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Upstream tRNA Genes Are Essential for Expression of Small Nuclear and Cytoplasmic RNA Genes in Trypanosomes

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An interesting feature of trypanosome genome organization involves genes transcribed by RNA polymerase III. The U6 small nuclear RNA (snRNA), U-snRNA B (the U3 snRNA homolog), and 7SL RNA genes are closely linked with different, divergently oriented tRNA genes. To test the hypothesis that this association is of functional significance, we generated deletion and block substitution mutants of all three small RNA genes and monitored their effects by transient expression in cultured insect-form cells of *Trypanosoma brucei*. In each case, two extragenic regulatory elements were mapped to the A and B boxes of the respective companion tRNA gene. In addition, the $tRNA^{Thr}$ gene, which is upstream of the U6 snRNA gene, was shown by two different tests to be expressed in *T. brucei* cells, thus confirming its identity as a gene. This association between tRNA and small RNA genes appears to be a general phenomenon in the family Trypanosomatidae, since it is also observed at the U6 snRNA loci in *Leishmania pifanoi* and *Crithidia fasciculata* and at the 7SL RNA locus in *L. pifanoi*. We propose that the A- and B-box elements of small RNA-associated tRNA genes serve a dual role as intragenic promoter elements for the respective tRNA genes and as extragenic regulatory elements for the linked small RNA genes. The possible role of tRNA genes in regulating small RNA gene transcription is discussed.

Protozoa of the family Trypanosomatidae include a diverse number of organisms, several of which are parasites of great medical and economical importance. Interest in these cells stems not only from their parasitic lifestyles but also from evolutionary considerations which place the trypanosomatids among the deepest branches of the eukaryotic lineage (23). In recent years, the analysis of trypanosome gene expression has been quite rewarding and has provided evidence for novel mechanisms, including RNA editing, *trans* splicing, and polycistronic transcription units. Notwithstanding many advances in our understanding of trypanosome biology, our knowledge of promoter structures in these organisms is still limited to a few examples. These include three RNA polymerase (pol) I promoters of the procyclic acidic repetitive protein (PARP) (1, 3), variant surface glycoprotein (27, 28), and rRNA (27) genes and one pol III promoter of the *trans*-spliceosomal U2 small nuclear RNA (snRNA) gene (4). For the protein-coding genes, it has become evident that the eukaryotic rule of one promoter for one gene does not apply. Rather, several protein-coding genes are transcribed as part of complex polycistronic transcription units. This functional economy in terms of usage of promoter signals is accompanied by a high density of genetic information in the nuclear genome, with protein-coding genes being separated only by a hundred to a few hundred nucleotides.

Another interesting feature of trypanosome genome organization was noted for some of the genes transcribed by pol III (4, 19). In *Trypanosoma brucei*, the genes coding for the *trans*-spliceosomal U6 snRNA, the U3 snRNA homolog (U-snRNA B [9]), and 7SL RNA (the RNA component of signal recognition particle [16]) have a divergently oriented tRNA gene in their 5′-flanking region, and the spacing between the companion tRNA and the small RNA gene is highly conserved in all three cases (95 to 97 bp). Moreover, the 7SL RNA and U-snRNA B genes are linked in the genome in a head-to-head configuration with the upstream tRNA genes sandwiched between them. In the study presented here, we have tested the hypothesis that this linkage between tRNA genes and small RNA genes is of functional significance. We show that (i) the A and B boxes of the companion tRNA gene provide extragenic regulatory elements for the U6 snRNA, 7SL RNA, and U-snRNA B genes; (ii) the $tRNA^{Thr}$ gene located upstream from the U6 snRNA gene is expressed in vivo; and (iii) the close association between tRNA genes and small RNA genes is also found in two other members of the family Trypanosomatidae, *Crithidia fasciculata* and *Leishmania pifanoi*, which are distantly related to *T. brucei* (5, 14). Taken together, these observations support the notion that the linkage of tRNA and small RNA genes is a widespread phenomenon in the trypanosomatid lineage and that the internal control regions of the small RNA-associated tRNA genes serve as bifunctional regulatory elements.

MATERIALS AND METHODS

Plasmid construction. The U6 snRNA gene contained in a 2.9-kb *Hin*dIII fragment was subcloned from a previously isolated genomic clone (24) into pT3T7. This construct (pU6FS1) contains 1.6 kb of 5′-flanking region, 98 bp of the U6 coding region, and 1.2 kb of 3′-flanking region. To mark the U6 snRNA gene, we used an *Eco*NI cleavage site at position 62 to insert a 19-nucleotide (nt)-long synthetic oligonucleotide (5′-TTCCATGGTATGGCGCCAG-3′). Deletions in the 5′-flanking region were done by using convenient restriction enzyme sites. A *Bgl*II site at position −215 was used to generate construct pU6Δ215, and a *Hae*III cleavage site at position −98 gave construct pU6Δ98. The deletion of the 3′-flanking region was generated by PCR, leaving 20 nt downstream of the putative U6 termination signal. Base substitu-

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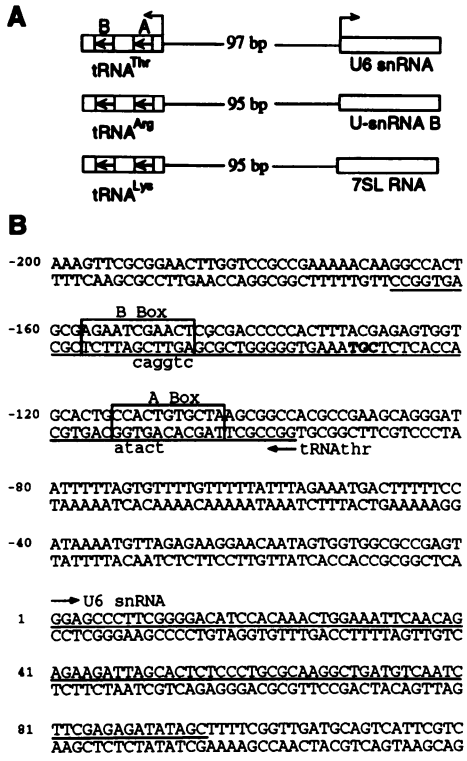

FIG. 1. Association of tRNA genes with small RNA genes in *T. brucei*. (A) Schematic representation of the locations and orientations of different genes. The large arrows indicate the transcription initiation sites, and the small arrows inside the boxes depict the orientations of the A- and B-block elements of the tRNA genes. The spacing between the small RNA and the tRNA gene is indicated. Drawings are not to scale. (B) Sequence of the *T. brucei* U6 snRNA/tRNA^{Thr} gene locus. Numbering is relative to the U6 snRNA gene transcription start site, which is taken as 1. The coding regions of the U6 snRNA and tRNA^{Thr} genes are underlined, and the direction of transcription is indicated by an arrow. The A- and B-box elements are indicated, and mutations are shown in lowercase letters under the respective box. The tRNA^{Thr} anticodon is highlighted (boldface).

tions in the A and B boxes of the tRNA^{Thr} gene in plasmid pU6Δ215 were introduced by two sequential PCRs using the following oligonucleotides (only the lower-strand sequence is shown as represented in Fig. 1B, and the mutated nucleotides are underlined): 6A-sub (5′-CCGCTTAGCACTCATACAGT GCACCAC-3′) and 6B-sub (5′-GGGGGTCGCGCTGGACA TTCTCGCAGTG-3′).

A genomic clone (λTB273) containing the 7SL RNA gene has previously been isolated in our laboratory (16). A 1-kb *Bam*HI-*Hin*dII restriction fragment was subcloned into the *Bam*HI-*Sma*I sites of pGEM-3. This construct contains 580 bp of 5′-flanking region, 273 bp of 7SL coding region, and 160 bp of 3′ flanking region. The 7SL RNA gene was marked at position 84 by the insertion of a 19-nt-long synthetic oligonucleotide (5′-TTCCATGGTATGGCGCCAG-3′) at the *Hin*dII site (pG7SL). A convenient *Alu*I site at position −107 was used to generate p7SLΔ107. Base substitutions in the A and B boxes of the tRNA^{Lys} gene were introduced as described above, using the following oligonucleotides (the mutations are underlined): 7A-sub (5′-CGGCTGCCCTTCACCATCGTACGGT AGAGCGC-3′) and 7B-sub (5′-CGTGTGGTCGTGTACGTC AGCCCCACG-3′).

A 1.1-kb *Eco*RI-*Hin*dII fragment derived from the genomic clone λTB273 (16) was subcloned into pT3T7 (construct p1.1RNAB). The U-snRNA B gene was marked at position 66 by the insertion of the sequence 5′-TTCCATGGTATGGCGC CAG-3′ by using two sequential PCRs. An *Nru*I site at position −25 was used to delete upstream sequences. The A and B elements of the tRNA^{Arg} gene were mutagenized with the following oligonucleotides (the mutations are underlined): RA-sub (5′-GATCACGTCCGTGCTCATCTCAGGAAGAG CATC-3′) and RB-sub (5′-CAGAGGGTTGCAAAGCTAAA TCCTGTCACG-3′).

To construct the suppressor tRNA, the insert of pU6Δ215 was transferred into pBluescript II KS(−), and mutagenesis was performed essentially as described previously (4, 12). In ptRNASu/am, the tRNA^{Thr} anticodon triplet was changed from CGT to CTA. In construct U6PI, following the procedure of Krieg et al. (11), the anticodon CGT of the tRNA^{Thr} gene was changed to TCA (small letters in the U6SYN-2 sequence) and a synthetic intron of 31 nt was inserted 1 nt after the anticodon (Fig. 1B) by using a pair of overlapping synthetic oligonucleotides (intron sequences are underlined): U6SYN-1 (5′-GCTGAGGCGCAGTAATCTGCGCAAAGTGGGGGT CGCGAG-3′) and U6SYN-2 (5′-CAGATTACTGCGCCT-CAGCATATTTGCtgaAGAGTGGTGCACTGCC-3′). Mutations were introduced in the A and B boxes of the intron containing tRNA^{Thr} gene as described above. The luciferase gene was expressed under the control of the PARP promoter (15). An amber stop codon was introduced into the luciferase gene by substituting the Thr codon at position 21 of the translated region with TAG. All constructs were verified by DNA sequencing using the Sequenase system (U.S. Biochemical).

Isolation of genomic clones. Genomic phage libraries from *L. pifanoi* and *C. fasciculata* (a generous gift of Diane McMahon-Pratt) were screened with ^{32}P-labeled antisense RNA probes complementary to *T. brucei* U6 and 7SL RNAs. Positive phages were characterized by restriction mapping and Southern blotting. Convenient restriction fragments were subcloned into the pT3T7 vector and sequenced by using oligonucleotide primers derived from the U6 or 7SL RNA coding region.

DNA transfection, RNA isolation, and primer extension analysis. Transient transfection of procyclic trypanosomes, RNA isolation, and primer extension analysis were done essentially as described by Fantoni et al. (4). The following RNA-specific primers were used as probes in primer extension analysis: U6-D, complementary to nt 66 to 83 of the U6 snRNA gene; 7SB, complementary to nt 101 to 120 of the 7SL RNA gene; and BR-19, complementary to nt 73 to 92 of the U-snRNA B gene. The chloramphenicol acetyltransferase (CAT) mRNA was primed with CAT-5, which is complementary to nt 26 to 39 of the coding region.

Northern (RNA) blot hybridization. Ten micrograms of total RNA was fractionated on a 6% polyacrylamide–7 M urea gel. The gel was electroblotted onto a Nytran membrane (Schleicher & Schuell) at 4°C in 0.5× Tris-borate-EDTA at 50 V for 2 h. The RNA was cross-linked to the membrane by exposure to UV light (312 nm) for 5 min. The blot was prehybridized in a buffer containing 5× SSPE (0.75 M NaCl, 50 mM NaH_2PO_4, 0.5 mM EDTA), 5× Denhardt's reagent, 0.1% sodium dodecyl sulfate (SDS), and 100 μg of tRNA per ml for at least 1 h at 37°C. The 5′-end-labeled oligonucleotides U6-D and THRINT (complementary to the synthetic intron) were added at a concentration of 10^6 cpm/ml, and hybridization was continued overnight. The blot was then washed twice in 6× SSPE–0.1% SDS for 15 min at room temperature and twice in 1× SSPE–0.1% SDS at 37°C.

Luciferase assay. Cells were collected 24 h after transfection

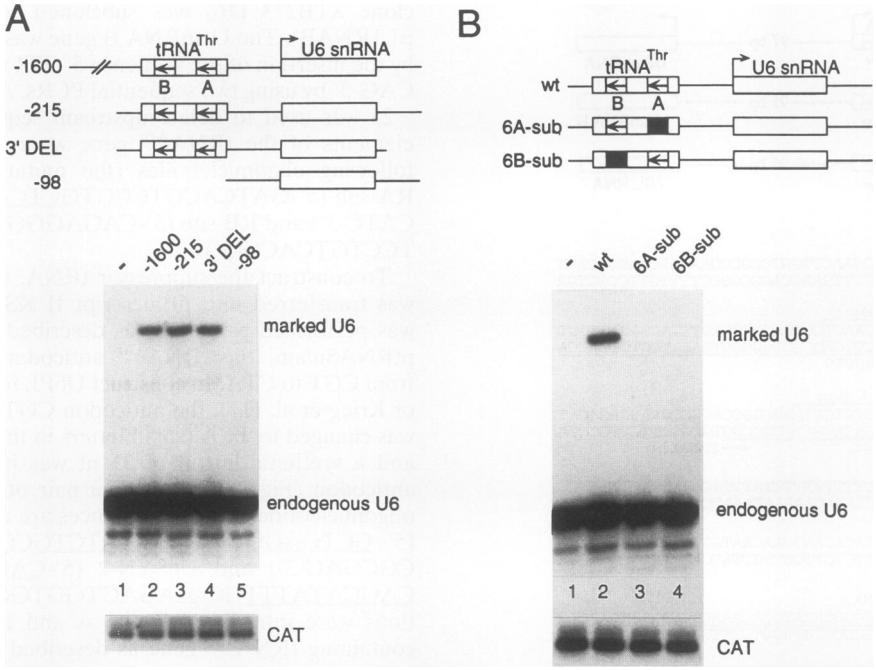

FIG. 2. U6 snRNA gene expression is dependent on upstream sequences. (A) Structures of the different constructs (not drawn to scale) are shown above the autoradiogram. Numbers refer to base pairs present upstream of the U6 snRNA gene transcription start site. Procyclic trypanosomes were transfected with the plasmid DNA shown above each lane together with the CAT control plasmid, and total RNA was subjected to primer extension analysis. The U6 snRNA was reverse transcribed with U6-D, complementary to nt 66 to 83 of the U6 snRNA gene. The cDNAs derived from the endogenous as well as from the marked transcripts are indicated. Primer extension analysis of the CAT mRNA from pJP44 (22), which served as an internal control for the efficiency of transfection, is shown below the autoradiogram. (B) Expression of the U6 snRNA gene mutated in the A and B boxes of the $tRNA^{Thr}$ gene. Mutations in the tRNA gene are indicated by black boxes, and the effects of these mutations were analyzed as described for panel A. wt, wild type.

and washed twice with a buffer containing 100 mM NaCl, 3 mM $MgCl_2$, and 20 mM Tris-HCl (pH 7.4). The pellet was resuspended in 100 mM K_2PO_4 (pH 7.8), and the samples were lysed with three cycles of freeze-thawing. The lysate was cleared by centrifugation at top speed in an Eppendorf centrifuge for 3 min. Twenty microliters of extract was added to 100 μl of reconstituted luciferase assay reagent from Promega, and luciferase activity was measured in a luminometer and expressed in light units.

RESULTS

In the genome of *T. brucei*, the genes coding for U6 snRNA, U-snRNA B, and 7SL RNA are closely linked to tRNA genes (4, 19) (Fig. 1A). The 5′ end of a $tRNA^{Thr}$ gene is located 97 bp upstream of the U6 snRNA gene, a $tRNA^{Arg}$ gene is found 95 bp upstream of the U-snRNA B gene, and a $tRNA^{Lys}$ gene is located 95 bp upstream of the 7SL RNA gene. To examine whether tRNA genes provide regulatory elements for expression of the associated small RNA genes, we first determined whether upstream sequences of the U6 snRNA, U-snRNA B, and 7SL RNA genes are required for transcription. To assay for expression, we generated a marked gene for each of the three small RNA genes by inserting a short linker sequence in the corresponding coding region. The various constructs were then cotransfected with a control plasmid (the CAT gene driven by the procyclic acidic repetitive protein gene promoter [22]) into insect-form trypanosome cells. Total cell RNA was assayed 5 to 7 h posttransfection by primer extension analysis using gene-specific oligonucleotides. Primer extension analysis with an oligonucleotide complementary to sequences downstream of the linker insertion will give two cDNA products, and the extension product derived from the marked gene will be longer by the size of the linker insertion.

The U6 snRNA gene with 1.6 kb of upstream sequences and 1.1 kb of downstream sequences was marked by inserting 19 nt at position 62 of the coding region. This construct (pU6FS1) was efficiently expressed in vivo following transfection of insect-form trypanosome cells (Fig. 2A, lane 2). Deletion of 5′-flanking sequences up to position −215 (lane 3) as well as of 3′-flanking sequences downstream of +120 (lane 4) did not affect U6 RNA accumulation. However, transfection of a construct containing only 98 bp of 5′-flanking sequences, and therefore lacking the upstream $tRNA^{Thr}$ gene (positions −99 to −167; Fig. 1B), resulted in undetectable levels of expression of the U6 snRNA gene (lane 5). We have previously shown that the promoter of the pol III-transcribed *T. brucei* U2 snRNA gene contains two extragenic elements, located 104 and 155 bp upstream, the complement of which closely resembles the consensus sequence of the A and B boxes of the internal control regions of tRNA genes (4). To test whether U6 snRNA gene expression requires the $tRNA^{Thr}$ A and B boxes at positions −104 and −147, respectively, we substituted 5 and 6, respectively, of the 11 conserved nucleotides. Both mutations reduced the accumulation of U6 snRNA to undetectable levels (Fig. 2B, lanes 3 and 4); no U6 transcripts could be detected even after longer exposures of the autoradiogram. These results clearly established that in vivo expression of the trypanosome U6 snRNA gene is dependent upon the A- and B-box elements located in the divergently oriented $tRNA^{Thr}$ gene.

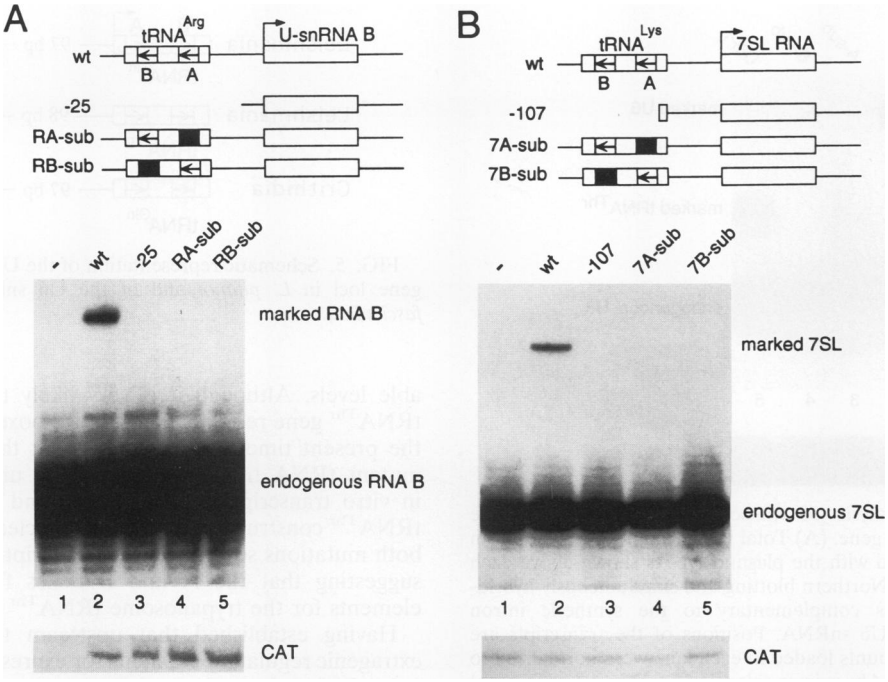

FIG. 3. Transcription of the U-snRNA B and 7SL RNA genes requires upstream A and B boxes. Total RNA was analyzed from cells transfected with the different constructs shown schematically above each autoradiogram. Drawings are not to scale. (A) U-snRNA B transcripts were reverse transcribed with BR-19, complementary to nt 73 to 92 of the U-snRNA B gene. The CAT control plasmid was omitted in lane 1. (B) 7SL RNA transcripts were primer extended with 7SB, complementary to nt 101 to 120 of the 7SL RNA gene. wt, wild type.

We next investigated the involvement of similar elements in the expression of the U-snRNA B and 7SL RNA genes, using the strategy described above for the U6 snRNA gene. As can be seen in Fig. 3A, the marked U-snRNA B is expressed in *T. brucei* cells (lane 2), and deletion of the companion $tRNA^{Arg}$ gene reduced expression of the marked U-snRNA B gene to undetectable levels (lane 3). Furthermore, when block substitutions were introduced in either the A or B box of the $tRNA^{Arg}$ gene, no U-snRNA B transcripts could be identified by primer extension analysis (lanes 4 and 5). Similarly, in a parallel set of experiments, when the $tRNA^{Lys}$ gene was deleted from the 5′-flanking region of the 7SL RNA gene, the resulting construct did not support 7SL RNA gene expression to a detectable level (Fig. 3B, lane 3), and mutations in the A- or B-box element had the same effect (lanes 4 and 5).

Taken together, our experiments showed that the A and B boxes of the companion tRNA genes provide essential regulatory elements for expression of the U6 snRNA, U-snRNA B, and 7SL RNA genes. In all three cases, the tRNA gene codes for a typical eukaryotic tRNA, as its sequence conforms in every respect to established consensus sequences and secondary structure requirements. Despite the conformity, we felt that it was important to address the possibility that these tRNA genes represent pseudogenes. To establish whether the small RNA-linked tRNA genes are transcriptionally competent, we selected the U6 snRNA gene locus for further analysis. The expression of the U6-associated $tRNA^{Thr}$ gene was assessed by two independent strategies. In our first approach, we used site-directed mutagenesis to alter the $tRNA^{Thr}$ to recognize and suppress amber (UAG) codons in vivo (construct ptRNASu/am). To test for nonsense suppression, we selected a threonine codon in the firefly luciferase gene at amino acid position 21 and changed it to the amber codon TAG (construct pLUCam21). Expression of the luciferase gene was placed under control of the PARP promoter, and nonsense suppression in *T. brucei* cells was assayed by cotransfecting various tRNA test constructs. When pLUCam21 was transfected along with the wild-type $tRNA^{Thr}$, no luciferase activity above background was detected (Table 1). However, when plasmid pLUCam21 was cotransfected with the suppressor tRNA gene (ptRNASu/am), luciferase activity was up 100-fold above background, to 0.4% of the activity of the wild-type luciferase construct (pLUC).

Since the suppressor activity described above is an indirect measure of $tRNA^{Thr}$ gene expression, in our second approach we marked the $tRNA^{Thr}$ gene by insertion of an intron-like sequence. For this experiment, we followed the strategy de-

TABLE 1. Suppression of a luciferase nonsense mutation in *T. brucei*[a]

Transfected plasmids		Luciferase activity (light units)	
Luciferase construct	tRNA construct	Expt I	Expt II
pLUC	None	12.96×10^6	5.4×10^6
None	ptRNASu/am	149	262
pLUCam21	None	446	215
pLUCam21	pU6Δ215	360	323
pLUCam21	ptRNASu/am	47,100	11,774
% Suppression		0.36	0.22

[a] Procyclic *T. brucei* cells were cotransfected with the various constructs as indicated, and luciferase activities were measured 24 h posttransfection. In seven independent experiments, the level of suppression varied between 0.1 and 0.5%, and the results of two such experiments are shown. pLUC, wild-type luciferase construct; pLUCam21, amber codon in luciferase gene; pU6Δ215, wild-type $tRNA^{Thr}$ gene; ptRNASu/am, suppressor tRNA gene.

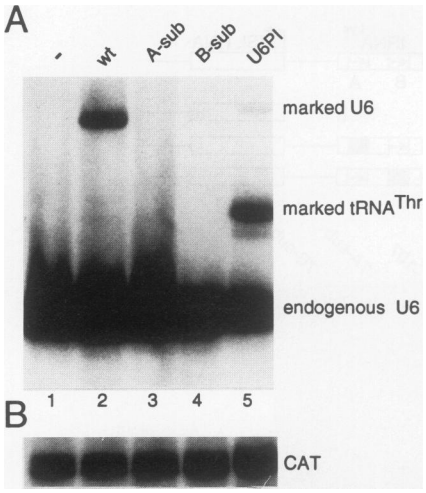

FIG. 4. Northern analysis of transcripts originating from the marked threonine tRNA gene. (A) Total RNA samples prepared from trypanosomes transfected with the plasmid DNAs shown above each lane were processed for Northern blotting and simultaneously hybridized to oligonucleotides complementary to the synthetic intron (THRINT) and to the U6 snRNA. Positions of the transcripts are indicated. The RNA amounts loaded in each lane were normalized to the CAT cDNA generated by primer extension analysis shown in panel B. Because of a gel artifact, the size of the marked U6 snRNA in lane 5 appears slightly larger than that in lane 2. wt, wild type.

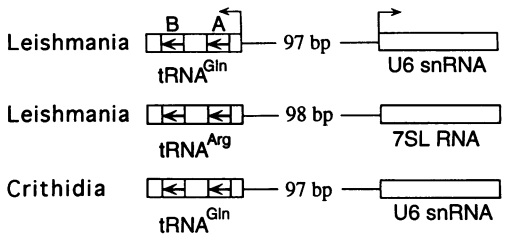

FIG. 5. Schematic representation of the U6 snRNA and 7SL RNA gene loci in *L. pifanoi* and of the U6 snRNA gene locus in *C. fasciculata*.

scribed by Krieg et al. (11) and introduced in the tRNA gene an artificial intron that cannot be spliced out. This allows accumulation of precursor tRNA molecules in vivo. We inserted a 32-nt synthetic sequence one base 3′ to the threonine anticodon triplet (CGU), at a position where a naturally occurring intron has been found in a trypanosome $tRNA^{Tyr}$ gene (21). This modification changed the spacing of the A and B boxes from 32 nt in the wild-type $tRNA^{Thr}$ gene (construct pU6Δ215) to 64 nt in construct pU6PI, which is within the optimal distance found in tRNA genes (30 to 90 bp). Expression of the intron-containing $tRNA^{Thr}$ gene was assayed by Northern blot analysis with an oligonucleotide probe complementary to the intron sequence (Fig. 4). The predicted size of the marked $tRNA^{Thr}$ is 106 nt, and the result of Fig. 4 showed that cells transfected with pU6PI produced an RNA whose size is consistent with it being the unspliced pre-$tRNA^{Thr}$ molecule (lane 5). Thus, these experiments established that the U6-associated $tRNA^{Thr}$ gene meets all of the requirements of a true gene, being expressed in *T. brucei* cells.

In the experiment of Fig. 4, we also monitored expression of the marked U6 snRNA gene present in the same construct. Although we observed a U6 transcript of the expected size (117 nt; lane 5), expression of the U6 snRNA gene was considerably reduced compared with a construct containing the wild-type $tRNA^{Thr}$ gene (compare lanes 2 and 5). It is likely that the spacing of the A and B boxes with respect to each other and to the U6 coding region or perhaps to other unidentified regulatory elements is of critical importance for U6 snRNA gene expression. This view is also supported by the high degree of conservation of the spacing between the 5′ ends of the tRNA and the associated small RNA gene (95 to 97 nt; Fig. 1A). A detailed study of the effects of changing the distances between the A and B boxes and the U6 snRNA gene is currently in progress. Finally, mutations that disrupted the A and B boxes abolished accumulation of the precursor $tRNA^{Thr}$ molecule, as well as of the marked U6 snRNA (lanes 3 and 4), to undetectable levels. Although it is very likely that the trypanosome $tRNA^{Thr}$ gene requires the A and B boxes for transcription, at the present time we cannot discount the possibility that the mutant tRNA transcripts are highly unstable. Nevertheless, in vitro transcription of wild-type and A- or B-box mutant $tRNA^{Thr}$ constructs in a HeLa cell nuclear extract showed that both mutations severely affect transcription (data not shown), suggesting that the A and B boxes function as promoter elements for the trypanosome $tRNA^{Thr}$ gene.

Having established that upstream tRNA genes provide extragenic regulatory elements for expression of three different small RNA genes, the question arose as to whether such an arrangement was peculiar to *T. brucei*. To investigate this issue, we isolated small RNA genes from other members of the family Trypanosomatidae, and in particular the U6 snRNA genes from *L. pifanoi* and *C. fasciculata* and the 7SL RNA gene from *L. pifanoi*. As schematically shown in Fig. 5, we found tRNA genes at all three gene loci, and their arrangements and orientations were identical to those found in *T. brucei* at the corresponding small RNA loci. Thus, our data demonstrate that the association of tRNA genes with small RNA genes is a widespread phenomenon in trypanosomatids and underscore the functional significance of this arrangement. However, the specific identities of the tRNA genes upstream of the U6 genes and 7SL RNA genes are different when the *T. brucei* loci ($tRNA^{Thr}$/U6 and $tRNA^{Lys}$/7SL) are compared with the corresponding loci in *L. pifanoi* ($tRNA^{Gln}$/U6 and $tRNA^{Arg}$/7SL) and in *C. fasciculata* ($tRNA^{Gln}$/U6). On the other hand, the *L. pifanoi* and *C. fasciculata* U6-associated tRNA genes are identical. It is possible that this latter observation is a reflection of the close evolutionary relationship between *L. pifanoi* and *C. fasciculata* (5, 14). How the association between tRNA and small RNA genes originated in trypanosomatids is at present only a matter of speculation. This evolutionary puzzle may become clearer once more sequences of small RNA genes from trypanosomatids and euglenoids (which presumably originated from a common ancestor) become available.

DISCUSSION

In the studies presented here, we have found that expression of the U6 snRNA, U-snRNA B, and 7SL RNA genes is dependent on A and B boxes located in an upstream tRNA gene. Furthermore, we demonstrated that the $tRNA^{Thr}$ gene linked to the U6 snRNA gene is expressed in vivo, suggesting that it represents a true gene. Although we have not formally demonstrated that this is the case for the other small RNA-associated tRNA genes, we believe that the tRNA genes upstream of the 7SL RNA and U-snRNA B coding regions are also functional. On the basis of the experiments reported here and of what is known about the requirements for pol III transcription of tRNA genes (reviewed in references 8 and 26),

we propose that the A and B boxes of the $tRNA^{Thr}$ gene have a dual role: they are bona fide promoter elements for the tRNA gene itself and are also required for expression of the companion U6 snRNA gene. At present, however, we cannot distinguish whether these elements affect U6 transcription directly via binding of transcription factors shared with the tRNA gene or indirectly by virtue of their requirement for transcription of the companion tRNA gene. Indeed, transcriptionally active tRNA genes exclude nucleosome assembly in their vicinities (18), and factor TFIIIC binding to the downstream B-box element of the yeast U6 snRNA gene interferes with assembly of an inhibitory chromatin structure (2). Thus, it is possible that similar mechanisms operate in trypanosomes and that the assembly of a functional transcription complex on the $tRNA^{Thr}$ gene is a prerequisite for transcription of the associated U6 snRNA gene. As mentioned above, another possibility is that the intragenic control region of the $tRNA^{Thr}$ gene functions in U6 snRNA gene transcription via binding of transcription factors. However, the fact that the tRNA and U6 snRNA genes are divergently oriented makes it difficult to rationalize how the A and B boxes might function in this scenario. This is because the assembly of transcription complexes at the A and B boxes takes place in an orientation-dependent manner; that is, initiation is always upstream from the A box. To circumvent this problem, we can hypothesize that upon binding of the transcription factors to the tRNA gene, the DNA separating the two genes is bent in such a way that the 5′ ends of the tRNA and U6 snRNA coding regions point in the same direction, and the bound transcription factors and the RNA polymerase can now interact in the proper orientation with the transcription start site of the U6 snRNA gene. Whatever the mechanism, it is remarkable that tRNA genes are endowed with yet another genetic function.

The requirement of A- and B-box elements for transcription of U6, 7SL, and U-snRNA B genes is reminiscent of what we have described for the pol III-transcribed *T. brucei* U2 snRNA gene (4). In this case, however, an additional promoter element located intragenically is required for positioning pol III at the correct start site. Results to be reported elsewhere indicate that this is also the case for the U6 snRNA gene (20). Thus, at first it would appear that the building blocks of the U2 and U6 snRNA promoters are similar. One major difference is the presence of a bona fide tRNA gene upstream of the U6 snRNA gene, whereas we can recognize only the vestiges of a tRNA gene at the U2 locus. It is possible that the ancestral U2 snRNA gene in trypanosomatids was associated with a tRNA gene which over evolutionary time accumulated deleterious mutations in the coding region with the exception of the A- and B-box elements.

The organization of small RNA genes in trypanosomes is reminiscent of divergently transcribed genes in other eukaryotic organisms. For instance, the genes encoding the enzymes responsible for galactose utilization have been extensively studied in the yeast *Saccharomyces cerevisiae*. Two of these genes, *GAL1* and *GAL10*, are divergently transcribed from a common promoter, and their activation is regulated through a complex array of multiple positive and negative control elements located in the intergenic region (6, 10, 25). In addition, two avian genes involved in de novo synthesis of purine nucleotides are controlled from a bidirectional promoter (7). However, genetic linkage is not a prerequisite for coordinate expression of a set of genes. For instance, when *Escherichia coli* is deprived of one or more amino acids, the synthesis of rRNA and tRNA is reduced 10- to 20-fold by a phenomenon called stringent response (13). A similar control mechanism has also been identified in *S. cerevisiae*, whereby amino acid starvation leads to increased expression of more than 30 biosynthetic enzymes (17). The genes characterized in our studies encode small stable RNAs that are required by the cell for essential functions, namely, RNA processing (U6 snRNA), translation (tRNAs), and targeting of membrane proteins to the endoplasmic reticulum (7SL RNA). It is tempting to speculate that the unorthodox promoter structure of these genes reflects regulatory strategies to coordinately express key components of crucial cellular processes.

Finally, it should be noted that we have constructed a mutant $tRNA^{Thr}$ gene that can function as a nonsense suppressor in *T. brucei*. Although the level of suppression was only 0.3%, it falls well within the range of suppression efficiencies measured in other eukaryotic systems (0.04 up to 50%). This genetic system not only will prove useful in the conditional expression of proteins but also provides a biological assay for a pol III-transcribed gene.

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