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tRNA^{Sec} is transcribed by RNA polymerase II in *Trypanosoma brucei* but not in humans

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ABSTRACT

Nuclear-encoded tRNAs are universally transcribed by RNA polymerase III (Pol-III) and contain intragenic promoters. Transcription of vertebrate tRNA^{Sec} however requires extragenic promoters similar to Pol-III transcribed U6 snRNA. Here, we present a comparative analysis of tRNA^{Sec} transcription in humans and the parasitic protozoa *Trypanosoma brucei*, two evolutionary highly diverged eukaryotes. RNAi-mediated ablation of Pol-II and Pol-III as well as oligo-dT induced transcription termination show that the human tRNA^{Sec} is a Pol-III transcript. In *T. brucei* protein-coding genes are polycistronically transcribed by Pol-II and processed by *trans*-splicing and polyadenylation. tRNA genes are generally clustered in between polycistrons. However, the trypanosomal tRNA^{Sec} genes are embedded within a polycistron. Their transcription is sensitive to α-amanitin and RNAi-mediated ablation of Pol-II, but not of Pol-III. Ectopic expression of the tRNA^{Sec} outside but not inside a polycistron requires an added external promoter. These experiments demonstrate that trypanosomal tRNA^{Sec}, in contrast to its human counterpart, is transcribed by Pol-II. Synteny analysis shows that in trypanosomatids the tRNA^{Sec} gene can be found in two different polycistrons, suggesting that it has evolved twice independently. Moreover, intron-encoded tRNAs are present in a number of eukaryotic genomes indicating that Pol-II transcription of tRNAs may not be restricted to trypanosomatids.

INTRODUCTION

All eukaryotes have at least three RNA polymerases (Pol-I, Pol-II and Pol-III) that are specialized to transcribe different sets of genes (1). With few exceptions, Pol-I synthesizes the rRNAs whereas Pol-II produces mRNAs, small nuclear (sn) RNAs, small nucleolar (sno) RNAs and microRNAs. Nuclear encoded tRNAs, on the other hand, are believed to be exclusively and universally transcribed by Pol-III, which also synthesizes 5S rRNA, U6 snRNA and some other small RNAs.

Transcription of most eukaryotic tRNA genes does not require control sequences outside of the gene but depends on two intragenic promoter elements termed Box A and B. However, the tRNA that specifies selenocysteine (Sec)—the focus of our study—represents an exception. tRNA^{Sec} mediates insertion of the rare amino acid Sec in response to a small number of UGA stop codons that have been recoded to Sec by the presence of a SECIS element in the 3′ UTR of a mRNA (2,3). Sec-containing proteins, termed selenoproteins, occur in all three domains of life but during evolution have been lost in some clades such as fungi and plants. Transcription of tRNA^{Sec} has so far only been studied in vertebrates, and in contrast to other tRNAs it was shown to depend on three upstream regulatory regions: the TATA box motif at −30, the PSE (proximal sequence element) located around position −70 and a distal AE (activator element) located further upstream (4–7). A consensus intragenic B box can still be found whereas a functional A box is absent (Supplementary Figure S3B). The PSE and AE promoter elements are not restricted to the tRNA^{Sec} gene but also occur in spliceosomal snRNA genes that are either transcribed by Pol-III (U6 snRNA) or Pol-II (U1, U2, U4 and U5 snRNAs), respectively. The PSE and the AE are known to recruit the same set of basic transcription factors for either Pol-III or Pol-II transcribed genes (8,9).

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Thus, it is the presence or absence of the TATA box that determines which polymerase transcribes these genes. If the TATA box is absent the gene is transcribed by Pol-II whereas if it is present, such as in the case of the $tRNA^{Sec}$ and U6 snRNA genes, it will be transcribed by Pol-III (10).

The notion that Pol-II and Pol-III independently transcribe distinct set of genes has recently been challenged. Chromatin immunoprecipitation studies have shown that in human cells Pol-II is physically associated with the upstream regions of the Pol-III transcribed U6 snRNA genes (11). Moreover, while U6 snRNAs were clearly transcribed by Pol-III their expression was inhibited by inactivation of Pol-II. More recently these results were confirmed by genome-wide study that showed that the association of Pol-II with the upstream region of Pol-III transcribed genes is not restricted to the U6 snRNA genes, but is also found for many other Pol-III transcribed genes, including tRNA genes. Furthermore, the study also showed that the same is true for many transcription factors that are usually associated with Pol-II transcription (12).

We have previously shown that the machinery and the mechanism of Sec-insertion into selenoproteins is conserved between mammals and the protozoan parasite *Trypanosoma brucei* (13). According to the recently revised eukaryotic phylogeny, eukaryotes are divided into six supergroups (14,15). Mammals and most of the popular model organisms such as yeast, *Drosophila* and nematodes belong to the supergroup of the *Opisthokonta*, whereas *T. brucei* is a representative of the supergroup of the *Excavata*. This suggests that the Sec-insertion machinery is conserved within eukaryotes. Thus, we wondered whether the same is true for the unusual way in which the $tRNA^{Sec}$ is transcribed. Transcription in *T. brucei* shows some substantial differences when compared to other eukaryotes (16–18). Trypanosomes have orthologs of all three eukaryotic polymerases (19). However, in addition to rRNAs, Pol-I also transcribes the mRNAs that code for the variant surface glycoproteins and for procyclins, the two major stage-specific surface proteins (20). Moreover, protein-coding genes are arranged in large clusters that are co-transcribed by Pol-II. Polycistronic transcription results in mRNA precursors that need to be processed into individual mature mRNAs, whereby the 5′-ends of mRNAs are formed by *trans*-splicing (21,22). Thus each open reading frame (ORF) of a polycistronic pre-mRNA has at least one 5′-splice acceptor site consisting of an AG dinucleotide that is preceded by a polypyrimidine tract of variable length. Both of these elements mediate the *trans*-splicing reaction in which a capped spliced leader (SL) sequence of 39 nt is added to the 5′-end of each mRNA [for review see (23)]. As a result, all mature mRNAs in *T. brucei* have an identical 39-nt SL-sequence at their 5′-end. To complete mRNA processing addition of a poly(A) tail is required. There is no consensus polyadenylation site in the 3′-untranslated region, rather polyadenylation occurs within a short region 100–400 nt upstream of the *trans*-splice acceptor site of the downstream gene. As in other organisms trypanosome Pol-III transcribes tRNA genes, which contain intragenic box A and B promoter elements. Unlike in other organisms, all U snRNA genes are transcribed by Pol-III, and the A and B boxes of a few tRNA genes, in addition to promoting transcription of the tRNAs, also function as upstream promoter elements for the U3 and U6 snRNA genes as well as for the 7SL RNA gene. Interestingly, in these cases the tRNAs are transcribed in the opposite directions than the three genes mentioned above (24).

In this study we performed a comparative analysis of $tRNA^{Sec}$ transcription in human cells and in *T. brucei*. We confirm that the human $tRNA^{Sec}$ is transcribed by Pol-III. However, transcription of the $tRNA^{Sec}$, unlike the otherwise very similar Pol-III-mediated transcription of the human U6 snRNA genes, does not seem to require Pol-II. Surprisingly and in contrast to all other tRNAs studied so far, transcription of the $tRNA^{Sec}$ of *T. brucei*, is mediated by Pol-II, indicating that the mode of transcription of the $tRNA^{Sec}$ is not conserved within eukaryotes.

MATERIALS AND METHODS

Production of transgenic HeLa cells

Human $tRNA^{Sec}$ (Chr. 19, GeneID 7234) and $tRNA^{Tyr}$ (accession M55612.1) with and without T-stretch insertion (Figure 1) were overexpressed in HeLa cells using the pGEM-T Easy Vector (Promega). Each tRNA gene was expressed carrying 600 bp of its own 5′ and 300 bp of its own 3′ flanking region. HeLa cells were grown at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin. Transgenic cells were obtained by seeding 1.5×10^5 HeLa cells each into six-well plates. Transfection was done the next day using 500 ng each of the tRNA expression plasmids mixed with 250 ng of the pcDNA3.1 plasmid (Invitrogen) containing an insert encoding HA-EGFP and 6 µl each of DreamFect (OZ Biosciences). One day later the efficiency of transfection (~60%) was checked by monitoring GFP expression and total RNA was isolated as described (25). The RNAi plasmids were generated by insertion of double-stranded oligonucleotides (19 bp in length) encoding short hairpin RNAs into the pSUPERpuro vector between the BglII and HindIII sites as described previously (26). Four RNAi constructs were produced: two of them were directed against either the sequence 5′TAAAGAAGGTGAAGAACAA3′ or the sequence 5′ACATAAAGATCCCGAACAA3′ of the core subunit of human Pol-II (NM_000938.1) and two other ones targeting the core subunit of human Pol-III (NM_007055.2), either the region 5′GAGGAAATCTCT CAGGAAA 3′ or the region 5′ACGCTGAGACAGTGA GATA3′, respectively. Transgenic HeLa cells were obtained by seeding 2×10^5 cells each into six-well plates. Transfection was done the next day using 400 ng of each of the RNAi plasmids and 4 µl each of DreamFect (OZ Biosciences). One day later the cells were put under antibiotic selection by addition of 1.5 µg/ml puromycin. After 2 days of selection the antibiotic was removed and

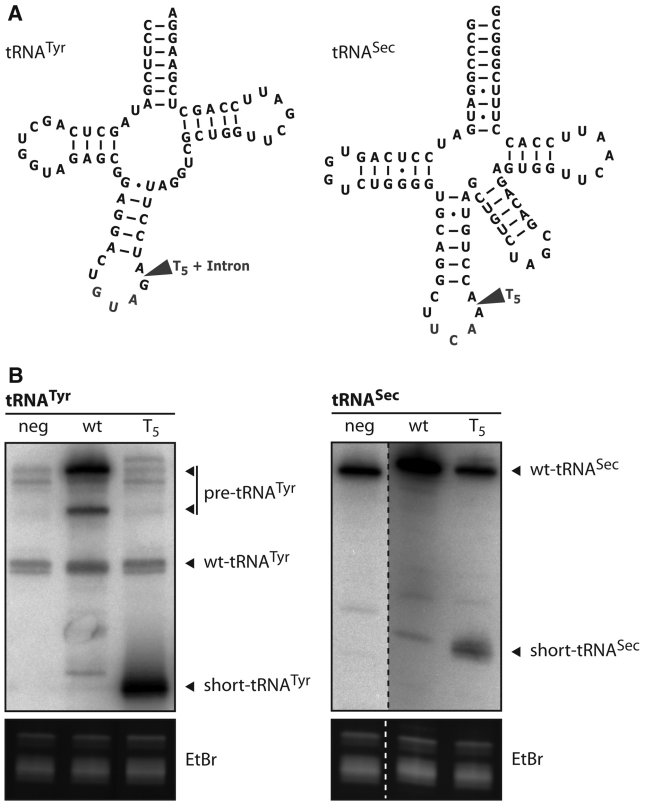

Figure 1. An intragenic T-stretch abolishes transcription of full-length human $tRNA^{Sec}$. (**A**) Predicted secondary structure of the human $tRNA^{Tyr}$ and $tRNA^{Sec}$, respectively. Arrows indicate the positions where a synthetic Pol-III termination signal consisting of five adjacent thymidine (T_5) has been inserted into the genes of the indicated tRNAs. In the case of the $tRNA^{Tyr}$ gene this position also corresponds to the position of the intron. (**B**) Left panel, total RNA samples isolated from control cells (neg), from transgenic cell lines overexpressing wild-type $tRNA^{Tyr}$ (wt) or the $tRNA^{Tyr}$ variant carrying the T-stretch were analyzed for the presence of $tRNA^{Tyr}$ by specific oligonucleotide hybridization on northern blots. The positions of the intron-containing $tRNA^{Tyr}$ (pre-$tRNA^{Tyr}$), the mature wild-type $tRNA^{Tyr}$ (wt-$tRNA^{Tyr}$) and the abortive transcript corresponding to the 5′-half of the $tRNA^{Tyr}$ (short-$tRNA^{Tyr}$) are indicated. Right panel, same as in the left panel but analysis was done for the human $tRNA^{Sec}$. The positions of the wild-type (wt-$tRNA^{Sec}$) and the abortive transcript corresponding to the 5′-half of the $tRNA^{Sec}$ (short-$tRNA^{Sec}$) are indicated. For the experiments on the left panel the cell line expressing the wild-type-$tRNA^{Sec}$ was used as a negative control (neg) whereas for the experiments on the right panel the control consisted of the cell expressing the wild-type $tRNA^{Tyr}$. The bottom panels serve as loading controls and show the ethidium bromide stained gel segment that contains the tRNAs.

after another 24 h of incubation total RNA was isolated as described above.

The relative RNAi-induced downregulation of Pol-II and Pol-III mRNAs when compared to the Pol-I transcribed 18S rRNA was quantified using real-time quantitative PCR (qPCR). One microgram of total RNA was reverse transcribed in 20 µl StrataScript 6.0 RT buffer containing 1 mM dNTPs, 300 ng random hexamers, 40 U RNasin (Promega), and 50 U StrataScript 6.0 reverse transcriptase (Stratagene) according to manufacturer's protocol. Reverse transcribed material corresponding to 25 ng RNA was amplified using Brilliant® II Fast SYBR® Green QPCR Master Mix (Stratagene) and the following primers: for Pol II 5′CCAGAGCTGGAGTATCTCAGGTGTT3′ (forward) and 5′TTGCTAGCTTGCCGTCTCTACC3′ (reverse); for Pol III 5′GCTGGCTCCTGTCTACCTGT CTAAC3′ (forward) and 5′CTTGTAGCCGGCATTCA GCA3′ (reverse); 18S rRNA was detected using a commercial available TaqMan® Gene Expression Assay (Applied Biosystems, catalog no. 431-9413E) and Brilliant® II Fast QPCR Master Mix (Stratagene).

Transcription in permeabilized *T. brucei* cells

Transcription was analyzed using *T. brucei rhodesiense* Ytat 1.1 maintained at 28°C in SM medium containing 10% fetal bovine serum. We used the Ytat 1.1 strain since the analysis of transcription in permeabilized cells was originally established in this strain. Lysolecithin-permeabilized cells (27) were incubated with ^{32}P-labeled UTP or CTP for 15 min at 28°C. Subsequently labeled RNA was isolated as described (25) and hybridized to denatured DNA spotted onto nitrocellulose membrane. Each spot contained 5 µg of DNA: the $tRNA^{Sec}$ gene and the $tRNA^{Ile}$ gene were cloned into pTZ18U, the U6 snRNA, the tubulin and the SL genes were prepared as described (27). Membranes were hybridized overnight at 68°C in an aqueous buffer (5× SET, 10× Denhardts, 1% SDS, 10 µg/ml yeast RNA), and then washed three times for 30 min each at 68°C in 2× SSC and 0.1% SDS. Blots were exposed to a PhosphorImager screen, developed and analysed using OptiQuant software (Perkin Elmer).

Production of transgenic *T. brucei* cells

Pol-III activity was ablated by RNAi using a stem loop construct based on a pLew 100 (28) derivative containing the puromycin resistance gene (29). As insert we used a 480-bp fragment (nt 301–780) of the largest subunit of trypanosomal Pol-III (Tb10.70.4870). The RPB9 RNAi cell line allowing ablation of Pol-II activity was obtained from L. Vanhamme (30). Ectopic expression of tagged $tRNA^{Sec}$ and $tRNA^{Met-i}$ (Figure 4) was based on the same pLew100 derivative. The tagged tRNA genes were prepared by PCR mediated site directed mutagenesis. The $tRNA^{Sec}$ gene encoded on the shorter intergenic region (Figure 3) was expressed in the context of 308 nt of its own 5′ and 205 nt of its own 3′-flanking region. $tRNA^{Met-i}$ served as a control; it was expressed in the context of 85 bp of its own 3′-flanking region and on the 5′-side was fused to 268 bp of the 5′-flanking region of a trypanosomal $tRNA^{Leu}$. It has previously been shown that the tagged $tRNA^{Met-i}$ can efficiently be expressed in this genomic context (31). The inserts of all constructs were verified by sequencing. All transgenic cell lines are based on procyclic *T. brucei* 29-13 that was grown at 27°C in SDM-79 (32) supplemented with 15% FCS and the required antibiotics. Transformation, cloning and selection of transgenic cell lines were done as described (33).

RT–PCR analysis in *T. brucei*

RT analysis to determine the splice acceptor and polyadenlyation sites was performed using the

ImProm-II system (Promega) following the manufacturers procedure. Primers for the 3′-RACE of the Tb09.160.1090 encoding cDNA were: 5′TTGAATTCGCATTGAGCAC CTGCTTTTTTTTTTTTTTTTTTVNN3′ (1. PCR, reverse), 5′TTGAATTCGCATTGAGCACCTGC3′ (2. PCR, reverse), 5′AAGAGCTAGAAGCACGCGG3′ (1. PCR, forward), 5′ACCAATTTCTTCATCCATTCACA 3′ (2. PCR, forward). Primers for the 5′-RACE of the Tb09.160.1070 encoding cDNA: 5′TGAAACTCCATGT ATTGCCGC3′ (1. PCR, reverse), 5′CTGAGGGACGAC AGAGCG3′ (2. PCR, reverse), 5′CGCTATTATTAGAA CAGTTTCTGTAC3′ (1. And 2. PCR forward).

Northern analysis

Denaturing polyacrylamide gels (Figures 1, 2, 4 and 6) were processed for northern blot analysis as described (34,35). The indicated ^{32}P-5′-end labeled oligonucleotides were used as probes (*T. brucei*: Tb, *H. sapiens*: Hs): 5′ AC CAGCTGAGCTCATCGTGGC3′(Tb-tRNA^{Sec}), 5′TAC GGGGTTGAATCCCGCA3′ (Tb-tagged tRNA^{Sec}) 5′T GCTCCCGGCGGGTTCGAA3′ (Tb-tRNA^{Ile}), 5′CGCT CTTCCCCTGAGCCA3′ (Tb-tagged tRNA^{Met-i}), 5′CA GATTCCCGCAGTATGCGG3′ (Tb-SL RNA), 5′ACC ACTGAGGATCATCCGGGC3′ (Hs-tRNA^{Sec}), 5′GCT CTACCAGCTGAGCTATCG3′ (Hs-tRNA^{Tyr}), 5′GTAT ATGTGCTGCCGAAGCGAG3′ (Hs-U6 snRNA), 5′GT GCACCGTTCCTGGAGGTACT3′ (Hs-U2 snRNA). Signals were quantitated as described above.

RESULTS

An intragenic T-stretch abolishes transcription of full-length human tRNA^{Sec}

Transcription of vertebrate tRNA^{Sec} and the multiple U6 snRNA genes (36) is very similar: both require a TATA box, the PSE and the AE and share several transcription factors. However, in the case of the tRNA^{Sec}

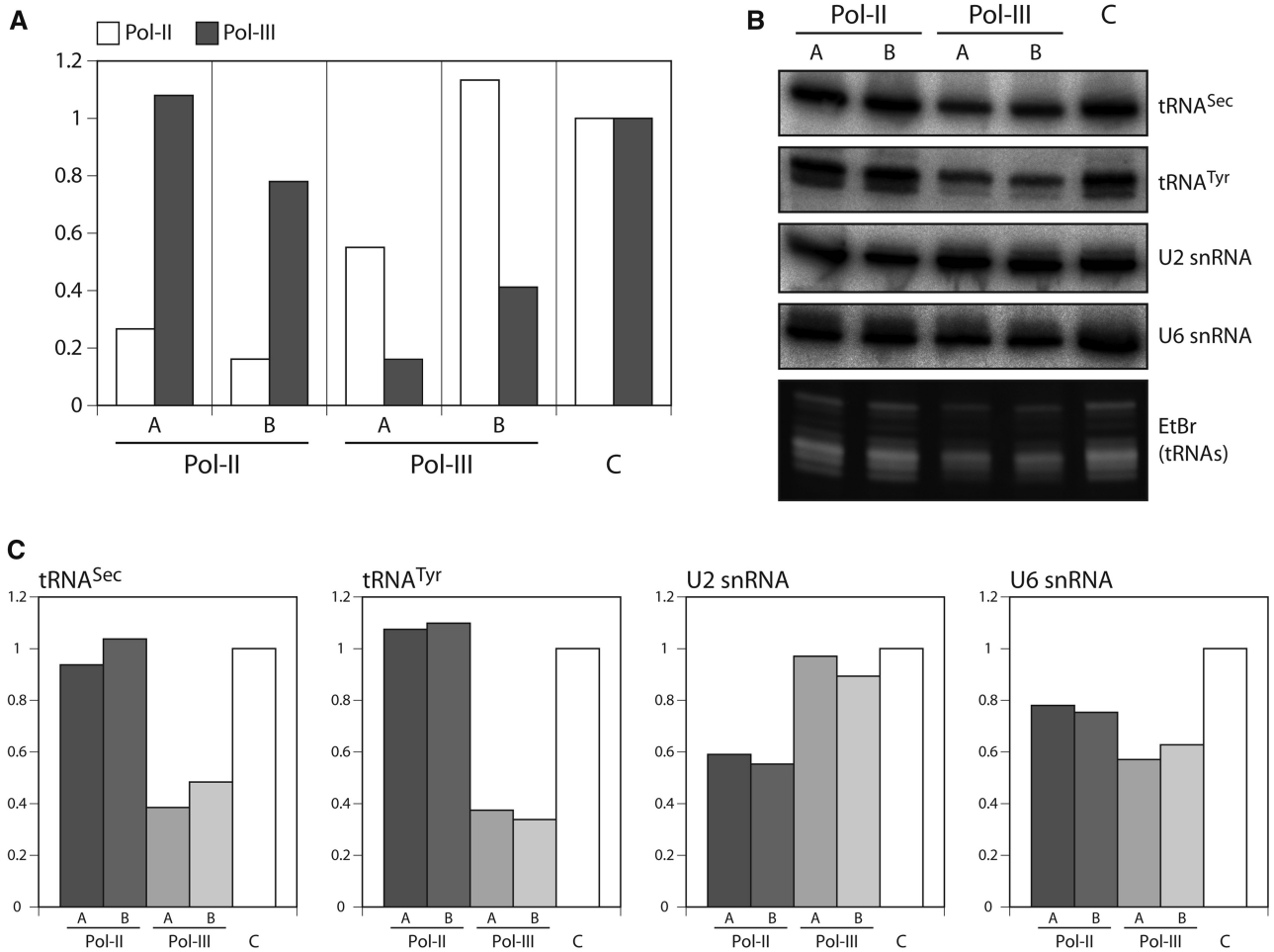

Figure 2. RNAi of Pol-III inhibits expression of human tRNA^{Sec}. (**A**) Real-time qPCR analysis of the mRNA levels of the core subunit of Pol-II (white bars) or Pol-III (grey bars) from HeLa cells transfected with either the empty plasmid (C) or with plasmids expressing shRNAs targeting the mRNAs of the core subunit of Pol-II or Pol-III, respectively. For both RNA polymerases, cells were transfected with two independent shRNA expressing plasmids that target distinct regions (A and B) of the corresponding mRNAs. All mRNAs levels have been normalized to the level of the 18S rRNA. The levels of mRNAs encoding the core subunits of Pol-II and Pol-III in the cell line transfected with the empty plasmid (C) was set to 1. (**B**) Northern analysis of 4 µg of total RNA isolated from control cells and from cells undergoing RNAi. Four days after transfection total RNA was isolated and analyzed for the presence of the indicated RNAs by specific oligonucleotide hybridization. The bottom panels serve as loading controls and show the ethidium bromide stained gel segment that contains the tRNAs. (**C**) Quantification of the results shown in (B). The signal in the control cells (C) that do not express shRNAs was set to 1.

surprisingly little attention has been paid to the question whether it is transcribed by Pol-III. Early experiments showed that transcription of the $tRNA^{Sec}$ is resistant to 1 μg/ml of α-amanitin suggesting that it is transcribed by Pol-III (4,6). However, it was not shown that under these conditions Pol-II-dependent transcription was inhibited. Moreover, it is known that short T-stretches act as termination signals for Pol-III. Consequently, tRNA genes are generally flanked at their 3′-end by short T-stretches. However, in case of the human $tRNA^{Sec}$ the first T-stretch (T_4) occurs only 45-nt downstream of the 3′-end of the gene. Finally, recent studies with human U6 snRNA have shown that even though its transcription is mediated by Pol-III, it also depends on active Pol-II (11). In summary, these observations underscore the complexity of $tRNA^{Sec}$ and U6 snRNA transcription modes and prompted us to reinvestigate whether human $tRNA^{Sec}$ is indeed transcribed by Pol-III. Practically this was achieved by using novel methods that for the most part were not available at the time the pioneering studies mentioned above were performed (4,6).

In order to identify the polymerase responsible for transcription of human $tRNA^{Sec}$, we designed a $tRNA^{Sec}$ gene variant containing a Pol-III termination signal consisting of five thymidine residues at position 37 in the region of the tRNA that encodes the anticodon loop (Figure 1A). If Pol-III transcribes the variant $tRNA^{Sec}$, transcription should prematurely terminate within the T-stretch, whereas if the tRNA is transcribed by Pol-II the full-length tRNA carrying the T-stretch insertion should be obtained. To show that the assay works as intended, we also designed a human $tRNA^{Tyr}$ gene variant containing five thymidines at the same relative position as in the variant $tRNA^{Sec}$ gene (Figure 1A). In the $tRNA^{Tyr}$ gene, this position coincides with the 5′-end of its intron. Constructs containing the corresponding wild-type tRNA genes and their variants containing the synthetic T-stretch insertions were transfected into HeLa cells. Twenty-four hours later expression of the transgenes was analyzed by northern blotting. Overexpression of wild-type $tRNA^{Tyr}$ gene resulted in the accumulation of large amounts of intron-containing precursor $tRNA^{Tyr}$ and, to a lesser extent, of the spliced form (Figure 1B). The accumulation of precursor rather than mature $tRNA^{Tyr}$ is most likely caused by overloading of the tRNA splicing machinery. Overexpressing the $tRNA^{Tyr}$ variant, on the other hand, resulted in the accumulation of large amounts of a shorter fragment, whose size is consistent with a molecule representing the 5′-half of the $tRNA^{Tyr}$, extended by an unknown number of T residues derived from termination within the T-stretch insertion (Figure 1B), as expected for a Pol-III transcript, The analogous experiments were also done for the human $tRNA^{Sec}$. The extent of overexpression was less than what was observed for the $tRNA^{Tyr}$. However, also in this case the T-stretch containing $tRNA^{Sec}$ variant gene led to the accumulation of a short fragment, whose length also corresponds to the expected length of the 5′-half of the $tRNA^{Sec}$ carrying the T-stretch nucleotides. Furthermore, no band corresponding to the full length $tRNA^{Sec}$ containing the added 5-nt long T-stretch was detected. These results show that human $tRNA^{Sec}$ responds to a typical Pol-III termination signal and therefore suggest that it is transcribed by Pol-III.

RNAi of Pol-III inhibits expression of human $tRNA^{Sec}$

It recently became clear that both Pol-III and Pol-II are required for efficient expression of the human U6 snRNA genes (11). In order to analyze whether this might also be the case for the human $tRNA^{Sec}$ gene, we performed RNAi experiments. HeLa cells were transfected with constructs allowing overexpression of short hairpin (sh) RNAs designed to induce RNAi-mediated ablation of the Pol-II or Pol-III core subunits, respectively. For each of the polymerases, the knockdown was performed independently with two shRNA expressing plasmids targeting different regions of the corresponding mRNAs. Following transfection, the cells were incubated for two days in the presence of puromycin in order to enrich for cells expressing the transgenic shRNAs. Figure 2A shows that, as expected, the Pol-II or Pol-III mRNA levels were specifically downregulated in the corresponding RNAi cell lines but not in cells transfected with a control plasmid. Pol-II and Pol-III are essential, and RNAi directed against the corresponding mRNAs is therefore expected to be lethal. Thus, RNA was isolated and analyzed 4 days after transfection at the time point when the cells were still completely viable. Figure 2 shows that ablation of Pol-II did neither affect the level of $tRNA^{Sec}$ nor the one of $tRNA^{Tyr}$. However, consistent with the notion that both tRNAs are transcribed by Pol-III their levels dropped to ~40% in cell lines downregulated for Pol-III. As control for a Pol-II transcript we analyzed the levels of the U2 snRNA which were reduced to ~55% in the Pol-II RNAi cell lines but unaffected by ablation of Pol-III. Finally, we also analyzed the Pol-III transcribed U6 snRNA. Interestingly, its expression was reduced to ~45% in Pol-III RNAi cell lines and also by 25% in the Pol-II RNAi cells. This latter result supports the recent suggestion that while human U6 snRNA is transcribed by Pol-III, its expression also requires active Pol-II (11). Moreover, the results confirm that human $tRNA^{Sec}$ is transcribed by Pol-III and suggest that, while there are many shared features between the transcription mode of the $tRNA^{Sec}$ and the U6 snRNA, the requirement for an active Pol-II is not one of them.

Unique $tRNA^{Sec}$ gene loci in *T. brucei*

Previous work has shown that the machinery and the mechanism of Sec-insertion into selenoproteins is conserved between mammals and *T. brucei* (13). This raises the question whether this conservation also extends to the transcription of $tRNA^{Sec}$ genes. The large majority of trypanosomal tRNA genes occurs in clusters of two to five or more genes separated by short intergenic regions. Within clusters, tRNA genes can be arranged in head-to-head, tail-to-tail or head-to-tail orientations (34,37). Trypanosomal tRNA gene clusters are confined to genomic regions of at least 5 kb in length which are devoid of protein-coding genes (this is also true for the few tRNA genes that do not occur in clusters). Some,

but not all, tRNA gene-containing loci coincide with strand switch regions where the transcription of two polycistronic transcription units either converges or diverges (38). The *T. brucei* genome encodes two identical $tRNA^{Sec}$ genes, which unlike all other tRNAs are found in the intergenic regions (1640 and 833 bp in length, respectively) between three adjacent protein-coding genes of a polycistronic transcription unit (Figure 3).

Sequence analysis indicates that the trypanosomal $tRNA^{Sec}$ genes appear to lack consensus sequences for the internal A and B boxes, as well as any upstream Pol-III promoter elements. The peculiar genomic arrangement, together with the apparent absence of functional promoter elements, suggest that the trypanosomal $tRNA^{Sec}$ genes might be transcribed as part of a polycistronic RNA in conjunction with the protein-coding genes.

This is further supported by the fact that no histone variants, that mark the boundaries of polycistronic transcription units in *T. brucei*, are found in chromatin associated with the $tRNA^{Sec}$ genes (39).

To analyze the situation in more detail for the $tRNA^{Sec}$ gene situated within the shorter intergenic region we determined the polyadenylation site of the upstream mRNA and the splice acceptor site of the downstream mRNA, respectively (Figure 3). Moreover, using RT–PCR we showed that the whole intergenic region together with the flanking ORFs is transcribed as one RNA molecule (Supplementary Figure S1). Interestingly, deep sequencing of poly(A)-enriched SL-containing cDNA libraries of procyclic and bloodstream *T. brucei* revealed a small number of molecules consisting of the SL sequence attached to the 5′-flanking region of the two $tRNA^{Sec}$ genes (data not shown). This suggests that the transcripts that contain the $tRNA^{Sec}$ are processed from a polycistronic precursor like mRNAs by *trans*-splicing and polyadenylation. The resulting *trans*-spliced RNA fragments could then be further processed like other tRNA precursors by RNAse P and a combination of endo- and exonucleases, respectively. This situation would be reminiscent of the Pol-II-transcribed snoRNAs of other organisms that are frequently encoded in introns and nucleolytically processed after splicing (40).

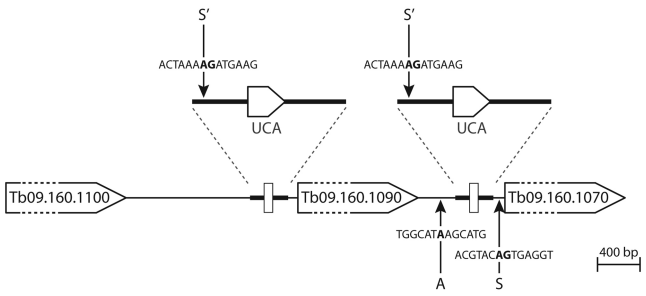

Figure 3. Schematic illustration of the two trypanosomal $tRNA^{Sec}$ genes and their genomic context (drawn to scale). The two $tRNA^{Sec}$ genes including the flanking sequences indicated in bold are identical. Tb09.160.1090 encodes a putative serine/threonine protein kinase, the two other ORFs are annotated as hypothetical proteins of unknown function. The position and sequence of the polyadenylation site (for Tb09.160.1090) and the splice acceptor site (for Tb09.160.1070) as determined by 3′ and 5′ RACE are indicated by A and S, respectively. The functional splice acceptor site detected by deep sequencing of a poly(A) enriched SL-containing cDNA library of procyclic and bloodstream *T. brucei* is indicated by S′.

Ectopic expression of trypanosomal $tRNA^{Sec}$ requires an external promoter

If the trypanosomal $tRNA^{Sec}$ genes lack a Pol-III promoter and instead are transcribed by Pol-II, they should be transcriptionally silent when integrated outside of a polycistron. To test this prediction we integrated a tagged version of one of the two $tRNA^{Sec}$ genes (Figure 4A) including its flanking regions and an upstream tetracycline (tet)-inducible Pol-I promoter into a non-transcribed intergenic rDNA region (Figure 4B). Figure 4C shows that in this genomic context expression of the tagged $tRNA^{Sec}$ depended on the presence of tet, indicating that this gene does not contain an internal promoter. A weak signal is detected in uninduced cells, which could be due to leaky Pol-I transcription in the absence of tet. It should be mentioned here that the intensity of this signal was always very weak and for unknown reasons somewhat variable. Supplementary Figure S4 shows an example of an experiment where $tRNA^{Sec}$ expression was strictly tet dependent. Moreover, the experiments in Figure 4C show that the Pol-I-transcribed $tRNA^{Sec}$ is correctly processed since it co-migrates with the endogenous wild-type $tRNA^{Sec}$ (Figure 4C, middle panel). As control we tested expression of a tagged initiator $tRNA^{Met}$ (31) in the context of the same construct. In this case, as expected for Pol-III-directed transcription, expression was constitutive and therefore independent of an active Pol-I promoter (Figure 4C).

Integration of the $tRNA^{Sec}$ gene into a polycistron is sufficient for its expression

The experiments described in Figure 4C raise the question whether the $tRNA^{Sec}$ gene would be expressed when placed in a different polycistron than the one it naturally resides in. In order to test this hypothesis we transplanted the same tagged $tRNA^{Sec}$ gene cassette that was used for the experiment in Figure 4C into the polycistron that encodes the Tb-VDAC (voltage-dependent anion channel) genes. This polycistron was chosen since previous studies have shown that the VDAC genes are not essential for normal growth of *T. brucei* in SDM-79 medium (41). The Southern analysis in the left panel of Figure 4D shows that, as expected, the cassette consisting of the $tRNA^{Sec}$ gene followed by the G418 resistance gene replaced one allele of the Tb-VDAC locus. Subsequent northern analysis using a specific oligonucleotide probe showed that the tagged $tRNA^{Sec}$ is expressed and correctly processed under these conditions (Figure 4D, right panel). Thus, whereas ectopic expression of the $tRNA^{Sec}$ gene outside of a polycistron requires an external promoter (Figure 4C) placing it into a polycistron allows it to be expressed. This observation strongly suggests that $tRNA^{Sec}$ is transcribed by co-expression with Pol-II transcribed protein-coding genes.

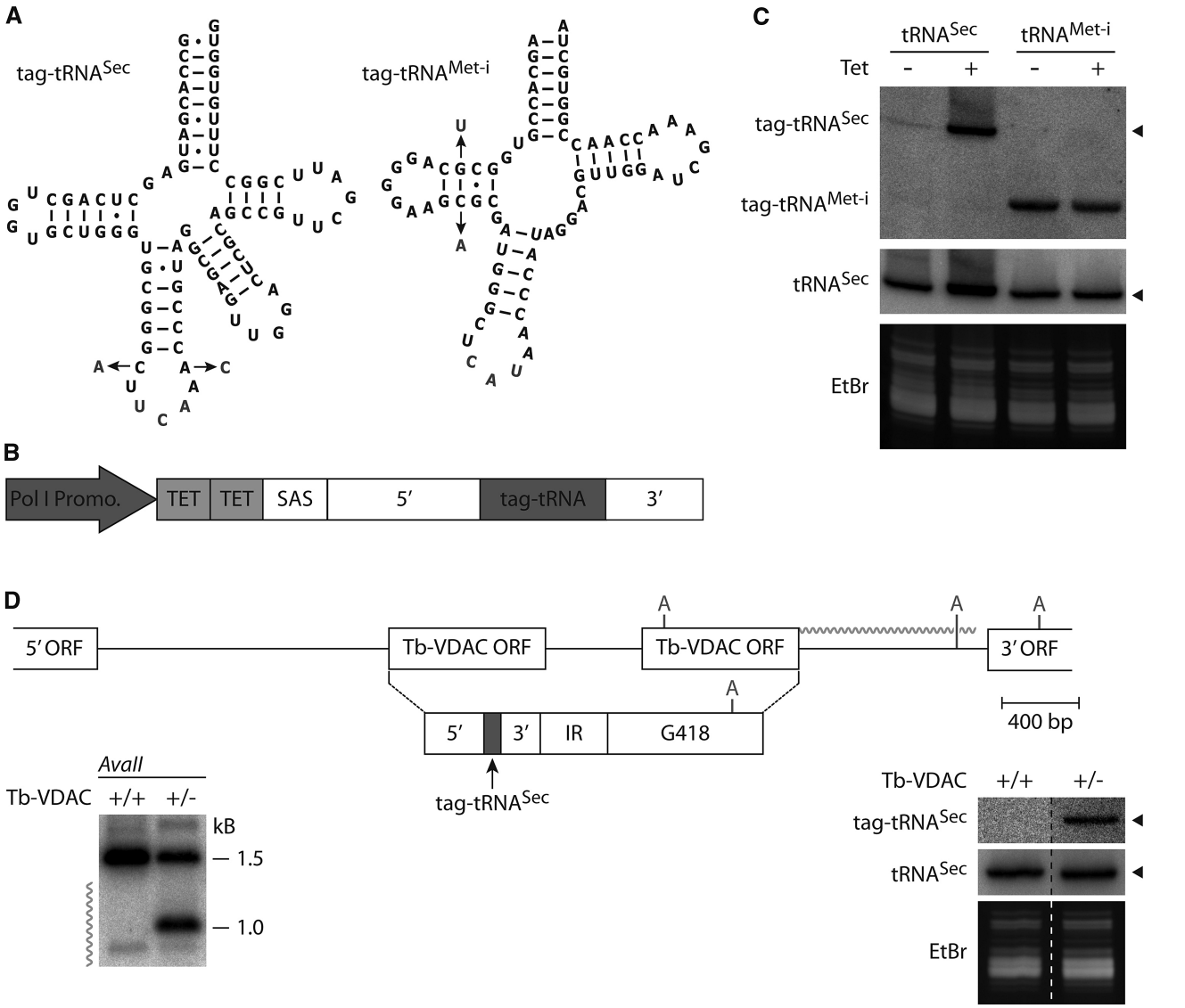

Figure 4. Ectopic expression of the trypanosomal tRNA^{Sec} gene requires an external promoter. (**A**) Predicted secondary structure of the tagged tRNA^{Sec} and tRNA^{Met-i}. The 2-nt changes introduced as tags are indicated. The tags allow the specific detection of the two tRNA variants by oligonucleotide hybridizations. (**B**) Cassette used for ectopic expression of the tRNA^{Sec} (the one encoded on the shorter intergenic region) and tRNA^{Met-i}, respectively. It contains the Pol-I procyclin promoter followed by two tetracycline operators and a splice acceptor site (SAS). The tagged tRNA^{Sec} was expressed in its own genomic context, whereas the tagged tRNA^{Met-i} was fused to the 5′-flanking region of a trypanosomal tRNA^{Leu} but retained its own 3′-flanking region (31). (**C**) Northern analyses of total RNA isolated from cell lines expressing the tetracycline repressor and transfected with the constructs shown in (A). tRNA^{Sec}, cell line expressing the tagged tRNA^{Sec}. tRNA^{Met-i} cell line expressing the tagged tRNA^{Met-i}. Top panel, hybridization with oligonucleotides that specifically recognize the tagged tRNA^{Sec} and the tagged tRNA^{Met-i}, respectively. Middle panel, same blot as above but reprobed with an oligonucleotide recognizing both the tagged and the endogenous tRNA^{Sec}. Bottom panel, ethidium bromide stained tRNA region of the gel used for the northern analyses. (**D**) To scale drawing of the wild-type Tb-VDAC locus (41) and the situation after homologous recombination leading to replacement of one allele by a tRNA^{Sec}/G418 resistance cassette. Relevant AvaII (A) restriction sites are indicated. The jagged line marks the probe used in the Southern analysis. Left panel: Southern analysis of genomic DNA isolated from the parental cell line (Tb-VDAC, +/+) and from a single knock out Tb-VDAC cell line (Tb-VDAC, +/−) containing the tRNA^{Sec}/G418 resistance cassette. Right panel, northern blot of total RNA isolated from the same cell lines that were analyzed by the Southern blot hybridized with a probe specific for the tagged tRNA^{Sec} (top panel). The same blot was reprobed with a probe recognizing both the tagged and the endogenous tRNA^{Sec} (middle panel). Bottom panel, ethidium bromide stained tRNA region of the gel used for the northern analyses.

α-Amanitin inhibits transcription of trypanosomal tRNA^{Sec}

To determine which RNA polymerase is responsible for transcription of the trypanosomal tRNA^{Sec} genes we performed nascent transcript labeling assays in lysolecithin-permeabilized cells (27). RNA from untreated and α-amanitin-treated cells was synthesized in the presence of radioactive UTP or CTP and hybridized to dot blots containing Pol-II and Pol-III transcribed genes. Figure 5 shows that maximum inhibition of transcription of the tRNA^{Sec} genes as well as of the Pol-II-transcribed tubulin genes is reached at 10 μg/ml α-amanitin. In contrast, expression of the Pol-III-transcribed tRNA^{Ile} and U6 snRNA is to a large

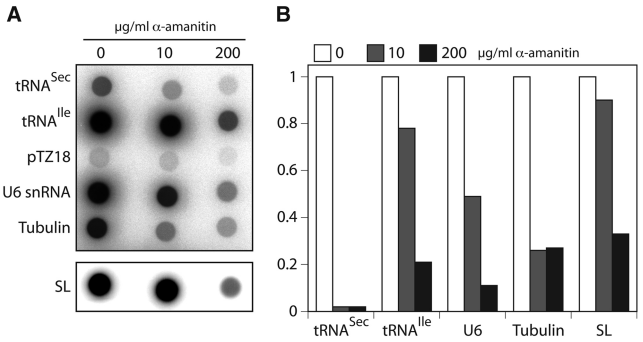

Figure 5. Transcription of the $tRNA^{Sec}$ gene is α-amanitin-sensitive. (**A**) UTP-labeled RNA was synthesized in permeabilized trypanosomes in the absence and presence of 10 and 200 μg/ml of α-amanitin and hybridized to nitrocellulose filters containing immobilized cloned DNA of the indicated genes (pTZ18: vector control; SL: spliced leader). (**B**) PhosphorImager quantitation of the results as shown in panel (A). The samples without α-amanitin were set to 1. Values represent means of two experiments. The variation between the values of two experiments was <15%.

extent resistant to 10 μg/ml α-amanitin but dramatically reduced at 200 μg/ml. Moreover, as shown previously, transcription of the SL RNA gene is less sensitive to α-amanitin than other Pol-II-transcribed genes (27). Sensitivity to low concentration of α-amanitin is a hallmark of Pol-II-mediated transcription and suggests that the trypanosomal $tRNA^{Sec}$ is transcribed by Pol-II.

RNAi of Pol-II but not of Pol-III affects transcription of trypanosomal $tRNA^{Sec}$

The question of which RNA polymerase transcribes trypanosomal $tRNA^{Sec}$ was also addressed in a manner analogous to the experiment with human cells shown in Figure 2. Thus, we used RNAi cell lines allowing inducible downregulation of subunit RPB9 of Pol-II (30) or of the largest subunit of Pol-III, respectively. Induction of the RPB9-RNAi cell line with tet leads to a growth arrest after ~24 h and subsequent cell death. Ablation of the largest subunit of Pol-III causes a slow growth phenotype after ~48 h (Figure 6A). Changes of the steady-state levels of $tRNA^{Sec}$, Pol-III-transcribed $tRNA^{Ile}$ as well as Pol-II-transcribed SL RNA were analyzed in uninduced and induced RNAi cell lines by northern blotting (Figure 6B). The steady-state levels of each of the tested RNAs were quantified after normalization to a Pol-I-transcribed small rRNA (M6 rRNA), which was not affected in the two RNAi cell lines. Figure 6C shows that the level of the $tRNA^{Sec}$ was reduced by ~25% in the RPB9-RNAi cell line but actually increased in the Pol-III RNAi cell line. The SL RNA showed a similar but more pronounced behavior: its level was reduced in the Pol-II RNAi cell line to ~30% and increased in the Pol-III RNAi cell line. The converse result was obtained for the $tRNA^{Ile}$: its level increased by ~30% in RPB9-RNAi cells and was reduced to ~30% after downregulation of Pol-III. The ethidium bromide stained gel (Figure 6B, bottom panel) shows that the bulk of tRNAs was downregulated by Pol-III knockdown. Unexpectedly, all tested RNA species showed an increase in their steady levels in the RNAi cell line ablated for the RNA polymerase that should not affect their transcription. This is potentially highly interesting since it may indicate an as yet unknown cross talk between the two RNA polymerases. It could, for example, be that both RNA polymerases compete for a common rate-limiting transcription factor. However, it is important to emphasize that ablation of either RNA polymerase is expected to have dramatic consequences on the physiology of the cells. The interpretation of these unexpected results is therefore very difficult and requires more elaborate investigations that are beyond the scope of this study.

However, in summary, the RNAi results are consistent with the experiments in permeabilized cells (Figure 5) and show that trypanosomal $tRNA^{Sec}$ is transcribed by Pol-II.

DISCUSSION

Eukaryotes are divided into six supergroups: humans belong to the *Opisthokonta* and *T. brucei* to the *Excavata*. Only a few species outside the opisthokont clade are known to have a $tRNA^{Sec}$. These include *Chlamydomonas reinhardtii*, *Dictyostelium discoideum*, *Tetrahymena* and members of the apicomplexans such as *Plasmodium falciparum* and *Toxoplasma gondii*. These species represent three supergroups: the *Archeaplastida* (*C. reinhardtii*), the *Amoebozoa* (*D. discoideum*) and the *Chromalveolata* (*Tetrahymena* and apicomplexans). In none of these species transcription of the $tRNA^{Sec}$ gene has been analyzed. However, sequence comparison shows that the putative $tRNA^{Sec}$ B box region (37,42) is conserved in most species, as would be expected if the gene is transcribed by Pol-III. The only exceptions are *T. brucei, L. major* and *D. discoideum* (Supplementary Figure S2). The fact that the $tRNA^{Sec}$ gene of *D. discoideum*, similarly to the ones of trypanosomatids, appears to have a deficient B box indicates that Pol-II transcription of $tRNA^{Sec}$ may occur in the *Archeaplastida*. However, without experimental evidence it remains possible that the B box of the *D. disciodeum* $tRNA^{Sec}$ is functional even though it deviates from B box consensus sequence. In summary, the sequence analysis shown in Supplementary Figure S2 suggests that Pol-III-mediated transcription of $tRNA^{Sec}$ represents the ancestral situation and that Pol-II-mediated transcription of $tRNA^{Sec}$, as observed in trypanosomatids, is a derived trait. It should be emphasized in that context that as far as we can tell all other trypanosomal tRNAs are transcribed by Pol-III.

Did this shift to Pol-II-mediated transcription of $tRNA^{Sec}$ occur by random genetic drift in the ancestor of trypanosomatids or does it reflect an adaptation that has functional importance? Selenoprotein synthesis requires at least four dedicated factors: phosphoseryl-$tRNA^{Sec}$ kinase, phosphoseryl-tRNA:selenocysteinyl-tRNA synthase, $tRNA^{Sec}$-specific elongation factor and selenophosphate synthetase (13,43). The synteny of the genes encoding these four proteins is conserved in the trypanosomatid species *T. brucei*, *T. cruzi* and *Leishmania major* (44). However, this is not the case for

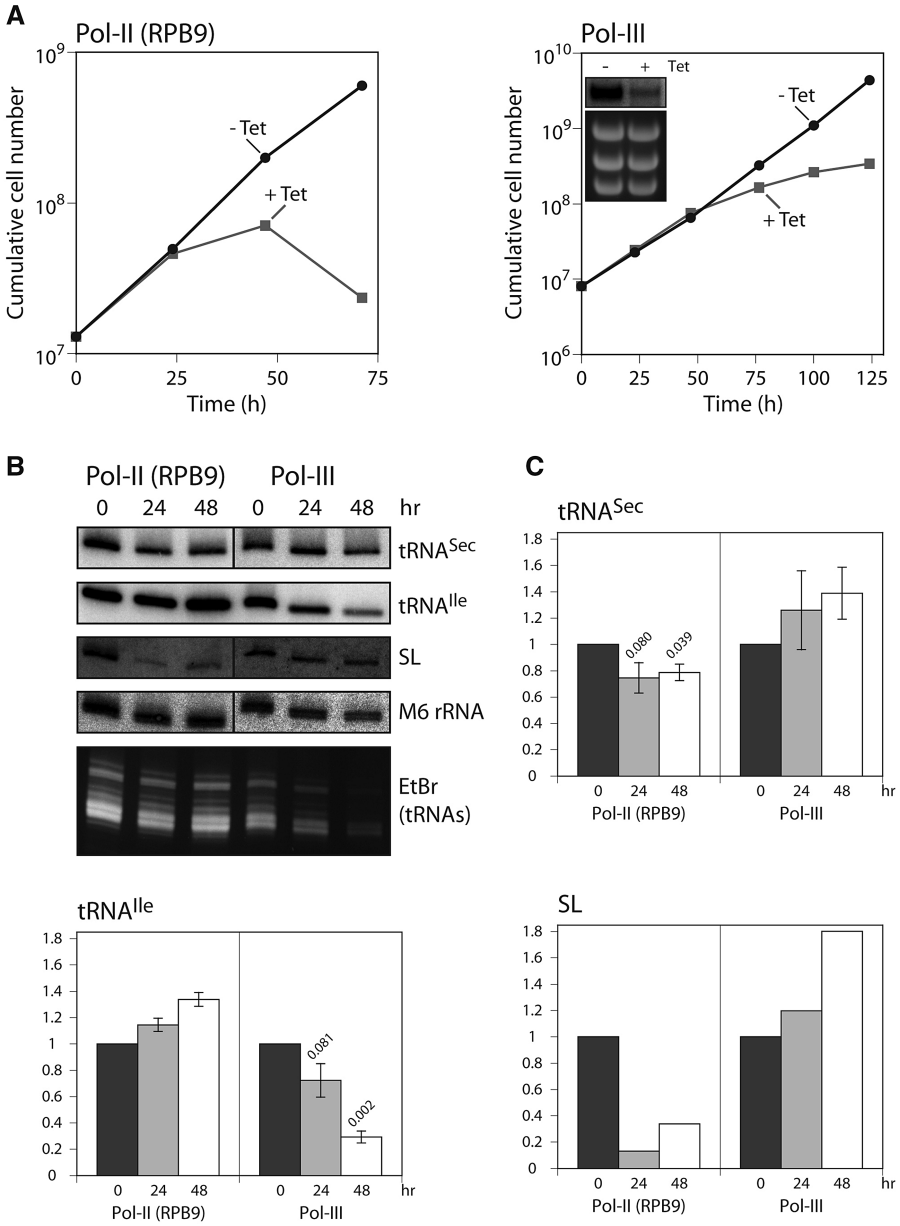

Figure 6. Effect of RNAi-mediated ablation of Pol-II and Pol-III activities on steady state levels of different RNAs. (**A**) Growth of uninduced and induced procyclic *T. brucei* RNAi cell lines downregulated for RPB9 (30) or the largest subunit of Pol-III. Inset: downregulation of the mRNA encoding the largest subunit of Pol-III was verified by northern blot analysis 24 h after induction of RNAi. (**B**) Northern analyses of total RNA isolated from uninduced (0) and induced (24, 48 h) RPB9 and Pol-III RNAi cell lines. RNA was resolved in 8 M urea on a 10% polyacrylamide gel and hybridized with oligonucleotides specifically detecting the transcripts indicated on the right. The tRNA region of the corresponding ethidium bromide stained gel is shown at the bottom. **(C)** Quantitation of the northern blots shown in (B). Signals were normalized to the cytosolic M6 rRNAs not affected by ablation of either RPB9 or Pol-III. For $tRNA^{Sec}$ and $tRNA^{Ile}$ the means of three experiments are shown. The signal in the uninduced cells was set to 1. Standard errors and the relevant *P*-values (Student's *t*-test, one-tailed, paired) are indicated.

the $tRNA^{Sec}$ genes: they are found within a polycistron in all three species, but the flanking genes in *L. major* are different from those in *T. brucei* and *T. cruzi* (Supplementary Figure S3A). This genetic arrangement shows that integration of the $tRNA^{Sec}$ gene into a polycistron is a relatively recent event that evolved twice independently in the trypanosomatid lineage and suggests that is has been positively selected for.

Pol-III-mediated transcription of eukaryotic $tRNA^{Sec}$ genes shows some peculiarities. Unlike other tRNA genes it requires upstream promoter elements (Supplementary Figure S3B) (5,45). These elements are very similar to the ones required for transcription of the U6 snRNA genes (9). It is interesting to note that transcription of the trypanosomal U6 snRNA gene, while still dependent on Pol-III, is different than in other eukaryotes since it requires an upstream inverted tRNA gene as a promoter element (Supplementary Figure S3C) (24). Why trypanosomal U6 snRNA is transcribed in a different way than in other systems is not known, but it should be

considered that trypanosomatids appear to lack many transcription factors that are conserved in other eukaryotes (46). Thus, it is possible that the Pol-II-mediated transcription of the trypanosomal $tRNA^{Sec}$ is connected to the fact that the standard eukaryotic U6 snRNA transcription mode is not operational in *T. brucei*. Most of the *T. brucei* genome codes for large Pol-II-transcribed polycistrons which may have predisposed the $tRNA^{Sec}$ gene to be transcribed by Pol-II. All that needs to be postulated for such a scenario is the integration of the $tRNA^{Sec}$ gene into a polycistron.

$tRNA^{Sec}$ is unusual in many respects, so we wondered if there was any evidence for Pol-II-mediated transcription of conventional tRNAs. Analyzing the genomic organization of tRNA genes in *Caenorhabdititis elegans* (http://www.wormbase.org, release WS201) and *Drosophila melanogaster* (http://flybase.org/) we found in both organisms a few cases where tRNAs are encoded within introns of Pol-II-transcribed genes. Interestingly, in *C. elegans* one of the tRNA genes that is found within an intron is $tRNA^{Sec}$ (http://www.wormbase.org/db/get?name=WBGene00023106;class=Gene), although unlike in trypanosomatids it is not present in a polycistron. Intron-encoded tRNA genes are embedded in Pol-II transcription units and therefore must be transcribed by Pol-II, although it is possible that they still contain internal Pol-III promoters and may be independently transcribed by Pol-III. That Pol-II-mediated transcription of tRNAs is in principle possible has recently been shown in mouse where insertion of a circularily permuted tRNA gene into a protein-coding gene yielded a functional tRNA, a large fraction of which was transcribed by Pol-II (47). Thus these findings indicate that Pol-II transcription of tRNA genes may not be restricted to trypanosomatids but be more widespread in eukaryotes.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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