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International Journal for Parasitology 35 (2005) 359–366
www.parasitology-online.com

The *Trypanosoma brucei* La protein is a candidate poly(U) shield that impacts spliced leader RNA maturation and tRNA intron removal

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Received 5 October 2004; received in revised form 17 December 2004; accepted 17 December 2004

Abstract

By virtue of its preferential binding to poly(U) tails on small RNA precursors and nuclear localisation motif, the La protein has been implicated for a role in the stabilisation and nuclear retention of processing intermediates for a variety of small RNAs in eukaryotic cells. As the universal substrate for *trans*-splicing, the spliced leader RNA is transcribed as a precursor with just such a tail. La protein was targeted for selective knockdown by inducible RNA interference in *Trypanosoma brucei*. Of three RNA interference strategies employed, a p2T7-177 vector was the most effective in reducing both the La mRNA as well as the protein itself from induced cells. In the relative absence of La protein *T. brucei* cells were not viable, in contrast to La gene knockouts in yeast. A variety of potential small RNA substrates were examined under induction, including spliced leader RNA, spliced leader associated RNA, the U1, U2, U4, and U6 small nuclear RNAs, 5S ribosomal RNA, U3 small nucleolar RNA, and $tRNA^{Tyr}$. None of these molecules showed significant variance in size or abundance in their mature forms, although a discrete subset of intermediates appear for spliced leader RNA and $tRNA^{Tyr}$ intron splicing under La depletion conditions. 5′-end methylation in the spliced leader RNA and U1 small nuclear RNA was unaffected. The immediate cause of lethality in *T. brucei* was not apparent, but may represent a cumulative effect of multiple defects including processing of spliced leader RNA, $tRNA^{Tyr}$ and other unidentified RNA substrates. This study indicates that La protein binding is not essential for maturation of the spliced leader RNA, but does not rule out the presence of an alternative processing pathway that could compensate for the absence of normally-associated La protein.

Keywords: Kinetoplastid; RNA interference; snRNA; tRNA splicing

1. Introduction

The 5′ end of all nuclear-derived mRNAs in kinetoplastid protozoa consists of a 39-nt spliced leader (SL) derived from a precursor transcript, the SL RNA (Liang et al., 2003). *Trans*-splicing of the SL represents the first step in the maturation of a polycistronic pre-mRNA to the mature capped and polyadenylated mRNA (LeBowitz et al., 1993; Matthews et al., 1994). The mechanism of *trans*-splicing closely resembles the mechanism of *cis*-splicing and uses similar small nuclear (sn) RNA and protein components (Tschudi and Ullu, 1990; Lücke et al., 1997). The SL RNA is a bifunctional transcript unique to *trans*-splicing. It is characterised by a phylogenetically-conserved secondary structure (Bruzik et al., 1988) and is found in a ribonucleoprotein (RNP) complex (Miller and Wirth, 1988). The 3′ end, or intron, of the SL RNA resembles a splicing-associated snRNA, while the 5′ end, the SL, is the common 5′ substrate in the splicing reaction that provides the mature mRNAs with a hypermethylated cap (Bangs et al., 1992) and a pseudouridine modification (Liang et al., 2002). Assembly of a functional SL RNP requires the addition of core and specific protein components to an SL RNA that has been trimmed and modified from the primary

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0020-7519/$30.00 © 2005 Published by Elsevier Ltd on behalf of Australian Society for Parasitology Inc.
doi:10.1016/j.ijpara.2004.12.012

transcript (Ismaïli et al., 1999; Palfi et al., 2000). The SL RNA precursor possesses a 3′ poly(U) tail (Sturm et al., 1999) that is removed in an Sm protein-dependent manner (Mandelboim et al., 2003; Zeiner et al., 2004a) after transport to the cytoplasm (Zeiner et al., 2003a).

Removal of the poly(U) tail for the SL RNA precursor appears to be a two-step process (Zeiner et al., 2004b). We propose that intermediate polyuridylated forms of the SL RNA are protected from 3′-exonucleolytic degradation by a 'poly(U)-shield' protein, consistent with the observations that the poly(U) tail is neither removed prior to nuclear egress (Zeiner et al., 2003a), nor before Sm-protein binding (Sturm et al., 1999). The most-abundant protein that performs such poly(U)-shielding function in eukaryotic cells is the homolog of the La autoantigen (Stefano, 1984; Maraia and Intine, 2002; Wolin and Cedervall, 2002). The La protein, which is dispensible for growth in two yeast genera (Yoo and Wolin, 1994; Van Horn et al., 1997), performs diverse functions including: transcription initiation and termination by RNA polymerase (pol) III (Maraia et al., 1994); binding to most nascent RNA pol III transcripts such as 5S rRNA and tRNA, where it protects precursor molecules (Preiser et al., 1993) and is necessary for tRNA maturation, like pre-tRNA folding (Chakshusmathi et al., 2003) and 3′ processing (Kufel and Tollervey, 2003); assembly of pol II- and pol III-transcribed snRNAs into snRNPs where it acts as a chaperone (Pannone et al., 1998; Xue et al., 2000); association with vault and telomerase complexes (Aigner et al., 2000; Kickhoefer et al., 2002); cap-dependent (Cardinali et al., 2003) and cap-independent Internal Ribosome Entry Site (IRES)-mediated (Pudi et al., 2003) translation of mRNA; and transposon mobility (Aye and Sandmeyer, 2003). If La protein acts as a poly(U) shield for precursor SL RNA, its absence could manifest itself in several molecular phenotypes: (i) SL RNA 3′-end maturation may be overzealous and/or occur in an incorrect temporal order, resulting in 3′-overexpressing with a possible loss of 5′ cap 4 formation; (ii) maturation may be slowed, resulting in the accumulation of 3′-extended SL RNA; or (iii) an alternative pathway may compensate for the absence of La, revealing no visible effect on SL RNA.

The gene for the La protein ortholog has been identified in *Trypanosoma brucei* (Marchetti et al., 2000; Westermann and Weber, 2000). Recombinant *T. brucei* La protein has been shown to bind poly(U) in vitro (Westermann and Weber, 2000; Dong et al., 2004) and the crystal structure of the La motif has been determined (Dong et al., 2004). To test whether the kinetoplastid La protein is involved in the SL RNA maturation pathway we have tested three different double-stranded RNA-interference (RNAi) plasmids for effects of La protein knockdown on a variety of polyuridylated small RNAs. In contrast to the yeasts, knockdown of La protein with the p2T7-177 vector yielded a lethal phenotype in *T. brucei*.

2. Materials and methods

2.1. Plasmid constructions

Three vectors for RNAi were constructed as follows. The 3′ part of the La gene spanning nts 315-1004 was amplified by PCR from *T. brucei* 427 genomic DNA using oligonucleotides LaF1 (5′-CCCACAGCAACA*CTCGAG*CA) and LaR1 (5′-CCC*TCTAGA*TTCACGTGACCGC T TGTGTC) or LaR2 (5′-CCC*GGATCC*TTCACGTGA CCGCTTGTGTC), with encoded *Xho* I and added *Xba* I or *Bam* HI recognition sites *underlined*. The amplified fragments were called La-A (product of LaF1 and LaR1 PCR) and La-B (product of LaF1 and LaR2 PCR) and were cloned into pCR2.1 TOPO® (Invitrogen). La-A was then subcloned in pZJM (Wang et al., 2000), resulting in plasmid La-Ai. La-B was subcloned in p2T7-177 (Wickstead et al., 2002), resulting in plasmid La-Bi.

The same fragment of the La gene was PCR-amplified using oligonucleotides LaF3 (5′-CCC*AAGCTTGGATCC*-CACAGCAACACTCGAGCA) and LaR3 (5′-GC*TCTA-GAGGATCC*TTCACGTGACCGCTTGTG), with added *Bam* HI, *Hin*d III or *Xba* I recognition sites *underlined*, and cloned in pCR2.1 TOPO®, resulting in plasmid La-C. *Bam* HI-digested La gene from La-C was subcloned into the pLew100-HX-GFP plasmid (Hutchings et al., 2002) and linearised with *Bam* HI, resulting in plasmid La-C-1. Next, the *Hind* III/*Xba* I-digested La fragment was cloned into La-C-1 and digested with the same enzymes to generate the La-Ci plasmid, containing the 'sense' and 'antisense' versions of the La gene fragment. All constructions were checked by sequence analysis.

2.2. Cell culture, transfection, RNAi induction and growth curves

The procyclic *T. brucei* strain 29-13, containing introduced T7 RNA polymerase and tetracycline (tet) repressor genes, was grown at 27 °C in SDM-79 medium supplemented with 10% foetal bovine serum in the presence of hygromycin (50 μg/ml) and G418 (15 μg/ml). Exponentially growing cultures were used for transfection (Wang et al., 2000). Electroporation with 10 μg linearised plamid DNA was performed in 4 mm cuvettes using a BTX electroporator with the settings of 1500 V, 50 μF and 500 Ω. Cells were transferred into fresh medium to which 5 μg/ml phleomycin was added after 24 h. Approximately 2 weeks later, only cells resistant to phleomycin survived, which were cloned using limiting dilution in 96-wells plates at 27 °C in the presence of 5% CO_2. After 3 weeks, several clones were transferred into larger volumes of SDM-79 and the synthesis of dsRNA was induced by the addition of 2 μg/ml tet. La protein levels were analysed in total cell lysates. Following confirmation of successful RNAi (see below), one out of three clones was used for further experiments. Growth curves obtained over a period of

10 days after the induction of RNAi were determined using the Beckman X2 Cell Counter. Five growth curves were performed for each cell line.

2.3. Preparation of antibodies and Western blotting

The entire coding region of La was PCR-amplified using LaF4 (5′-*GAGCTC*CCACTTTCCTCCGAGAACAAG) and LaR4 (5′-*CTGCAG*TTCACGTGACCGCTTGTGTC), with added *Sac* I and *Pst* I sites *underlined*, cloned into pCR2.1 TOPO®, subcloned into pRSET-A (Invitrogen) incorporating an N-terminal His_6-tag, and transfected into *Escherichia coli* BL21(DE3)pLysS cells. The resulting clone was verified by sequencing of the insert. The bacterial cells were grown in the presence of 35 μg/ml chloramphenicol and 100 μg/ml ampicillin at 37 °C, and induced with 0.05 mM IPTG. Soluble protein was obtained by purification under native conditions following the manufacturer's instructions (Xpress™ System Protein Purification, Invitrogen). The over-expressed protein was purified from the supernatant by two Ni-nitrilotriacetic acid affinity chromatography steps.

Polyclonal antibodies were prepared by immunising rabbits at 2-week intervals with four subcutaneous injections of 0.5 mg of purified recombinant La protein that was over-expressed in *E. coli* (data not shown) emulsified with complete (first injection) and incomplete (following injections) Freund's adjuvant. Sera were collected 7 days after the fourth injection and were tested by Western blotting, aliquoted and stored at −80 °C. Cell lysates were prepared from 1×10^7 cells/10 μl, analysed on a 12% SDS-PAGE gel, blotted and probed with polyclonal anti-La antibodies (1:1000). The secondary anti-rabbit IgG antibody (1:1000) (Sevapharma) coupled to horseradish peroxidase was visualised according to the manufacturer using the ECL kit (Amersham Biosciences). The preparation of recombinant *T. brucei* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been described elsewhere (Hannaert et al., 1995).

2.4. RNA analysis

Total RNA was isolated using TriZOL reagent (Invitrogen) with ethanol versus isopropanol precipitation of RNA as described previously (Zeiner et al., 2003a). Approximately 8 μg RNA/lane were loaded on a 1% formaldehyde agarose gel, blotted and cross-linked onto nylon membranes. After pre-hybridisation in Church-Gilbert solution (0.5 M Na_2HPO_4, pH 7.2; 1 mM EDTA; 7% SDS, 1% BSA) for 2 h at 55 °C, hybridisation with the La gene fragment labelled by random priming (Fermentas) with [α^{32}P]dATP (ICN) was performed overnight in the same solution at 60 °C. A wash in 2×SSC + 0.1% SDS at room temperature for 20 min was followed by two washes in 0.2×SSC + 0.1% SDS for 20 min each at 55 °C. The signal was quantified using a Typhoon PhosphoImager (Amersham).

For the medium resolution analysis of small RNAs, total RNA 6 days post-induction was resolved on an 8% polyacrylamide gel containing 8 M urea; sequencing gels were used for high resolution RNA blots as described previously (Sturm et al., 1999). The RNA was transferred to Biodyne® B membrane (Gelman Laboratory) and probed. Oligonucleotide probes were end-labelled with [γ^{32}P]ATP (ICN) by polynucleotide kinase (New England Biolabs): U1 snRNA = 5′-CCCCTCAAAATATGCTG; U2 snRNA = 5′-AGCTAAAAAGCCGAGAAGATAT; U3 snoRNA = 5′-TGCCGTTCATCGAAC; U4 snRNA = 5′-GTACCGGATATAGT; U6 snRNA = 5′-AGCTATATCTCTCGAA; 5S rRNA = 5′-TAACTTCACAAATCGGACGGGAT; SLA1 RNA = 5′-TCTYGCTCTCCAGTTTCRTG; $tRNA^{Tyr}$-3′ = 5′-GTGGTCCTTCCGGCCGGAATCGAA; $tRNA^{Tyr}$-intron = 5′-CCCGCATACTCTACAGTCA; TbSL stem-loop I = 5′-CTACTGGGAGCTTCTCATCA. Primer extension analysis on the SL RNA was performed as described previously (Sturm et al., 1998) using specified oligonucleotides. Sequence ladders were generated using the pUC8-TbSL plasmid with the corresponding extension oligonucleotides.

3. Results

3.1. RNAi effects vary by vector

Three plasmids for inducible RNAi were used to knock-down the expression of La protein due to empirical differences found for other targets. pLew100, pZJM and p2T7-177 were chosen as representative vectors with variation in transcription strategies and/or integration site targeting: pLew100 uses the *T. brucei* PARP promoter to transcribe a single transcript containing two opposing fragments of the target gene separated by a stuffer sequence and integrates into the rRNA locus, while pZJM and p2T7-177 use opposing T7 promoters to transcribe a fragment of the target gene and integrate into the chromosomal rRNA locus and the minichromosomal 177-nt repeat region, respectively.

After induction of the RNAi response, total protein was isolated from the various clonal cell lines at different time points and analysed on Western blots with polyclonal antibodies generated against the recombinant *T. brucei* La protein. Western analysis with polyclonal antibodies generated against recombinant La protein revealed reduction of the target protein only in cultures and clones transfected with the plasmids La-Bi (p2T7-177) and La-Ci (pLew-100), while the La protein was not eliminated in the La-Ai (pZJM)-bearing cells (Fig. 1A). Secondary challenge of the membranes with anti-GAPDH antibodies confirmed this observation. The failure of pZJM to produce the RNAi effect was caused by the absence of double stranded (ds) RNA in the transfectants, despite confirmation by Southern hybridisation of integration in the rRNA locus (data not shown). Based on this result, the La-Ai cells were excluded from

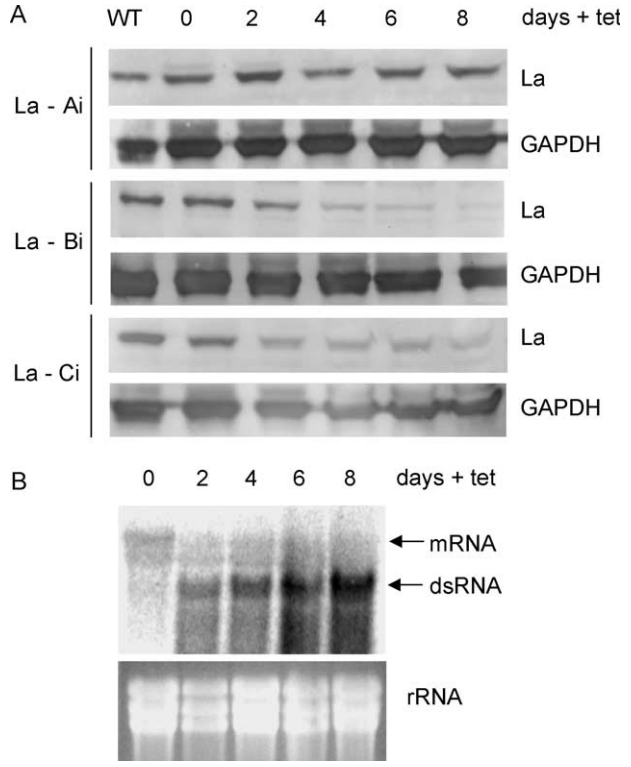

Fig. 1. Effect of La RNAi on protein and mRNA levels. (A) La protein levels were analysed by Western blot analysis in extracts from 29-13 cells (WT), non-induced cells (day 0), and in extracts isolated 2, 4, 6, and 8 days after induction of La-Ai (pZJM), La-Bi (p2T7-177), and La-Ci (pLew100) cells. Each lane was loaded with protein from $\sim 10^7$ cells and blots were immunostained using anti-La polyclonal rabbit antibody. Anti-GAPDH antibody was used as a loading control. (B) La mRNA levels were analysed by blotting total RNA extracted from non-induced La-Bi cells (day 0), and La-Bi cells harvested 2, 4, 6 and 8 days after induction. The positions of the targeted mRNA and the dsRNA synthesised following induction are indicated with arrows. As a control, the gel was stained with ethidium bromide to visualise rRNA bands.

further studies. Significant elimination of the target protein occurred 6 days after induction in the La-Ci cells. However, at day 8 residual La protein was present. In the La-Bi cells there was a strong decrease of the same protein after 4 days of induction, followed by virtually total loss at day 8. To follow the RNAi phenomenon in cells where the most efficient ablation of the La protein occurred, we continued with analysis of total RNA from the La-Bi cells (Fig. 1B). In these cells the target La mRNA was eliminated after 2 days of induction, and remained absent through day 8.

The three vectors tested showed a spectrum of efficacy for the desired knockdown of La protein. The variance among the vectors suggests that the integration site is key to success of RNAi. We next examined the cell lines showing knockdown of La protein for effects on cell growth.

3.2. La elimination is lethal to T. brucei cells

La protein is not required for cell growth in yeast, thus a growth phenotype of La protein RNAi in *T. brucei* was not anticipated. However, the difference in the dynamics and level of La protein elimination was reflected by the growth curves (Fig. 2). Upon RNAi induction growth of the La-Bi and La-Ci cell lines were identical to wild type and non-induced cells until day 4. Thereafter, the La-Bi cells stopped dividing and formed large aggregates. Inspection of these cells by light and electron microscopy did not reveal any morphological changes (data not shown). Only an insignificant growth defect was observed for the La-Ci cells. Non-cloned La-Bi and La-Ci cultures behaved in the same manner as the representative clones. In order to examine the cause of lethality, the phenotype of small RNAs in induced La-Bi cells was determined.

3.3. La elimination results in reduced cis-splicing efficiency for $tRNA^{Tyr}$

The validity of our hypothesis that La acts as a poly(U) shield protein for small RNAs was tested by examination of several candidate substrates containing 3′-oligo(U) stretches, and the control SLA RNA that lacks a 3′ poly(U), under La knockdown conditions in the La-Bi clonal line. The predicted fate of unbound substrate molecules would depend on the functional role that La plays in their maturation, and could result in a variety of phenotypic outcomes including precursor destabilisation, loss of 3′ processing due to the inability to recruit

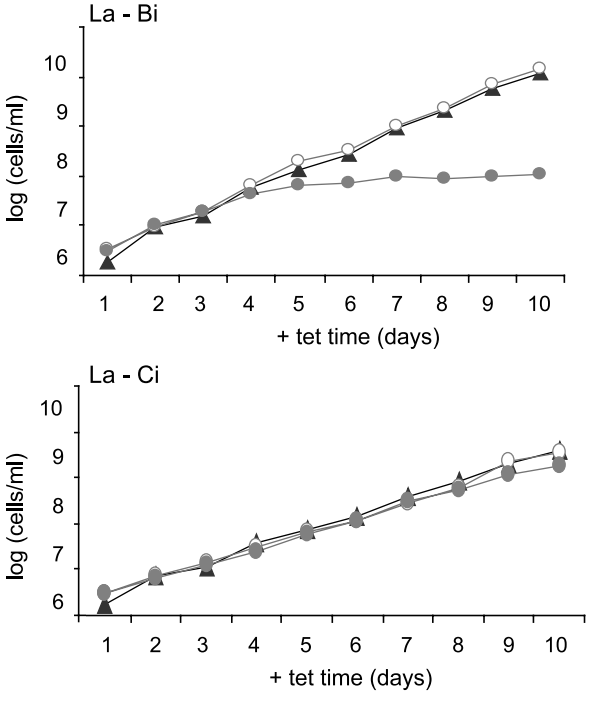

Fig. 2. Effect of La RNAi on cell growth. Growth effect following induction of La dsRNA in La-Bi (p2T7-177) and La-Ci (pLew100) cells. The numbers of 29-13 cells (triangles), non-induced cells (open circles), and those after RNAi induction by the addition of 2 μg/ml tet (filled circles) were plotted as the product of cell density and total dilution. Growth curves are one representative set from five experiments.

interacting nucleases, and accumulation of unspliced forms of the substrate (Van Horn et al., 1997; Yoo and Wolin, 1997; Intine et al., 2002; Kufel and Tollervey, 2003).

The steady-state forms of small RNAs involved in *cis*- and *trans*-splicing, RNA modification and translation were determined by medium-resolution acrylamide gel analysis from the La-Bi cells 6 days after induction (Fig. 3A). None of the mature RNAs showed any significant alterations in size or abundance when normalised to ethidium bromide-stained small ribosomal RNAs in the same gel (data not shown); oligo(U) precursor was only evident in the case of the SL RNA, which was not altered upon tet induction at this resolution. Due to the association of La protein with tRNA maturation in other systems, we also probed for effects on an intron located in $tRNA^{Tyr}$ (Schneider et al., 1993) that represents one of the rare introns found in *cis* in the kinetoplastid genome. Hybridisation of a probe against the 3′ end of $tRNA^{Tyr}$ revealed similar levels of the 76 nt tRNA, form IV defined in Schneider et al. (1993). The hybridisation intensity of an ~115 nt band increased after induction with tet. The identity of this band, which also appeared to be detected by the intron probe (see below), was not determined in this study or elsewhere. A probe directed against the $tRNA^{Tyr}$ intron detected two main bands of unequal intensity in each lane and, faintly, the ~115 nt band. The abundance of the larger band, which corresponds to the poly(U)-containing intermediate I (Schneider et al., 1993), was markedly reduced following knockdown of the La protein. In contrast, the shorter band that corresponds to intermediate form II and lacks a 3′ poly(U) increased in abundance upon the disappearance of the La protein. These data are consistent with stabilisation of the intermediate form I by bound La protein, and a requirement for the La protein for progression to the next step of intron removal.

In order to examine the SL RNA intermediates at the finest possible level, high resolution RNA blots were prepared and probed with oligonucleotide TbSL stem-loop I. Upon equal loading of the SL RNA in induced and uninduced samples, the pattern of intermediates below the mature form of SL RNA was altered (Fig. 3B). Two new sets of smaller forms appear in the +tet samples. The appearance of truncated forms of the SL RNA is consistent with the anticipated phenotype of the loss of the SL RNA poly(U) shield. The exonucleases responsible for 3′-end

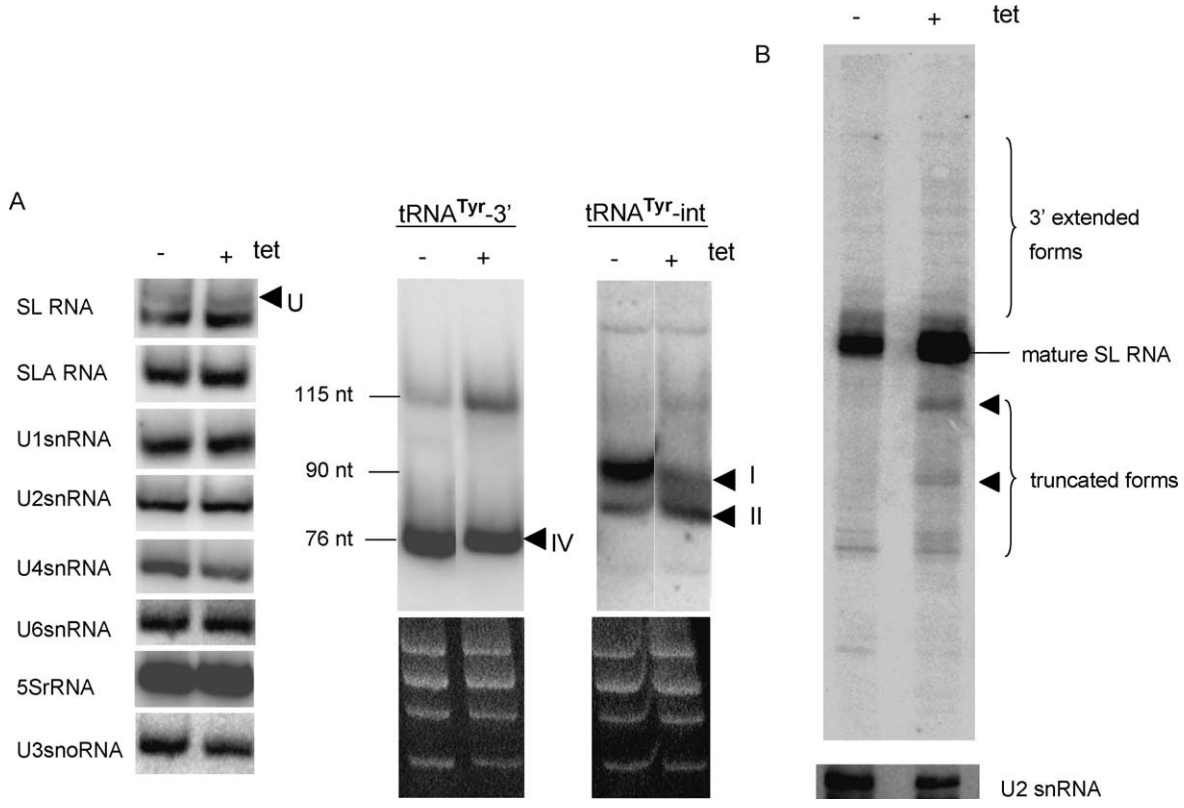

Fig. 3. RNA analysis of the La RNAi line La-Bi. (A) Left panel: Medium-resolution Northern blots for small RNAs involved in *cis*- and *trans*-splicing and RNA modification as indicated in the left column. 'U' indicates the poly(U)-extended SL RNA precursor. The size of the bands was determined from comigrating, ethidium-bromide stained, small ribosomal RNA molecules. Right panels: Medium-resolution Northern blot analysis of the $tRNA^{Tyr}$ mature (-3′) and intermediate (−int) forms. Segment of the same gels stained with ethidium bromide showing equal loading of RNA. Roman numerals indicate the forms defined by (Schneider et al., 1993). RNA was isolated from non-induced cells (−tet) and cells 6 days after the addition of tet (+tet). (B) High resolution Northern blot for SL RNA isolated from non-induced cells (−tet) and cells 6 days after the addition of tet (+tet). Multiple 3′ U-extended and -truncated forms are indicated; bands of greater relative intensity following addition of tet are indicated with arrowheads. To control for RNA loading, the membrane was rehybridized with U2 snRNA.

formation may be overenthusiastic without the physical limitation provided by the La protein, thus reducing the efficiency of mature SL RNA production. 5′-end maturation was examined by primer extension for the extensively methylated SL RNA. No evidence of decreased 5′-methylation of the U1 snRNA and SL RNA was detected (data not shown). Based on the results from similar experiments in yeast where a reduction in the levels of RNA-binding proteins resulted in lower levels of the cognate bound RNA, the observed relative increase in SL RNA abundance was unexpected and counterintuitive. The accumulation may be due to a lack of, or delayed, entry of the abnormal SL RNA into the *trans*-splicing pathway. Consistent within *T. brucei*, increased levels of SL RNA were observed also upon knockdown of the core ribonucleoprotein component SmD1 (Mandelboim et al., 2003; Zeiner et al., 2004a) suggesting the mechanisms of defective-RNA recognition and degradation are not identical in trypanosomes and yeast.

As in other systems, a reduction of *cis*-splicing was detected for a tRNA, consistent with the conclusion that splicing normally precedes 3′ processing (Schneider et al., 1993) and/or that La may play a role in the recruitment of spliceosomal machinery to the substrate. These data indicate that La is not required for the mediation of 3′-end formation, but it does suggest the association of La with the oligo(U) tails of the SL RNA and $tRNA^{Tyr}$ precursors.

4. Discussion

We examined the role of La protein in the maturation of small RNA molecules. Using RNAi to reduce the presence of La mRNA and protein in *T. brucei* procyclic cells, we show an anticipated effect on the removal of an intron located in $tRNA^{Tyr}$ analogous to the splicing inhibition seen for tRNAs in other systems upon La knockout. Superficially, no phenotypic changes were evident in a host of other potential RNA substrates containing poly(U) stretches at their 3′ ends, however, the SL RNA showed variation in the accumulation of 3′-processing intermediates. No deficiency in 5′ methylation of either SL RNA or U1 snRNA was detected. Contrary to knockout experiments in yeast where the La gene was demonstrated to be non essential, in *T. brucei* the RNAi knockdown of La was ultimately inhibitory to cell division. The lethal phenotype due to reduction of La protein was not attributable directly to the observed effects on SL RNA and $tRNA^{Tyr}$ and could have been due to more rapid inhibition of another process or possibly secondary effects due to cumulative inhibition of multiple substrates.

In the course of this study, three RNAi vectors were constructed and compared, with widely variable effects. The p2T7-177 scenario, employing opposing promoters to transcribe a segment of the target gene and integration into a DNA repeat element, was the most successful in the reduction of La protein and the generation of a perceivable phenotype; the pZJM arrangement was ineffective due to the apparent lack of dsRNA production, while the pLEW100 vector resulted in an eventual reduction of protein but no slowing in cell division through 10 days of induction. Only in the La-Bi cells the La protein drop below a critical threshold. A key variable among the transfections is the genomic integration site, and it has been speculated that the numerous and widely-distributed rRNA loci may be differentially effective as successful RNAi integration locales (Wickstead et al., 2002). All three vectors have been used successfully in previous studies, thus there may also be an empirical difference among the genes targeted that accounts for the spectrum of results.

A phenotypic change in maturation was discerned in two out of nine potential La-binding substrates: U1, U2, U4 and U6 snRNAs; SL and SLA RNAs; 5S rRNA, U3 snoRNA and $tRNA^{Tyr}$. In choosing the candidate substrates for examination two characteristics were common to all or most of the transcripts: (i) size of the mature product of ≤ 150 nt, and (ii) the presence of a poly(U) stretch in the 3′ region of the unprocessed or mature transcripts. The SLA RNA was included as a control short transcript that lacks 3′ poly(U). RNA pol III is responsible for driving transcription of the substrate genes, with the exception of the SL and SLA RNAs, which are transcribed by RNA pol II (Roberts et al., 1996). The products are involved in a variety of roles, including splicing, RNA modification and translation. Likewise, the substrates reside in discrete compartments throughout the cell, from the nucleolus to the cytosol to the mitochondrion, with several likely undergoing multiple nucleus-cytosol-nucleus trafficking in the course of their function.

The cause of the lethality of La protein knockdown in *T. brucei* is not likely due to the direct inhibition of $tRNA^{Tyr}$ splicing and/or SL RNA processing. Steady-state levels of the mature $tRNA^{Tyr}$ and SL RNA transcripts appear normal at 6 days post-induction, indicating that the loss of functional $tRNA^{Tyr}$ or SL RNA were not primary defects. The accumulation of unspliced $tRNA^{Tyr}$ forms is consistent with the phenotype of La protein knockout seen in yeast (Yoo and Wolin, 1994), however indicators for other affected substrates are not apparent. In the case of yeast, back-up processing systems appear to compensate for the lack of La protein function (Yoo and Wolin, 1997), thus trypanosomes may be lacking in an alternative pathway. It is also possible that the indirect lethality to trypanosomes is due to the accumulation of $tRNA^{Tyr}$ intermediates that are blocking the processing, or translational, machinery and inhibiting the function of other tRNAs. Of the other roles ascribed to La protein in eukaryotes, a loss of nuclear retention may account for the accumulation of unspliced forms that prematurely escape the splicing machinery, yet not eliminate splicing of all nascent transcripts. We have not performed the kinetic studies necessary to comment on this definitively. Due to its rapid consumption in *trans*-splicing

(Laird et al., 1987a,b; Ullu and Tschudi, 1990) maturation defects in the SL RNA should have been readily evident, and since no significant reduction in the mature form of SL RNA was manifest, we can conclude that La protein is not essential for maturation of the universal *trans*-splicing substrate. A possible cause of lethality that could have gone undetected in this study is a specific reduction in complete cap 4 formation on the SL RNA. The primer extension assay employed here could give a misleading result for partial cap 4 formation; our studies on translation of mRNAs containing undermethylated SLs indicate that this state could result in loss of polysome loading (Zeiner et al., 2003b). Likewise, we did not assay for SL RNA pseudouridinylation, however, this has been demonstrated to be a dispensable modification that is not required for *trans*-splicing or translation (Sturm et al., 1998; Zeiner et al., 2003b).

The $tRNA^{Tyr}$ intermediates that accumulate in the absence of La protein are consistent with the scenario in which *cis*-splicing precedes 3′ processing in tRNAs (Schneider et al., 1993). This processing pathway dictates that La protein is bound to the poly(U)-containing 3′-extension while splicing occurs. The presence of La protein is also thought to direct an endonuclease to the 3′ extension, which is replaced by an alternative exonuclease activity in the absence of La protein in yeast (Yoo and Wolin, 1997). Analysis of $tRNA^{Met}$ maturation intermediates in *T. brucei* also implicated La protein participation in the absence of an intron and indicated that 3′ trimming preceded 5′-end formation (Arhin et al., 2004).

The slow death phenotype following RNAi against La protein indicates that a key role is played by La protein in *T. brucei*, although the role itself is not immediately apparent. Our interest in La protein is as a component in the maturation of the SL RNA. As such, the accumulation of truncated SL RNA in the absence of La protein is consistent with La protein interaction with the 3′ end of this molecule, but that is not required for accurate 3′ maturation. While recombinant trypanosome La protein binds poly(U) in vitro by gel mobility-shift assay (Westermann and Weber, 2000; Dong et al., 2004) we have been unable to detect the in vivo bound RNA substrates by immunoprecipitation due to insufficiently high-titre antiserum. Additional experiments are necessary to determine whether the essential La-protein function is one of its previously reported properties or an as-yet undescribed activity. The importance of a poly(U) shield in the maturation of SL RNA remains an outstanding issue. The role of this interaction may initiate in the nucleoplasm immediately upon release of the nascent SL RNA transcript, and could remain in association until the final stages of its maturation by serving to mediate the 3′-processing events prior to the final polish provided by the snRNA incomplete processing exonuclease (SNIP) (Zeiner et al., 2004b), back in the nucleoplasm after cytosolic trafficking. Consistent with its role in the maturation of other small RNA molecules, the La protein could act as a facilitator for intracellular transport and interaction with other processing enzymes, as well as providing a physical barrier to spurious processing events by opportunistic exonucleases.

Acknowledgements

We thank Paul T. Englund, Keith Gull, Klaus Ersfeld, and Kent L. Hill for providing RNAi vectors, Paul A.M. Michels for providing the GAPDH antisera, and Gus M. Zeiner and members of our laboratories for stimulating discussions and helpful comments. This work was supported by NIH grants AI34536 and AI056034 to NRS and DAC, and by grants A5022302 and Z60220518 from the Grant Agency of the Czech Academy of Sciences to JL.

References

Aigner, S., Lingner, J., Goodrich, K.J., Grosshans, C.A., Shevchenko, A., Mann, M., Cech, T.R., 2000. *Euplotes* telomerase contains an La motif protein produced by apparent translational frameshifting. Eur. Mol. Biol. Org. J. 19, 6230–6239.

Arhin, G.K., Shen, S., Irmer, H., Ullu, E., Tschudi, C., 2004. Role of a 300-kilodalton nuclear complex in the maturation of *Trypanosoma brucei* initiator methionyl-tRNA. Eukaryot. Cell 3, 893–899.

Aye, M., Sandmeyer, S.B., 2003. Ty3 requires yeast La homologous protein for wild-type frequencies of transposition. Mol. Microbiol. 49, 501–515.

Bangs, J.D., Crain, P.F., Hashizume, T., McCloskey, J.A., Boothroyd, J.C., 1992. Mass spectrometry of mRNA cap 4 from trypanosomatids reveals two novel nucleosides. J. Biol. Chem. 267, 9805–9815.

Bruzik, J.P., Van Doren, K., Hirsh, D., Steitz, J.A., 1988. *Trans* splicing involves a novel form of small nuclear ribonucleoprotein particles. Nature 335, 559–562.

Cardinali, B., Carissimi, C., Gravina, P., Pierandrei-Amaldi, P., 2003. La protein is associated with terminal oligopyrimidine mRNAs in actively translating polysomes. J. Biol. Chem. 278, 35145–35151.

Chakshusmathi, G., Kim, S.D., Rubinson, D.A., Wolin, S.L., 2003. A La protein requirement for efficient pre-tRNA folding. Eur. Mol. Biol. Org. J. 22, 6562–6572.

Dong, G., Chakshusmathi, G., Woplin, S.L., Reinisch, K.M., 2004. Structure of the La motif: a winged helix domain mediates RNA binding via a conserved aromatic patch. Eur. Mol. Biol. Org. J. 23, 1000–1007.

Hannaert, V., Opperdoes, F.R., Michels, P.A., 1995. Glycosomal glyceraldehyde-3-phosphate dehydrogenase of *Trypanosoma brucei* and *Trypanosoma cruzi*: expression in *Escherichia coli*, purification, and characterization of the enzymes. Protein Expr. Purif. 6, 244–250.

Hutchings, N.R., Donelson, J.E., Hill, K.L., 2002. Trypanin is a cytoskeletal linker protein and is required for cell motility in African trypanosomes. J. Cell Biol. 156, 867–877.

Intine, R.V., Dundr, M., Misteli, T., Maraia, R.J., 2002. Aberrant nuclear trafficking of La protein leads to disordered processing of associated precursor tRNAs. Mol. Cell 9, 1113–1123.

Ismaïli, N., Pérez-Morga, D., Walsh, P., Mayeda, A., Pays, A., Tebabi, P., Krainer, A.R., Pays, E., 1999. Characterization of a SR protein from *Trypanosoma brucei* with homology to RNA-binding *cis*-splicing proteins. Mol. Biochem. Parasitol. 102, 103–115.

Kickhoefer, V.A., Poderycki, M.J., Chan, E.K.L., Rome, L.H., 2002. The La RNA-binding protein interacts with the vauly RNA and is vault-associated protein. J. Biol. Chem. 277, 41282–41286.

Kufel, J., Tollervey, D., 2003. 3′-processing of yeast $tRNA^{Trp}$ precedes 5′-processing. RNA 9, 202–208.

Laird, P.W., ten Asbroek, A.L., Borst, P., 1987a. Controlled turnover and 3′ trimming of the *trans* splicing precursor of *Trypanosoma brucei*. Nucleic Acids Res. 15, 10087–10103.

Laird, P.W., Zomerdijk, J.C., de Korte, D., Borst, P., 1987b. In vivo labelling of intermediates in the discontinuous synthesis of mRNAs in *Trypanosoma brucei*. Eur. Mol. Biol. Org. J. 6, 1055–1062.

LeBowitz, J.H., Smith, H.Q., Rusché, L., Beverley, S.M., 1993. Coupling of poly(A) site selection and *trans*-splicing in *Leishmania*. Genes Dev. 7, 996–1007.

Liang, X.-h., Xu, Y.-x., Michaeli, S., 2002. The spliced leader-associated RNA is a trypanosome-specific sn(o) RNA that has the potential to guide pseudouridine formation on the SL RNA. RNA 8, 237–246.

Liang, X.-h., Haritan, A., Uliel, S., Michaeli, S., 2003. *Trans* and *cis* splicing in trypanosomatids: mechanisms, factors, and regulation. Eukaryot. Cell 2, 830–840.

Lücke, S., Klöckner, T., Palfi, Z., Boshart, M., Bindereif, A., 1997. *Trans* mRNA splicing in trypanosomes: cloning and analysis of a PRP8-homologous gene from *Trypanosome brucei* provides evidence for a U5-analogous RNP. Eur. Mol. Biol. Org. J. 16, 4433–4440.

Mandelboim, M., Barth, S., Biton, M., Liang, X.-h., Michaeli, S., 2003. Silencing of Sm proteins in *Trypanosoma brucei* by RNAi captured a novel cytoplasmic intermediate in SL RNA biogenesis. J. Biol. Chem. 278, 51469–51478.

Maraia, R.J., Intine, R.V., 2002. La protein and its associated small nuclear and nucleolar precursor RNAs. Gene Exp. 10, 41–57.

Maraia, R.J., Kenan, D.J., Keene, J.D., 1994. Eukaryotic transcription termination factor La mediates transcript release and facilitates reinitiation by RNA polymerase III. Mol. Cell. Biol. 14, 2147–2158.

Marchetti, M.A., Tschudi, C., Kwon, H., Wolin, S.L., Ullu, E., 2000. Import of proteins into the trypanosome nucleus and their distribution at karyokinesis. J. Cell Sci. 113, 899–906.

Matthews, K.R., Tschudi, C., Ullu, E., 1994. A common pyrimidine-rich motif governs *trans*-splicing and polyadenylation of tubulin polycistronic pre-mRNA in trypanosomes. Genes Dev. 8, 491–501.

Miller, S.I., Wirth, D.F., 1988. *Trans* splicing in *Leishmania enriettii* and identification of ribonucleoprotein complexes containing the spliced leader and U2 equivalent RNAs. Mol. Cell. Biol. 8, 2597–2603.

Palfi, Z., Lücke, S., Lahm, H.-W., Lane, W.S., Kruft, V., Bragado-Nilsson, E., Séraphin, B., Bindereif, A., 2000. The spliceosomal snRNP core complex of *Trypanosoma brucei*: Cloning and functional analysis reveals seven Sm protein constituents. Proc. Natl Acad. Sci. USA. 97, 8967–8972.

Pannone, B.K., Xue, D., Wolin, S.L., 1998. A role for the yeast La protein in U6 snRNP assembly: evidence that the La protein is a molecular chaperone for RNA polymerase III transcripts. Eur. Mol. Biol. Org. J. 17, 7442–7453.

Preiser, P., Vasisht, V., Birk, A., Levinger, L., 1993. Poly(U)-binding protein inhibits *Drosophila* pre-5 S RNA 3′-exonuclease digestion. J. Biol. Chem. 268, 11553–11557.

Pudi, R., Abhiman, S., Srinivasan, N., Das, S., 2003. Hepatitis C virus internal ribosome entry site-mediated translation is stimulated by specific interaction of independent regions of human La autoantigen. J. Biol. Chem. 278, 12231–12240.

Roberts, T.G., Dungan, J.M., Watkins, K.P., Agabian, N., 1996. The SLA RNA gene of *Trypanosoma brucei* is organized in a tandem array which encodes several small RNAs. Mol. Biochem. Parasitol. 83, 163–174.

Schneider, A., McNally, K.P., Agabian, N., 1993. Splicing and 3′-processing of the tyrosine tRNA of *Trypanosoma brucei*. J. Biol. Chem. 268, 21868–21874.

Stefano, J.E., 1984. Purified lupus antigen La recognizes an oligouridylate stretch common to the 3′ termini of RNA polymerase III transcripts. Cell 36, 145–154.

Sturm, N.R., Fleischmann, J., Campbell, D.A., 1998. Efficient *trans*-splicing of mutated spliced leader exons in *Leishmania tarentolae*. J. Biol. Chem. 273, 18689–18692.

Sturm, N.R., Yu, M.C., Campbell, D.A., 1999. Transcription termination and 3′-end processing of the spliced leader RNA in kinetoplastids. Mol. Cell. Biol. 19, 1595–1604.

Tschudi, C., Ullu, E., 1990. Destruction of U2, U4, or U6 small nuclear RNA blocks *trans* splicing in trypanosome cells. Cell 61, 459–466.

Ullu, E., Tschudi, C., 1990. Permeable trypanosome cells as a model system for transcription and *trans*-splicing. Nucleic Acids Res. 18, 3319–3326.

Van Horn, D.J., Yoo, C.J., Xue, D., Shi, H., Wolin, S.L., 1997. The La protein in *Schizosaccharomyces pombe*: a conserved yet dispensable phosphoprotein that functions in tRNA maturation. RNA 3, 1434–1443.

Wang, Z., Morris, J.C., Drew, M.E., Englund, P.T., 2000. Inhibition of *Trypanosoma brucei* gene expression by RNA interference using an integratable vector with opposing T7 promoters. J. Biol. Chem. 275, 40174–40179.

Westermann, S., Weber, K., 2000. Cloning and recombinant expression of the La RNA-binding protein from *Trypanosoma brucei*. Biochim. Biophys. Acta 1492, 483–487.

Wickstead, B., Ersfeld, K., Gull, K., 2002. Targeting of a tetracycline-inducible expression system to the transcriptionally silent minichromosomes of *Trypanosoma brucei*. Mol. Biochem. Parasitol. 125, 211–216.

Wolin, S.L., Cedervall, T., 2002. The La protein. Annu. Rev. Biochem. 71, 375–403.

Xue, D., Rubinson, D.A., Pannone, B.K., Yoo, C.J., Wolin, S.L., 2000. U snRNP assembly in yeast requires the La protein. Eur. Mol. Biol. Org. J. 19, 1650–1660.

Yoo, C.J., Wolin, S.L., 1994. La protein from *Drosophila melanogaster* and *Saccharomyces cerevisiae*: a yeast homolog of the La autoantigen is dispensible for growth. Mol. Cell. Biol. 14, 5412–5424.

Yoo, C.J., Wolin, S.L., 1997. The yeast La protein is required for the 3′ endonucleolytic cleavage that matures tRNA precursors. Cell 89, 393–402.

Zeiner, G.M., Sturm, N.R., Campbell, D.A., 2003a. Exportin 1 mediates nuclear export of the kinetoplastid spliced leader RNA. Eukaryot. Cell 2, 222–230.

Zeiner, G.M., Sturm, N.R., Campbell, D.A., 2003b. The *Leishmania tarentolae* spliced leader contains determinants for association with polysomes. J. Biol. Chem. 278, 38175–38269.

Zeiner, G.M., Foldynová, S., Sturm, N.R., Lukeš, J., Campbell, D.A., 2004a. SmD1 is required for spliced leader RNA biogenesis. Eukaryot. Cell 3, 241–244.

Zeiner, G.M., Hitchcock, R.A., Sturm, N.R., Campbell, D.A., 2004b. 3′-end polishing of the kinetoplastid spliced leader RNA is performed by SNIP, a 3′ to 5′ exonuclease with a motley assortment of small RNA substrates. Mol. Cell. Biol. 24, 10390–10396.