). The *L. tarentolae* TBR17 homolog has a box D element located at its 3′-end as well (52). We also observe a potential box C element located about 95 nucleotides from the 3′-end of the molecule (Fig. 5*B*). The snoRNA search algorithm could not find potential guide regions to the *T. brucei* rRNAs or any of the other RNAs tested. Taken together, these results suggest that TBR17 is a genuine box C/D fibrillarin-associated snoRNA.

DISCUSSION

To explore the nature of methylation guide snoRNAs in an ancient eukaryotic organism, we characterized the box C/D snoRNAs of *T. brucei* by direct sequencing of RNAs immunoprecipitated with anti-fibrillarin antibodies. The *T. brucei* fibrillarin cDNA was cloned, and the predicted protein sequence was found to bear the GAR and methyltransferase domains and to be conserved across species. The fibrillarin protein was expressed in and purified from *E. coli* and was used to raise polyclonal rabbit antibodies. Immunoprecipitation of *T. brucei* extracts with these antibodies indicated that there are at least thirty fibrillarin-associated snoRNAs in this trypanosomatid. We have identified and characterized seventeen of them and have named them TBR1–17. Sixteen have conserved box C and D elements at their 5′- and 3′-ends, respectively, a hallmark of box C/D snoRNAs in other studied eukaryotes. Box C of TBR17 is located interiorly, similar to the box C location in all known U3 snoRNAs, vertebrate U8, and U13 snoRNAs (25). Our snoRNA search algorithm predicts that 15 of the 17 snoRNAs are putative guide RNAs for 2′-*O*-ribose rRNA methylation. We have confirmed that eight of the snoRNAs carry out methylation of their 2′-*O*-ribose target sites according to the box D + 5 rule. Six of the fifteen guide snoRNAs are potential functional homologs to yeast and vertebrate snoRNAs, suggesting that these specific box C/D snoRNAs existed in early eukaryotes and were maintained as eukaryotes evolved. Conservation of their target methylation sites in rRNA from trypanosomes to metazoans suggests that preservation of methylation on the ribose of these nucleotides is important for ribosome function.

In higher eukaryotes, box C and D elements have roles in snoRNA maturation, stability, 5′-TMG cap formation, and fibrillarin association (65–69). All seventeen trypanosome snoRNAs that we have identified possess the conserved box C and D motifs. Tabulation of nucleotide usage in boxes C and D indicates that the first three nucleotides of box C and the last two nucleotides of box D are invariant among all seventeen snoRNAs (Table III). This pattern of box C and D nucleotide usage is generally similar to that observed in yeast and vertebrates (30, 70). One exception is that TBR1 has an atypical box D, CCGA, not seen before in any organism. Because trypanosomes are considered ancient eukaryotes, these results suggest that strong evolutionary constraints have been placed on the box C and D nucleotides throughout evolution.

The vast majority of box C/D snoRNAs function in ribose

TABLE II

Guide regions of potential snoRNA homologs between trypanosomes, yeast, and humans showing rRNA complementary nucleotides

The asterisk indicates the rRNA methylated nucleotide according to the box D + 5 rule of yeast and vertebrate box C/D guide snoRNAs (37–39).

trypanosome snoRNA	yeast snoRNA	human snoRNA
LSU*3722 5′- AGACCGUCGUGAG 28S 3′- AGUAUUCUGGCAGCAUUC TBR1 box D′	LSU*2956 5′- UAGACCGUCGUGA 25S 3′- CGUCAACUGGCAGCACU snR73 box D′	LSU*4496 5′- AGACCGUCGUGAGA 28S 3′- AGGCGUCUGGUAGCACUCU U35 box D′
SSU*1867 5′- GCAGGUCUGUG 18S 3′- AGUCACGUCCAGACAC TBR8 box D	SSU*1425 5′- ACAGGUCUGUG 18S 3′- AGUGAUGUCCAGACAC snR56 box D′	SSU*1490 5′- ACAGGUCUGUGA 18S 3′- AGUCUUGUCCAGACACU U25 box D′
LSU*740 5′- GAAACACGGACCA 28S 3′- AGACUUUGUGCCUGGU TBR9 box D′	LSU*647 5′- AAACACGGACCAAAG 25S 3′- AGUAUUUGUGCCUGGUUUC U18 box D′	LSU*1306 5′- GAAACACGGACCA 28S 3′- AGUCUUUGUGCCUGGU U18 box D′
LSU*3556 5′- AGAGGUGUCAG 28S 3′- AGUCUUCUCCACAGUC TBR11 box D′	LSU*2790 5′- UAGAGGUGCCAGAAA 25S 3′- UGUAAUCUCUACGGUCUUU snR48 box D′	LSU*4330 5′- AGGAGGUGUCAGAA 28S 3′- AGUAUGCUCCACAGUCUU U60 box D′
LSU*3578 5′- ACAGGGAUAACUG 28S 3′- AGUCUAUCCCUAUUGAC TBR11 box D	LSU*2812 5′- ACAGGGAUAACUG 25S 3′- AGUCUGUCCCUAUUGAC snR38 box D	
SSU*652 5′- GGUAAUUCCAG 18S 3′- AGUCUACCAUUAAGGUC TBR12 box D′	SSU*578 5′- CGGUAAUUCCAGCU 18S 3′- AGUCGGUCAUUAAGGUCGA snR77 box D′	
LSU*2853 5′- CAAAUGCCUCG 28S 3′- AGUAUGUUUACGGAGC TBR13 box D′	LSU*2279 5′- GCCAAAUGC 25S 3′- AGCUGGUUUACG snR13 box D′C	LSU*3754 5′- CAAAUGCCUC 28S 3′- AGUCUGUUUACGGAG U15 box D

methylation in all other eukaryotes studied. Our computer search algorithm predicts that fifteen of the seventeen snoRNAs are potential guide RNAs for 2′-*O*-ribose methylation of rRNA (Fig. 3). Because the related trypanosome *Crithidia fasciculata* contains 95–100 sites of 2′-*O*-ribose methylation (71), it is likely that the rRNA of *T. brucei* is also extensively methylated. Previously, three rRNA methylation sites were either mapped in *T. brucei* RNA or can be inferred from mapping of rRNA in a related trypanosomatid: Gm75 in 5.8 S rRNA, Gm1867 in 18 S rRNA (erroneously specified as 1868), and Gm3968 in large subunit rRNA (51, 52). Levitan *et al.* (51) sequenced the *L. collosoma* snoRNA that is likely to guide methylation of the 5.8 S rRNA at a position that would be complementary to the fifth nucleotide upstream of the box D′ sequence. We mapped 7 additional methylation sites in *T. brucei* rRNA: 28 S mA740, 28 S mA2713, 18 S mC18, 18 S mU652, 5.8 S mG75, 28 S mC3573, and 28 S mG3578. We have identified snoRNAs that can mediate the methylation reactions of eight of the mapped sites. Furthermore, 6 of the 15 guide snoRNAs are potential functional homologs to snoRNAs from yeast and/or vertebrates. In each case, the nucleotide whose ribose undergoes methylation is the fifth nucleotide upstream of either box D or box D′, within the snoRNA-rRNA complementarity (31, 72). This strongly suggests that trypanosome

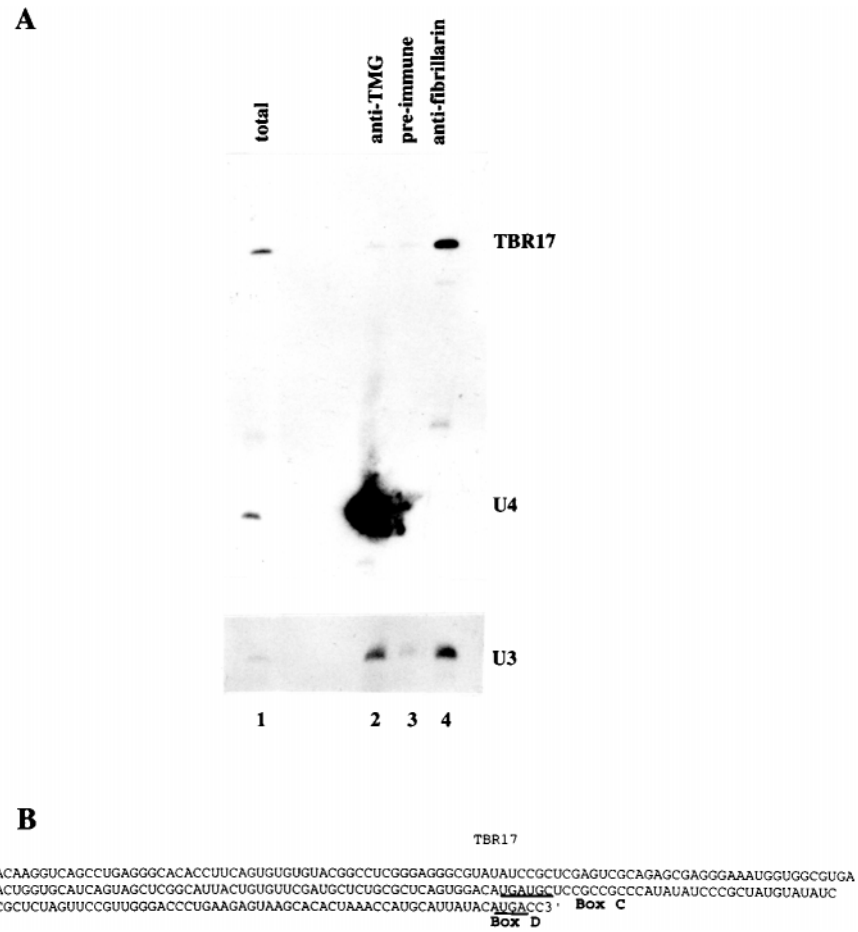


FIG. 5. **TBR17 is a fibrillarin-associated box C/D snoRNA.** *A,* immunoprecipitations were performed on *T. brucei* whole cell extracts using anti-TMG cap antibodies (*lane 2*), rabbit preimmune serum (*lane 3*), or anti-fibrillarin rabbit immune serum (*lane 4*) in NET-5 buffer. *Lane 1* contains total RNA isolated from a whole cell extract representing 1/100 the amount used in the immunoprecipitations. RNAs from immunoprecipitations were resolved on a 10% denaturing polyacrylamide gel, transferred to a Zeta-probe membrane, and hybridized to labeled PCR probes to U4, U3, or TBR17. *B,* full-length sequence of TBR17. The conserved boxes C and D are indicated.

TABLE III
T. brucei snoRNA box C and D nucleotide usage
Box C and D nucleotide usage of the 17 characterized snoRNAs.

	U	G	A	U	G	A
Box C consensus sequence						
C				18%		12%
U	100%			82%	12%	18%
G		100%			88%	6%
A			100%			65%

	C	U	G	A
Box D consensus sequence				
C	88%	5%		
U		95%		
G			100%	
A	12%			100%

rRNA methylation does indeed follow the box D + 5 rule, in contrast to a previous proposal (52). Our results and those of Levitan *et al.* (51) suggest that methylation guide snoRNAs originated early in eukaryotic evolution and that the strategy for targeting methylation according to the box D + 5 rule evolved with them.

Studies in several eukaryotic organisms indicate that the biogenesis of the box C/D snoRNAs occurs by a number of strategies (17, 20). In vertebrates, the majority of the box C/D snoRNAs are processed from introns of pre-mRNA transcripts. The yeast box C/D snoRNAs are transcribed as monocistronic or polycistronic RNAs or as introns of pre-mRNAs (73). All known plant box C/D snoRNAs are also processed from polycistronic transcripts (74, 75). It will be interesting to determine the mode of biogenesis of the trypanosome box C/D snoRNAs. Based on the work of Roberts *et al.* (52, 64), it is likely that at least some are clustered in the trypanosome genome. Are they also processed from polycistronic transcripts? Which RNA polymerase transcribes the newly identified trypanosome snoRNAs? So far it is known that the trypanosome U3 snoRNA, like the trypanosome U2, U4, and U6 small RNAs, is transcribed by RNA polymerase III (76, 77). Are the other trypanosome box C/D snoRNAs also transcribed by RNA polymerase III or by a different RNA polymerase?

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