Elucidating the Role of H/ACA-like RNAs in trans-Splicing and rRNA Processing via RNA Interference Silencing of the Trypanosoma brucei CBF5 Pseudouridine Synthase

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Most pseudouridinylation in eukaryotic rRNA and small nuclear RNAs is guided by H/ACA small nucleolar RNAs. In this study, the Trypanosoma brucei pseudouridine synthase, Cbf5p, a snoRN, a snoRNA, was identified and silenced by RNAi. Depletion of this protein destabilized all small nuclear RNAs of the H/ACA-like family. Following silencing, defects in RNA processing, such as accumulation of precursors and inhibition of cleavages to generate the mature rRNA, were observed. snR30, an H/ACA RNA involved in rRNA maturation, was identified based on prototypical conserved domains characteristic of this RNA in other eukaryotes. The silencing of Cbf5p also eliminated the spliced leader-associated (SLA1) RNA that directs pseudouridylation on the spliced leader RNA (SL RNA), which is the substrate for the trans-splicing reaction. Surprisingly, the depletion of Cbf5p not only eliminated the pseudouridine on the SL RNA but also abolished capping at the fourth cap-4 nucleotide. As a result of defects in the SL RNA and decreased modification on the U small nuclear RNA, trans-splicing was inhibited at the first step of the reaction, providing evidence for the essential role of H/ACA RNAs and the modifications they guide on trans-splicing.

The rRNA of all eukaryotes undergoes extensive covalent modifications. Post-transcriptional endonucleolytic cleavages of the large precursor generate the rRNAs, whereas covalent modification takes place co-transcriptionally (1). These modifications include methylation of the 2'-hydroxyl residue (2'-O-methylation) and isomerization of the uracil to pseudouridine (Ψ). Most of these modifications are guided by sitespecific base pairing of small nucleolar RNAs (snoRNAs) (2–5). The snoRNAs that guide these modifications are designated by their specific sequence motifs; C/D boxed RNA guides the 2'-O-methylation, and H/ACA RNA guides pseudouridylation (6, 7). Most relevant to this study are the H/ACA RNAs (2, 3, 5, 6, 8).

In most eukaryotes studied so far, the H/ACA snoRNAs are composed of two hairpin domains connected by a single-stranded hinge, the H domain, and a tail region, the ACA box (3). Four core proteins (Gar1p, Nop10p, Nhp2p, and Cbf5p/dyskerin) were identified in eukaryotic H/ACA snoRNP. All of these proteins except Gar1p are essential for snoRNA stability (9). Two short RNA recognition motifs of the snoRNA base-pair with the rRNA sequences flanking the uridine to be converted to pseudouridine. The pseudouridine is always located 14–16 nt upstream from the H or ACA box of the snoRNA (3, 5, 7). Surprisingly, all of the 34 H/ACA-like RNAs identified in Trypanosoma brucei can form only a single stem-looop structure and carry an AGA instead of ACA box at the 3' end (10). These 34 H/ACA RNAs can potentially guide pseudouridylation on 32 rRNA positions (10). Interestingly, 84 methylations guided by 57 C/D snoRNAs were also identified in the same study, suggesting that trypanosome represents an organism in which the amount of nucleotide modification (2'-O-methylation) may exceed that of Ψs (10).

The most interesting H/ACA-like RNA in trypanosomes is the spliced leader-associated RNA1 (SLA1). This RNA was first discovered based on its efficient cross-linking to the spliced leader RNA (SL RNA) and was proposed at that time to be the U5 snoRNA homologue (11). However, since the discovery of “authentic” U5 snoRNA (12–14), the identity of this RNA has not been resolved. Based on its genomic localization together with C/D snoRNAs and the finding that in trypanosomes both C/D and H/ACA RNA exist in genomic clusters carrying the two type of RNAs, it was proposed that SLA1 is a special H/ACA that directs modification at position –12 with respect to the 5′ splice site on the SL RNA. Indeed, the SLA1/SL RNA interaction domain complies with the canonical rule for guiding pseudouridylation on the SL RNA. Position –12 with respect to the 5′ splice site is pseudouridylated in all of the trypanosomatid SL RNA, and SL RNA mutants that cannot interact with SLA1 by extensive base pairing are not pseudouridylated (15). The SLA1 is localized both to the nucleolus and the nucleoplasm (15). More recently, it was demonstrated that pseudouridylation takes place early during SL RNA biogenesis before Sm assembly, since SL RNA synthesized in SmD1-depleted cells was properly pseudouridylated in all of the trypanosomatid SL RNA, and SL RNA mutants that cannot interact with SLA1 by extensive base pairing are not pseudouridylated (16). Interestingly, however, SL RNA mutants lacking a U in this conserved position do not show splicing defects (17, 18).

Not all of the H/ACA snoRNAs function in guiding modifications, since several are required for endonucleolytic cleavages of pre-rRNA (19, 20). One such RNA is snR30 in yeast and U17 in mammals, which functions early in RNA maturation (21, 22). snR30 inactivation in yeast prevents the synthesis of 18S rRNA, since cleavages at A0, A1, and A2 of the pre-rRNA precursor are inhibited (21, 22). Another special H/ACA-like RNA is telomerase RNA in mammals (23). Indeed, patients with the disease dyskeratosis cogenita, caused by mutation in the pseudouridine synthase, have reduced telomerase activity and shorter telomeres (24). Relatively little is known about trypanosome rRNA maturation. This...
process seems to differ from maturation in other eukaryotes. The small subunit rRNA (SSU) in trypanosomes is the largest one known so far, and the large subunit rRNA (LSU) is processed into six fragments (25, 26). Whereas in most eukaryotes, the first cleavage of LSU is in the 5′ external transcribed spacer (5′ETS), in T. brucei the first cleavage takes place at the internal transcribed spacer, ITS1 (position B1), that separates the pre-SSU from the pre-LSU (27). Cleavages at the 5′ETS in sites A′, A0, and A1 generate the 5′ end SSU rRNA. The only trypanosome snoRNA involved in rRNA maturation identified so far is U3 (27–30). The T. brucei U3 snoRNA is unique in having three 5′ETS complementary sequences, two of which are essential for SSU production (30).

CBF5, the pseudouridine synthase of the H/ACA snoRNP, is essential in yeast, mostly because it is required for stabilizing H/ACA RNAs that are essential for rRNA endonucleolytic cleavages (31). Note that most of the snoRNAs that direct modification are not essential for viability (5, 20). Recent studies in rats demonstrate that mutation in Cbf5p elicits the disease dyskeratosis cogenita. This disease, introduced above, is characterized by premature aging and increased tumor susceptibility. The study further suggests that all of the defects associated with this disease appear before the defects in telomere length caused by reduced telomerase activity, suggesting that deregulated ribosome function is important for the onset of the disease (24).

Little is known about the linkage between RNA modification and splicing, compared with rRNA maturation. Modifications on U2 snoRNA are essential for U2 snoRNP biogenesis and pre-mRNA splicing in Xenopus oocytes (32) and mammals (33). The pseudouridine at the U2 snoRNA domain that interacts by base pairing with the pre-mRNA not only stabilizes the branch point interaction as compared with an unmodified uridine, but the pseudouridine induces a structural change in the RNA that helps to position the branch site adenosine more precisely for recognition and subsequent activity in splicing (34).

In this study, the effect of depletion of H/ACA-like RNA on RNA maturation was studied upon silencing CBF5 by RNAi in T. brucei. The gene is essential for viability, and its depletion elicited major defects in rRNA maturation. The first trypanosome H/ACA RNA involved in rRNA maturation, the snoR30 homologue, was identified in this study, and the defects in rRNA maturation during CBF5 silencing can be attributed in part to its absence. As opposed to the well documented effect of CBF5 depletion on rRNA maturation and modification, this study also describes defects in trans-splicing associated with the Cbf5p depletion. The defects in trans-splicing may stem from the elimination of SLA1, which has a critical role as an SL RNA chaperone during SL rRNA maturation, the snR30 homologue, was identified in this study, including an HindIII site (underlined), was used for PCR amplification of the 540 bp for cloning into the pLew100 vector (36) to generate the stem-loop construct (36).

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MATERIALS AND METHODS

All of the oligonucleotides were specific for T. brucei. Oligonucleotides for the Silencing Construct—0924 (5′-CCG CTC GAG TGA GGC ACA TGG TAA AGA GG-3′, sense) from position 21 to 40 of the Cbf5 coding region, including the Xhol site (underlined), was used for PCR amplification of the 480 bp for cloning into the pZJM vector (35) to generate the T7 opposing construct. 0943C (5′-CCC AAG CTT ATT AAC GGT GGT CTT TGA AA-3′, antisense) from position 481 to 500 of the Cbf5 coding region, including the HindIII site (underlined), was used for PCR amplification of the 480 bp for cloning into the pZJM vector (35) to generate the T7 opposing construct. 9485E1 (5′-GCT CTA GAT CGA CCA TGC ATG ACG TTC T-3′, sense), from position 721 to 740 of the Cbf5 coding region, including an Xbal site (underlined), was used for PCR amplification of the 540 bp for cloning into the pLew100 and pM326 vectors (35) to generate the stem-loop construct. 9485E2 (5′-CGA GGC GTA GGG CGC TTA TTC TTC TT C-3′, antisense), from position 1241 to 1260 of the Cbf5 coding region, including an MluI site (underlined), was used for PCR amplification of the 540 bp for cloning into the pLew100 vector (36) to generate the stem-loop construct. 9485E3 (5′-GCC AGG CTT GCC TCC ATT CTT TC-3′, antisense) from position 1241 to 1260 of the Cbf5 coding region, including an HindIII site (underlined), was used for PCR amplification of the 540 bp for cloning into pM326 vector to generate the stem-loop construct (36).

Oligonucleotides for Analysis of Small RNAs—TB5H1 (5′-CGC AGG TGC TTC GTA CCG-3′, antisense) was complementary to TB10cs3h1 snoRNA from position 41 to 58; 2-CH-2 (5′-GGG AGG TCC GAT TGA G-3′, antisense) was complementary to TB6cs1h2 snoRNA from position 51 to 66; 2-CH-3 (5′-AAC TTC AGG TAA GGT TAT C-3′, antisense) was complementary to TB6cs1h4 snoRNA from position 54 to 71; TB5hno-H-1 (5′-ATTCTCGGACACCGTGA-3′, antisense) was complementary to TB9cs2h1 snoRNA from position 58 to 75; 35987 (5′-ATA TGA TCG ACG TTC ATC TG-3′, antisense) was complementary to snoRNA 92 (Tb11C2C2) from position 37 to 54; TB5H3 (5′-TGCCTTTGACAC-3′, antisense) was complementary to U3 snoRNA from position 109 to 123; 31253 (5′-TCTCTGTCTCAGTTTCTTG-3′, antisense) was complementary to SLA1 RNA from position 67 to 9091 (5′-TGGGCACTTTGTTGCC-3′, antisense) was complementary to SL RNA from position 65 to 79; anti-SL (5′-TGG TCT TCA TAC CCT TT-3′, antisense) was complementary to the SL RNA from position 36 to 52; 56759 (5′-CCA TTT CCC TGC TCT GCG AC-3′) was complementary to the 270 snoRNA (from position 70 to 91); U2–81 (5′-GGT CTA TGC CCG GTT TCC GG-3′, antisense) was complementary to the U2 snoRNA from position 59 to 81.

Oligonucleotides for Analysis of rRNA—44252 (5′-GGG TGA ACA ATC CAA CCC TT-3′, antisense) was complementary to the rRNA LSU-β from position 1302 to 1321; srRNA-1 (5′-TAA TGC GCC GAA CTC ACA AC-3′, antisense) was complementary to the srRNA-1 from position 177 to 196; sn01 (5′-GAT CTT GTA AAA ATG AAG GTA TGT GGC GCC GAC A-3′, antisense) was complementary to the srRNA-6 from position 53 to 76; SSU1923 (5′-ATT GTA GCG GTG TGC TCG-3′, antisense) was complementary to the SSU rRNA from position 1923 to 1940; A2–2738 (5′-CCT TAA CTG AAG ACG GTG TA-3′) was complementary to the ETS pre-rRNA from position 1058 to 1080; A0–5470 (5′-AGT GTA GAC GCG TGA TCC GCT GT-3′, antisense) was complementary to the ETS pre-rRNA from position 1110 to 1130; A1–2729 (5′-GGC TAA GTC TTT GAA ACA AGC A-3′, antisense) was complementary to the SSU rRNA from position 29 to 50; A2–5472 (5′-CCT TAA CTG AAG ACG TGT CA-3′, antisense) was complementary to the ITS1 pre-rRNA from position 36 to 63.

Oligonucleotides Used for Northern Analysis of Tubulin mRNA—Tubulin-3A (5′-ATG CAG ATA GCC TCA GCC-3′, antisense) was complementary to the a-tubulin gene, from position 3 to 20, relative to the start codon.

Cell Growth and Transfection—Procylic T. brucei strain 29-13 (obtained from the laboratory of Paul England, a gift from George Cross laboratory), which carries integrated genes for T7 polymerase and the tetracycline repressor, was grown in SDM-79 medium supplemented with 10% fetal calf serum in the presence of 50 µg/ml hygromycin and 15 µg/ml G418. Cells were transfected as previously described (16). For cloning, the transfected cells were diluted onto microtiter plates in the presence of the parental T. brucei 29-13, which served as feeder cells.
Next, the microtiter plate was incubated in a humid chamber at 27 °C in a 5% CO₂ atmosphere. After 2–3 weeks, clonal populations were obtained in the microtiter plates, and the cells were transferred to medium for propagation. Cells from cultures that showed typical growth arrest upon tetracycline induction were grown and frozen. Every 2 weeks, a new culture was started from the original frozen stock.

**Northern Analysis**—Total RNA was prepared with TRIzol reagent (Sigma), and 20 µg/lane was fractionated on a 1.2%/0.8% agarose, 2.2 M formaldehyde gel. The RNA was visualized with ethidium bromide. The CBF5 and tubulin mRNAs were detected with randomly labeled probes (Random Primer DNA Labeling Mix, Biological Industries Co.). rRNA subunits were detected with [γ-32P]ATP-labeled oligonucleotides. For analyzing small rRNAs (srRNAs), total RNA was fractionated on a 10% polyacrylamide gel containing 7 M urea. The RNA was transferred to a nylon membrane (Hybond; Amersham Biosciences) and probed with [γ-32P]ATP-labeled oligonucleotides.

**Primer Extension and Mapping the Pseudouridine on the SL RNA, U2 snRNA, and rRNA**—Primer extension was performed as described previously (12). Total RNA (5–10 µg) was used in a primer extension reaction with 1 × 10⁵ cpm of [γ-32P]ATP-labeled oligonucleotide. For determining the ψ on the SL RNA, U2 snRNA, and rRNA, RNA from 5 × 10⁸ cells was treated with 50 µl of N-cyclohexyl-N'-β-(4-methylmorpholinium)ethyl-carbodiimide p-tosylate (CMC) buffer composed of (0.17M NaCl, 0.1% Nonidet P-40, 1% bovine serum albumin, 5 µg/ml leupeptin). Next, 1% Triton X-100 was added, and the extract was incubated on ice for 20 min. The extract was then centrifuged at 10,000 × g for 15 min, and the supernatant was subjected to affinity selection using IgG-Sepharose beads for 2 h. RNA was extracted from the beads with TRIzol (Sigma) reagent and was subjected to primer extension analysis with specific probes.

**RESULTS**

Silencing of CBF5 Suggests That the Gene Is Essential for Growth—To elucidate the role of H/ACA RNA in RNA maturation and especially to better understand the role of SLA1 in trans-splicing, the trypanosome CBF5 homologue was identified in the *T. brucei* genome (TB10.100.0060). The putative protein is 427 amino acids in length. The first ~310 residues share 61% identity with the mammalian NAP57 and 65% identity with the yeast Cbf5p. The N terminus of the trypanosome protein is shorter than its homologous proteins in metazoans and plants by ~20 amino acids, and the trypanosome sequence lacks the highly charged region that is found mainly in mammalian and fly proteins. In yeast, the C terminus of Cbf5p is composed of highly charged amino acids, and 67 of the 83 residues are either Asp, Glu, Lys, or Arg (30 are Asp/Glu, and 37 are Arg/Lys), including 10 consecutive KK/E/D repeats (indicated in purple in supplemental Fig. 1S). The fly protein does not have KK/E/D repeats, but 45 of the last 73 amino acids are Asp, Glu, Lys, or Arg (22 are Asp/Glu, and 23 are Arg/Lys) (39). The trypanosome C terminus, on the other hand, is weakly charged; it carries 25 conserved amino acids but only 5 Asp/Glu and 5 Arg/Lys. These amino acids are probably involved in centromere binding activity, since *S. cerevisiae* expressing a C-terminal truncated CBF5 gene lacking the KK/E/D repeats showed a delay at G2/M arrest, which links this protein with function in cell cycle control, most probably via its effects on centromeres. In addition, CBF5 lacking these repeats also failed to bind microtubules *in vitro* (40). Furthermore, mutation of specific amino acids in the C-terminal region of the *Aspergillus nidulans* homologue affects the polar growth of these cells but not pseudouridylation or RNA maturation, also suggesting that this region of the protein is involved in centromere binding (41). The absence of the charged domains in both the N terminus and C terminus of the trypanosome protein suggests that the trypanosome protein may not be associated with centromere function as it is in other eukaryotes but rather mainly functions in pseudouridylation and RNA maturation.

To elucidate the function of the trypanosome CBF5, the gene was silenced by RNAi. To silence the gene, a construct that is expressed from the T7 opposing cassette was prepared (35). A 480-bp fragment was amplified from genomic DNA and cloned into the plZM vector (35). Since one of the characteristics of the T7 opposing system is its instability, especially under prolonged culture, a stem-loop silencing construct was also prepared, as described under “Materials and Methods.” The constructs were linearized for integration into the nontranscribed RNA spacer region, and clonal populations were prepared, as previously described (16). After 3 weeks, the cloned cells were used to establish a pure culture. A typical growth curve of the silenced cells carrying
the stem-loop construct is presented in Fig. 1. Four days after tetracycline induction at 26 °C, the cells stopped growing. Interestingly, during culture at 37 °C, growth arrest occurred sooner and to a greater degree (Fig. 1B). Such a phenotype was not observed in yeast depleted of this protein (31), suggesting that the growth inhibition at the restrictive temperature may stem from the effect of the depletion on splicing (see below). The clear growth arrest upon silencing suggests that CBF5 is an essential gene in trypanosomes, as was demonstrated in yeast (31).

To further demonstrate the relationship between growth arrest and silencing of these genes, the level of CBF5 mRNA was examined upon silencing. The results in Fig. 2 suggest that in both silenced cell lines using the T7 opposing system (Fig. 2A) and the stem-loop silencing (Fig. 2B), the mRNA was eliminated as early as 2 days after silencing. Since the size of the CBF5 mRNA overlaps that of the transcript produced from the silencing construct, we used a random labeled PCR product from the gene located outside the domain that was used to prepare the silencing construct. The probe therefore recognizes only the mRNA transcript (Fig. 2B). The reduction in the level of CBF5 mRNA was determined by densitometry. The reduction of the CBF5 mRNA in the T7 opposing cell line was estimated as 95.23 ± 3.22%, and that of the stem-loop cell line was estimated as 97.02 ± 2.87%. Silencing with the stem-loop construct was more stable, whereas the growth of the cells carrying the T7 opposing system was arrested only for 6 days and then resumed at the normal rate (data not shown).

CBF5 Silencing Destabilizes H/ACA-like RNAs, Including SLA1—To examine whether CBF5 functions in stabilizing the snoRNP complex, as in other systems, the levels of different H/ACA-like RNA species were monitored by primer extension following silencing. The results presented in Fig. 3A suggest that the levels of all H/ACA-like snoRNAs tested were significantly reduced. The reduction of the different H/ACA snoRNAs levels was subjected to densitometry and was estimated to be in the range of 97–100% (Fig. 3A). Some of the H/ACA snoRNA were completely eliminated (TB9Cs2H1 and TB6Cs1H2), but in two other cases, silencing was less efficient; TB10Cs3H1 and Tb6Cs1H4 were reduced by 97.32 ± 1.79 and 98.48 ± 1.34%, respectively. The results in Fig. 3A also indicate a major difference in the abundance of different H/ACA-like RNAs. These variations stem from either a difference in the number of genes encoding for each snoRNA species or the efficacy of processing of the snoRNA within its cluster (42). Indeed, we have recently demonstrated that the numbers of different snoRNA clusters vary from 1 to 10, and therefore the copy numbers of individual H/ACA genes differ considerably (10). In addition, we noticed a difference in the level of snoRNAs emerging from the same polycistronic RNA, most probably as a result of differential processing efficacy (43). The reduction in the level of H/ACA due to the CBF5 silencing is specific, since no effect on the most abundant C/D snoRNA, the U3, and another C/D (TB11Cs2C2-92 nt snoRNA) was observed (Fig. 3B).

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**FIGURE 2. Reduction of CBF5 mRNA upon RNAi silencing.** A. Northern analysis of CBF5 mRNA from cells expressing the T7-opposing silencing construct. Total RNA (30 μg) was separated on 1.2% agarose, 2.2 M formaldehyde gel. The RNA was blotted and hybridized with a randomly labeled probe specific for the CBF5 gene (positions 21–500 of the coding sequence). The level of 7SL RNA (used as a control for equal loading) was determined using a randomly labeled probe specific for 7SL RNA as described under “Materials and Methods.” The marker is indicated in Kb. B. Northern analysis of CBF5 mRNA from cells expressing the stem-loop construct was performed as described in A.

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**FIGURE 3. Effect of CBF5 silencing on the level of snoRNAs.** A. effect of CBF5 silencing on the level of H/ACA snoRNAs. Total RNA was prepared from cells carrying the stem-loop RNAi construct without induction (− Tet) or after 2 days of induction (+ Tet). Total RNA (10 μg) was subjected to primer extension with radiolabeled oligonucleotides complementary to each of the H/ACA snoRNAs: TB10Cs3H1, TB9Cs2H1, TB6Cs1H2, and TB6Cs1H4. The oligonucleotides and their sequences are summarized under “Materials and Methods.” cDNA was separated on a 6% sequencing gel. To control the level of RNA of each sample, primer extension was performed with primer 35987, specific to 92-nt C/D snoRNA (TB11Cs2C2). B, effect of CBF5 silencing on the level of C/D snoRNAs. Total RNA was prepared from the same cells described in A before induction (− Tet) and after 2 days of induction (+ Tet). Total RNA (10 μg) was subjected to primer extension with radiolabeled oligonucleotide TB13 specific to U3. C, effect of CBF5 silencing on the levels of SLA1. Total RNA was prepared from the same cells as in A and B before silencing (− Tet) and after 2 days of induction (+ Tet). Total RNA (10 μg) was subjected to primer extension with radiolabeled oligonucleotide 31253 specific to SLA1.
The major reduction of SLA1 during this silencing, presented in Fig. 3C, supports the notion that SLA1 is an H/ACA RNA. The results were quantified by densitometry from four independent experiments, and the results indicate 98.08 ± 1.73%. These data join our previous results, which demonstrated that the level of SLA1 did not change with the depletion of either Sm or Lsm proteins (16, 44).

SLA1 Depletion Eliminates the Ψ28 of the SL RNA and Also Affects cap-4 Modification and trans-Splicing—To examine whether the elimination of SLA1 affects the pseudouridylation of the SL RNA, the RNA prepared from uninduced (−Tet) cells and cells 2 days after induction (+Tet) was treated with CMC followed by alkaline treatment and primer extension using an oligonucleotide complementary to the SL RNA. In this method, the reverse transcriptase stops 1 nt before the modified base. The position of the pseudouridylated nucleotide is marked with an asterisk next to a partial DNA sequence on the left.

FIGURE 5. Effect of CBF5 silencing on U2 snRNA modification. Mapping of the pseudouridine on U2 snRNA before and after CBF5 silencing. Total RNA from cells carrying the Cbf5 RNAi stem-loop construct without induction (−Tet) and after 2 days of induction (+Tet) (10 μg) was treated with CMC, as described under “Materials and Methods.” The treated RNA (+CMC) and control RNA (−CMC) were subjected to primer extension with oligonucleotide U2-81 complementary to the U2 snRNA. The products were separated on a 6% polyacrylamide denaturing gel alongside a DNA sequencing ladder of the corresponding U2 snRNA gene. The arrow designates the authentic pseudouridine, but the reverse transcriptase stops 1 nt before the modified base. The position of the pseudouridylated nucleotide is marked with an asterisk next to a partial DNA sequence on the left.
control for equal loading). These results provide evidence for the direct effect of Cbf5p depletion on trans-splicing. Interestingly, however, the complete elimination of SLA1 during this silencing, splicing was not abolished but only partially inhibited.

**Depletion of CBF5 Affects the Pseudouridylation of U2 snRNA**—The defects in trans-splicing could also emerge from the lack of Ψ on the U snRNAs, since in Xenopus oocytes, U2 snRNA lacking Ψs is not assembled properly to the 17 S U2 snRNP and cannot join the spliceosome (32). To examine the effect of Cbf5 depletion on U2 pseudouridylation, the RNA from silenced cells (+Tet) and uninduced cells (−Tet) was subjected to primer extension in the presence of CMC as described above. The results (Fig. 5) demonstrate elimination of Ψs at position 14 upon induction of silencing (+Tet).

To examine the significance of this reduction, the experiment was repeated four times, and reduction was estimated as 74.73 ± 5.87%. Interestingly, no changes due to silencing were observed in Ψ33, Ψ34, Ψ36, and Ψ37 (results not shown), suggesting that, as in yeast, these modifications are most probably carried out by non-small RNA-containing enzymes (45, 46).

**Effect of Cbf5p Depletion on rRNA Modification**—To examine the effect of CBF5 silencing on rRNA modifications, the level of pseudouridylation was determined in the highly functional and important LSU domain, the peptidyltransferase region of LSU rRNA. The results, obtained by CMC mapping (Fig. 6), indicate reduction of Ψ1272. The significance of this reduction was estimated from four independent experiments to be 74.76 ± 5.92%. Interestingly, no difference was observed in these four experiments in modification at Ψ1257. However, no H/ACA RNA to guide this modification was identified in our recent genome search for H/ACA, whereas Ψ1272 is guided by TB9Cs1H1 (10). One possible explanation for this differential effect on Ψ formation at the two positions is that Ψ1257 modification is mediated by a non-small RNA-containing enzyme, as in the case of snRNAs (46). However, there is currently no evidence for non-small RNA pseudouridylation on eukaryotic rRNA. Another possible explanation for the differential effect of the depletion on different rRNA positions is that the modifications, which are not altered during CBF5 silencing, are guided by H/ACA RNAs that are very abundant and stable and hence are not completely eliminated during silencing (as in the case of TB10Cs3H1 in Fig. 3A). The residual level of such snoRNAs may be sufficient to maintain almost normal modifications on their targets.

The results presented in Fig. 6 are representative of many additional mappings performed on other domains of rRNA. In all of the mapping experiments, reduction in most but not all Ψs was observed (data not shown). Note that also in yeast, depletion of Cbf5p also did not completely eliminate the Ψs at all positions (31).

**Cbf5p Depletion Results in the Accumulation of Pre-rRNA and Reduction in Mature rRNA**—In yeast and mammals, the major growth defects associated with CBF5 inactivation stem from the perturbation in rRNA processing. In trypanosomes, rRNA processing is very complex compared with yeast and mammals, and additional cleavages are introduced into the LSU to produce the 28 S α and β and four srRNAs (25, 26). It was therefore of interest to examine the defects in rRNA endonucleolytic cleavages in the CBF5 knockdown cells and investigate whether snoRNA of the H/ACA family is involved in the special cleavage of the trypanosome LSU.

To examine such defects, two approaches were taken. First, the accumulation of precursors and reduction of the mature RNAs were examined by Northern analysis (Fig. 7), and then fine mapping of the cleavage sites was performed by primer extension (Fig. 8). RNA was extracted from cells before and after induction of silencing and subjected to Northern analysis with a probe specific to LSU-β (marked as a in Fig. 7C). The exact structure of these precursors is presented in Fig. 7C. The results (Fig. 7A) demonstrate accumulation of four precursors ~9.6, 5.9, 5.1, and 4.3 kb. The ~9.6 kb is the full-length pre-rRNA transcript. The ~5.9-kb fragment emerges from cleavage of the 9.6-kb precursor at the B1 site. This cleavage is not affected by silencing of the H/ACA pathway or by silencing of the C/D pathway, suggesting that it is not mediated by these snoRNAs families but most probably by MRP as in yeast (47). The ~5.1 kb is most probably generated by cleaving the precursor at a position immediately upstream to LSUα. The identity of these precursors (9.6, 5.9, and 5.1 kb) was confirmed by hybridization with 5.8 S probe (marked as b in Fig. 7C) (results not shown). The ~4.3-kb transcript is most probably generated by cleavage at ITS7 upstream to srRNA-4. This precursor did not appear after hybridization with probe complementary to srRNA-4 (marked as c in Fig. 7C) but did appear after hybridization with probe complementary to srRNA-6 (marked as d in Fig. 7C) (data not shown).

To examine the rRNA endonucleolytic cleavage defects in the SSU part of the precursor, the Northern was probed with a primer specific for the SSU sequence. The results are presented in Fig. 7D, and the schematic presentation of the precursors is shown in Fig. 7F. The results indicate the accumulation of two major precursors, a ~3.7-kb and a ~2.6-kb species. Reduction in the mature 18 S rRNA presented in Fig. 7E was also observed. The 3.7-kb precursor is a result of cleavage at the B1 site, releasing the entire pre-SSU portion of the precursor that was not cleaved at site A0, A1, or A2. The ~2.6-kb fragment is the most abundant and represents a precursor that is cleaved at A0 sites but not at the A2 site. The identity of these precursors was confirmed by hybridization with 5′ ETS and ITS1 probes (marked as f and g, respectively, in Fig. 7F) (data not shown).

4 S. Barth and S. Michaeli, unpublished observations.
To examine defects in LSU endonucleolytic cleavage to generate the srRNA molecules, the levels of srRNA-1 and srRNA-6 were examined by Northern analysis following RNA separation on polyacrylamide denaturing gels. The results (Fig. 7G) indicate no effect on their level, suggesting that no H/ACA RNA is involved in the endonucleolytic cleavages of these small RNAs.

To further confirm the rRNA defects suggested by the hybridization data, primer extension was performed to fine map the precursors and cleavages in the pre-SSU. Primer extension was performed with oligonucleotides situated in regions downstream from A0, A1, and A2 (indicated in Fig. 8B). An increase in the primer extension products using primer situated downstream to A0 (Fig. 8B, b) and A2 (Fig. 8B, d) was observed, suggesting accumulation of pre-rRNA covering these sites since cleavage at these sites was inhibited during silencing. The accumulation of RNA carrying the A0 site (Fig. 8B, a) suggests defects in removing the ETS, which may stem from defects in cleavage at A0 and A1. Indeed, cleavage at A1 was inhibited, as can be seen in Fig. 8B, c, demonstrating reduction in the stop at the A1 position.

The data also suggest that cleavage at A0 was not abolished completely, since the ~2.6-kb precursor was also observed (Fig. 7D), and the
The 270-nt RNA Present in the SLA1 Locus Is the Trypanosome snR30 Homologue—The 270-nt RNA was described as part of the SLA1 locus (48) and was shown to be immunoprecipitated by anti-fibrillarin antibody, suggesting that it belongs to the C/D family (49). However, this RNA can be folded, as can the canonical structure of snR30 (illustrated in Fig. 9B). The structure is composed of three stem-loop structures, the 5’ hairpin, the internal hairpin, and the 3’ hairpin. The H box suggested for the yeast snr30 RNA is located between the 5’ hairpin and the internal one. This sequence 5’-AGAGCGAG-3’ is located in the same position in the trypanosome RNA but deviates from the canonical H box (ANANNA) in other eukaryotes. The 270-nt RNA is the first trypanosome H/ACA RNA described that is composed of more than a single hairpin structure, as in other eukaryotes (22). Strikingly, it is also the first H/ACA-like RNA that possesses an ACA box instead of AGA at the 3’-end. The trypanosome homologue is much shorter than the yeast homologues (270 compared with 600-nt RNA), but it resembles in size the homologous RNA identified in Schizosaccharomyces pombe and Tetrahymena thermophila (22). All snR30 homologues have a common and highly conserved 3’ hairpin loop, which contains the sequences m1 and m2, comprising the most terminal loop with respect to the 3’-end of the molecule (indicated in Fig. 9B). Indeed, the trypanosome 270-nt RNA carries these boxes. Whereas the m2 sequence (5’-AAACCCAU-3’) is fully homologous with the box present in other eukaryotes, the 3’ m1 box (5’-AUUCCUG-3’) deviates from the consensus sequence (5’-AUUCCUA-3’) by a missing U in the fifth position. Like the T. thermophila snR30, sequence m1 has a G instead of an A at the terminal position. Support for the notion that the 270 nt is indeed the snR30 homologue are additional conserved features that can be found in this RNA. As in all snR30, the first residues of the T. brucei m1 motif (AU) and the last nt of the m2 motif (UA) can base-pair, whereas the other conserved nt of m1 and m2 occupy unpaired positions that form the internal loop. The distance between the conserved m1-m2 structural motif and the ACA box is also at 7 nt in the trypanosome RNA. To examine whether the 270 RNA is indeed an H/ACA RNA, the level of the RNA was examined in Cbf5p-depleted cells. The results (Fig. 9A) demonstrate the elimination of this RNA during Cbf5p depletion (reduction of 98.1 ± 0.8%). The level of this RNA was not affected during Nop58p RNAi silencing (data not shown), suggesting that 270 is indeed an H/ACA RNA.

The Nhp2p H/ACA-specific Protein Binds to SLA1 and snR30—To obtain independent evidence that SLA1 and 270-nt RNAs are indeed H/ACA RNA, we expressed a TAP tag version of Nhp2p, a protein that constitutes the H/ACA snoRNP (8). The protein was identified in the T. brucei genome (GeneDB Tb927.4.750). The T. brucei homologue shares 33% identity and 65% similarity with S. cerevisiae protein and 34% identity and 68% similarity with the human protein. The sequence alignment of the T. brucei proteins with homologues from other eukaryotes is presented in supplemental Fig. 2S. The cell line expressing the TAP tag version of the protein was prepared as described under “Materials and Methods.” The expression of the tagged protein was examined by Western analysis upon induction, and fusion protein of 37.3 kDa was observed. Extracts were prepared from the cell line and were subjected to affinity selection using IgG-coated beads. The RNA bound to the beads was examined by primer extension. The results are presented in Fig. 10 and indicate the specific selection of H/ACA RNPs, since among the selected RNA, the SLA1 and snR30 homologue were detected, but the most abundant C/D snoRNA and the SL RNA were absent. It should be noted that the specific efficacy of the selection varied and was dependent on the expression of the tagged protein, which was reduced during prolonged culture.

DISCUSSION

This study in T. brucei highlights the importance of the H/ACA RNA pathway for RNA processing. Whereas in most organisms the necessity of the H/ACA RNAs for life was explained mainly based on their role in ribosomal RNA maturation and modifications, in trypanosomes, H/ACA RNAs play a special role in trans-splicing, and growth is most probably also affected because of trans-splicing defects. By eliminating the H/ACA pathway, the modifications guided by these RNAs were
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FIGURE 9. A, effect of CBF5 silencing on the level of snoRNA 270. Total RNA was prepared from cells carrying the CBF5 RNAi construct without induction (−Tet) and after 2 days of induction (+Tet). Total RNA was subjected to primer extension with radiolabeled oligonucleotide 56759 complementary to snoRNA 270. cDNA was separated on a 6% denaturing gel. To ensure equal levels of RNA in each sample, primer extension was performed with primer 35987, specific to snoRNA 92 (TB11Cs2C2). B, predicted secondary structure of snoRNA 270 (GenDB: Tb927.11_01_v3 511599–511868). Conserved boxes (AGA or ACA) are indicated within the loop. Conserved motifs m1 and m2 are also marked.

![Diagram](image)

FIGURE 10. Specific affinity selection of SLA1 and 270-nt RNA (snR30). Affinity selection with IgG beads. Whole cell extracts were prepared from induced cells (500 ml, 10^7 cells/ml) carrying the NHP2-TAP construct as described under "Materials and Methods." The extracts were subjected to affinity selection using IgG-Sepharose beads. RNA from the cell extract (one-thirtieth), supernatant (one-thirtieth), and all of the selected RNA (designated as T, S, and P, respectively) was subjected to primer extension analysis with radiolabeled oligonucleotides complementary to SLA1, 270-nt RNA, 92-nt RNA (TB11Cs2C2), and SL RNA genes. The RNA was separated on a 6% denaturing gel.

reduced on their rRNA and snRNA targets. The silenced cell line was also used to identify the unique H/ACA snR30 homologue that is involved in rRNA maturation and is described in this study. The major RNA processing defects observed in cells depleted of the H/ACA pathway as well as the high level of modifications on its RNAs suggest a pivotal role in trypanosomes for these RNAs and the modification they guide.

The Function of SLA1 in trans-Splicing—The special role of the H/ACA-like RNAs in regulating splicing in trypanosomes is of particular interest. The function of SLA1 was an enigma for many years before it was suggested to be an authentic H/ACA-like RNA (15). This study strongly supports the notion that SLA1 is a member of the H/ACA family. SLA1 was discovered based on its efficient cross-linking to the SL RNA. Based on the presence of the sequence resembling the invariant loop sequence of U5 snRNA in all eukaryote 5'-CUUUUA-3', it was proposed to be the trypanosome U5 homolog. The first observation that did not support this suggestion was the finding that in Leptomonas symouri, SLA1 lacks this invariant sequence (50). Interestingly, however, SLA1 was found to be associated with core proteins common to snRNAs, suggesting that SLA1 associates with other spliceosomal RNAs and may be part of the trans-spliceosome (50). However, we found that neither Sm nor Lsm proteins associate with SLA1, since the level of this RNA was not affected during RNAi silencing of these proteins (16, 44). Thus, it is still puzzling how this RNA was immunoprecipitated via the anti-core Sm proteins (50).

The major question that remains is the role of SLA1 in trans-splicing. The observation that under Sm depletion LRNA is pseudouridylated suggests that SLA1 associates with LRNA early in its biogenesis (16). This observation strongly supports the notion that SLA1 functions in regulating the biogenesis of LRNA and is not part of the network of snRNAs constituting the catalytic center of the trans-splicing reaction. What then is the main role of SLA1? Is it to direct the pseudouridylation, or does it mainly function as a chaperone? We do not yet have sufficient data to answer this interesting question. The ψ is located in a strategic position of the LRNA, which is involved in both intramolecular base pairing and/or intermolecular base pair interaction with U5 and U6 snRNAs (51). These complex base pair interactions, which involve the same domains, are most probably mutually exclusive (51). However, since pseudouridylation does not take place during spliceosome assembly but rather during SL RNP assembly, it most probably does not function to trigger the switch between the intramolecular interactions and the intermolecular ones. Interestingly, mutations that disrupted this U had only a minor effect on trans-splicing (17, 18).

Based on the arguments listed above, we favor the possibility that the main function of SLA1 is as an LRNA chaperone. As can be seen in Fig. 4B, during silencing, the LRNA not only lacks the ψ at position 28 but also the cap +4 modification. It is therefore possible that SLA1 forces the LRNA to fold in a configuration essential for interacting with the methyltransferase that mediates this +4 modification. The fact that trans-splicing is only at most ~30% inhibited despite the complete elimination of SLA1 may be explained if we consider that trans-splicing defects emerge mainly from the defects elicited at the +4 cap-4 modification. Indeed, mutations at the +4 cap nt do not abolish trans-splicing completely relative to mutations introduced in the 1−3 cap nt (17). It remains to be determined where modification on LRNA by SLA1 takes place in the nucleus and whether SL/SLA1 LRNA interactions take place only before Sm assembly. Our hypothesis is that SLA1 functions mainly as the LRNA chaperone that directs the molecule during early steps of its biogenesis. Since RNAi inhibition results only in knockdown and not knockout of expression, we could not rule out the possibility that a very minute amount of SLA1 is what still supports trans-splicing in the Cbf5p-depleted cells.
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Modifications on U1snRNAs Are Carried Out in Part by H/ACA—It is well documented that at least the modification on U2 snRNA is essential for splicing (32, 33). More recently, it was shown that three pseudouridines and five 2’-O-methyl groups within the first 20 nt of the U2 snRNA are required for efficient pre-mRNA splicing in mammals (33). This might also be the case for the trypanosome U2 snRNA, since during CBF5 knockdown, the Ψ at position 14 was also partially eliminated. Interestingly, the Ψ14 is conserved also in mammals and yeast (52). The fact that, in trypanosomes, U2 modification is mediated by H/ACA RNAs is of special interest in light of the finding that in yeast S. cerevisiae all of the U2 modifications are catalyzed by an RNA-independent mechanism assisted by enzymes (45, 46). We noticed that the level of Ψ at positions 33, 34, 36, and 37, which are complementary to branch point sequences 33, 34, 36, and 37, which are complementary to branch point positions 33, 34, 36, and 37, which are complementary to branch point RNAs is of special interest in light of the finding that in yeast S. cerevisiae such RNAs do exist. Recently, we described a genome-wide analysis of their role in U2 pseudouridylation. We failed, so far, to identify the guide RNAs that direct modifications on U2 snRNA. However, we expect that such RNAs do exist. Recently, we described a genome-wide analysis of H/ACA RNA in T. brucei (10). However, among the 34 H/ACA-like molecules identified, we detected no small Cajal body-specific RNA (scRNAs) or conventional RNAs that could guide Ψ modification on U snRNAs (10). Note that all of the H/ACA identified so far in T. brucei are present in clusters also containing C/D snoRNAs, since our search used the SnoScan tool (available on the World Wide Web at rna.wustl.edu/snoRNAdb/code) that specifically identifies C/D snoRNAs. These H/ACA RNAs were identified based on their co-localization in the same clusters carrying the C/D snoRNAs. However, H/ACA RNAs may also exist in clusters unaccompanied by C/D snoRNAs, which would have been missed in our search (10). These missing guide RNAs will hopefully be identified in a library prepared from RNA immunoprecipitated with tagged H/ACA-binding proteins. In addition, we are in the process of developing an algorithm able to detect the trypanosome H/ACA-like prototype in a whole genome search.

The 270-nt Species Is the snR30 Homologue and Guides RNAs with Special Functions—The finding of the 270-nt species in the SLA1 locus is fascinating H/ACA RNA family. The line described in this study can be used as an excellent tool to determine small RNAs not only in rRNA maturation but also in splicing. The cell fusion of single stem-loop RNAs (53).

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Elucidating the Role of H/ACA-like RNAs in trans-Splicing and rRNA Processing via RNA Interference Silencing of the *Trypanosoma brucei* CBF5 Pseudouridine Synthase

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