Molecular & Biochemical Parasitology 164 (2009) 137–146

Contents lists available at ScienceDirect

Molecular & Biochemical Parasitology

Functional characterization and protein–protein interactions of trypanosome splicing factors U2AF35, U2AF65 and SF1

Martin P. Vazquez[a,b], David Mualem[c], Natalia Bercovich[a,b], Michael Zeev Stern[c], Benson Nyambega[a,d], Omer Barda[c], Dan Masiga[d], Sachin Kumar Gupta[c], Shulamit Michaeli[c], Mariano J. Levin[a,e,*]

[a] Laboratorio de Biología Molecular de la Enfermedad de Chagas, INGEBI- CONICET, Vuelta de Obligado 2490 2P, 1428, Buenos Aires, Argentina
[b] Departamento de Fisiología y Biología Molecular y Celular, Facultad de Ciencias Exactas y Naturales, University of Buenos Aires, Argentina
[c] The Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan 52900, Israel
[d] International Center for Insect Physiology and Ecology, P.O.Box 30772-00100, Nairobi, Kenya
[e] Institut Cochin, Departement Maladies Infectieuses, INSERM U567, Paris F-75014, France

ARTICLE INFO

Article history:
Received 17 August 2008
Received in revised form
24 November 2008
Accepted 30 December 2008
Available online 15 January 2009

Keywords:
Trypanosoma cruzi
Trypanosoma brucei
Trans-splicing
U2 auxiliary factor

ABSTRACT

Early in the assembly of the spliceosome of eukaryotes the branch-point binding protein (BBP, also called SF1) recognizes the branch point sequence, whereas the heterodimer U2AF consisting of a 65 and a 35 kDa subunit, contacts the polypyrimidine tract and the AG splice site, respectively. Herein, we identified, cloned and expressed the *Trypanosoma cruzi* and *Trypanosoma brucei* U2AF35, U2AF65 and SF1. Trypanosomatid U2AF65 strongly diverged from yeast and human homologues. On the contrary, trypanosomatid SF1 was conserved but lacked the C-terminal sequence present in the mammalian protein. Yeast two hybrid approaches were used to assess their interactions. The interaction between U2AF35 and U2AF65 was very weak or not detectable. However, as in other eukaryotes, the interaction between U2AF65 and SF1 was strong. At the cellular level, these results were confirmed by fractionation and affinity-selection experiments in which SF1 and U2AF65 co-fractionated in a complex of approximately 400 kDa and U2AF65 was affinity-selected with TAP tagged SF1, but not with TAP tagged U2AF35. Silencing of the three factors affected growth and *trans*-splicing in the first step of this reaction. Trypanosomes are the first described example of eukaryotic cells in which the interaction of two expressed U2AF factors seemed to be very weak, or even undetectable.

1. Introduction

The genome sequences of the three trypanosomatid species suggest that in these parasites entire chromosomes are transcribed as long polycistronic transcripts that are maturated by the concerted action of *trans*-splicing and polyadenylation [1,2]. Through *trans*-splicing, the first 39-nt (miniexon or spliced leader, SL) of a 139 nt long capped RNA, the SL RNA, is joined at appropriate sites to each of the protein-coding exons of the primary transcripts. Thus, SL addition serves two functions: together with polyadenylation, it dissects mRNAs from polycistronic primary transcripts, and it provides the cap structure to all mRNAs. In contrast to *cis*-splicing, *trans*-splicing joins exons derived from two independently transcribed RNAs. Mechanistically, however, *trans*- and *cis*- splicing share several common features, both require the same motifs, the GU at the 5′ splice site (SS), the adenosine branch point, a polypyrimidine tract (Py) and the AG at the 3′ SS [1,2].

Early in the assembly of the spliceosome of higher eukaryotes the branch point sequence is recognized by the branch-point binding protein (BBP, also called SF1) [3]. Human U2AF was initially identified as an activity necessary for the recruitment of U2 snRNP to the branchpoint [4] and consists of a 65 [5] and a 35 kDa subunit [6]. The large subunit contacts the Py [5], while the 35 kDa subunit (U2AF35) binds to the 3′SS AG [7]. Thus, splicing at the first AG downstream of the branch point is a consequence of the combined interactions between these two factors. In eukaryotes, U2AF65 and U2AF35 form a heterodimeric complex in which the RRM of U2AF35 and a central polyproline segment of U2AF65 interact via reciprocal "tongue in groove" involving tryptophan residues [8]. The latter is composed by an N-terminal Arg-Ser (RS) rich domain followed by three RRM motifs. The N-terminal domain helps to strengthen the interaction between the U2 snRNP and the branch point region [9], whereas the first two RRM domains mediate the interaction with the Py tract [10]. The third RRM domain, RRMIII, is engaged in a strong unidirectional "tongue in groove" interaction with a tryptophan residue of the N-terminal portion of SF1

* Corresponding author at: Laboratorio de Biología Molecular de la Enfermedad de Chagas, INGEBI- CONICET, Vuelta de Obligado 2490 2P, 1428, Buenos Aires, Argentina. Tel.: +54 11 4783 2871; fax: +54 11 4786 8578.
E-mail address: mlevin@dna.uba.ar (M.J. Levin).

0166-6851/$ – see front matter © 2009 Published by Elsevier B.V.
doi:10.1016/j.molbiopara.2008.12.009

[10]. The U2AF35-65 heterodimer has been described in humans [11], *Caenorhabditis elegans* [12], *Drosophila melanogaster* [13] and *Schizosaccharomyces pombe* [14], and in these species its existence is essential for life. However, in *S. cerevisiae* no homologue of U2AF35 exists, and U2AF65 is not essential [14].

In trypanosomes, no homologue of U2AF65 has been yet identified and the only protein of the U2AF complex that has been characterized is the U2AF35 of *Trypanosoma cruzi* [15]. This protein shared 38% identity with its human homologue, and immunolocalization demonstrated its distribution in nuclear speckles [15]. Interestingly, the central RRM motif of TcU2AF35 was surrounded by two CCCH zinc finger motifs (Cx8Cx5Cx3H) and instead of the classical C-terminal SR domain it accommodated a third and peculiar zinc finger motif, CCHC. Residues Thr 45, Leu 47 and Tyr 114, known to be directly involved in RNA recognition were conserved. On the contrary, only two of the seven residues that were essential for the interaction with U2AF65 were present in the *T. cruzi* protein. Notably, Trp 134, necessary for the reciprocal "tongue in groove" heterodimerization with U2AF65, was changed to Lys, suggesting that, in trypanosomes, the U2AF35–U2AF65 interaction could be altered.

In this study we identified, cloned and expressed the *T. cruzi* and *T. brucei* U2AF65 and SF1, together with the U2AF35 of *T. brucei*. As predicted from sequence analyses, the interaction between U2AF35 and U2AF65 was very weak or not detectable. However the typical U2AF65, unidirectional "tongue in groove" interaction with SF1 was as strong as the one described for other organisms. Silencing of all three factors affected growth and *trans*-splicing in the first step of this reaction [2,16,17,18]. Trypanosomes are the first described example of eukaryotic cells in which both U2AF factors are expressed but do not engage in a strong protein–protein interaction. This feature may be related to the very flexible distance requirements between the 3′ splice site and branch point of trypanosome pre-mRNAs [19].

2. Materials and methods

2.1. Cloning of Tc/Tb U2AF35, Tc/Tb U2AF65 and Tc/TbSF1 in the gateway system

The coding sequences of these genes were PCR amplified from 100 ng of total genomic DNA of *T. cruzi* CL-Brener and *T. brucei* 927 strain using the primers specified in Supplemental Material.

PCR products were cloned into pGEM-T (Promega), subsequently digested with *Kpn*I and *Eco*RI, *BamH*I and *Not*I or *BamH*I and *Eco*RI, respectively and subcloned in frame into the Gateway entry vector pENTR2B (Invitrogen).

Regions and domains were amplified from the corresponding full protein pGEM-T clones. Amino-terminal U2AF65 was made by using primer 65up and 65-Nterm 5′CTAATCGCGTGGCCGACGGATTG 3′. U2AF65RRM2 was made by using primer 65RRM2up 5′GGATCCCAGACGCAAGGTTGTTATC 3′ and 65RRM2down 5′CT AGCTGCCGTACAACGTTGA 3′. pRRM3 was made by using primer 65pRRMup 5′GGATCCCACGGTGCCTATTTACCCCA and 65down.*T. brucei* coding sequences were amplified from gDNA using the following primers: TbSF1 with 5′ GCTAGCGATGGGGGAGAACCGTCGAC 3′ up containing a NheI site and 5′ GCGGCCGCCTACAACTCGTCCAGAAA 3′ down containing a NotI site for direct cloning into the Binding Domain vector pDBLeu (Invitrogen). TbU2AF35 with 5′GGATCCCATGTATCAAGACCGTTGC 3′ up containing BamHI and 5′ TTATTTTAAGGGGCATTCGCG 3′ down. TbU2AF65 with 5′ GGATCCAATGGGGCGTGATAGTCGCG 3′ up containing BamHI and 5′ TTAACCGTCAATACCTGCGATAC 3′ down with NotI site.

Construction of modified proteins. The TcSF1PPRR was made by PCR amplification using a sense primer containing the mutations CCG (Pro) to CGG (Arg) and CCT (Pro) to CGT (Arg), SF1PPRR 5′GGATCCGGAGGCGAAACGGAGGCG TTCGC 3′. The TcSF1SRDA was made by PCR amplification where the sense primer contain the mutation CGC (Arg) to GAC (Asp) and TGG (Trp) to GCG (Ala), RWDA 5′GGATCCGGCGAAACCGAGGCCTTCGGACGCGAGCAAAGAG3′.
SF1down was used as antisense primer in both amplifications. TcU2AF35WF was generated by SOEing PCR [20] as follows, two complementary internal primers were designed containing the mutations AAG (Lys) to TGG (Trp) and TTA (Leu) to TTT (Phe) in the RRM domain, tc35WF-s 5′GGAACTAAAGGCGAAATGGTTTAACGAAAT 3′ and tc35WF-as 5′ATTTCGTT AAACCATTTCGCCTTTAGTTCC 3′. These primers were used in combination with the external primers 35down and 35up, respectively, to produce the PCR hemi-products. These hemi-products were gel purified and combined in equal amounts to perform a second PCR with the external primers to generate the final product that was Gateway cloned as described.

The deletion mutant pRRM3Δnors was also generated by SOEing PCR using the internal complementary primers pRRMdel-up 5′CCGCCGTGCCCCACCACCGATGCATCCAATTCACGGTGGTTTTGG 3′ and pRRMdel-down 5′CATCGGTGGTGGGG CACGGCGG 3′ and the external primers 65down and 65pRRMup, respectively.

All PCRs were performed using the proofreading AccuTaq enzyme (Sigma–Aldrich). All products were sequenced on a MegaBACE 500 (Amersham Biosciences) capillary sequencer to verify the mutations introduced.

2.2. Yeast two-hybrid pair wise analysis

The ProQuest Yeast Two-Hybrid Gateway compatible System (Invitrogen) was used for protein–protein interaction analysis as previously described [21].

Identification of proteins that interact with U2AF35 and U2AF65 by yeast two-hybrid (Y2H) screening. Bait cloning and Y2H screening were performed by Hybrigenics, S.A., Paris, France (http://www.hybrigenics.com/services.html).

DNA from *Trypanosoma brucei* 927 strain, prepared by Dr. M. Turner, Glasgow Biomedical Research Centre, University of Glasgow, Scotland, UK, was randomly sheared and used to construct genomic library into the Y187 yeast strain. The library contained 7.5 million independent fragments, and was used for screening [22]. Forty-four million interactions were actually tested with U2AF35 and 97 million with U2AF65. After selection on medium lacking Leu, Trp and His, 190 positive clones were picked from U2AF35 full-scale screen and 36 positive clones from U2AF65 full-scale screen.

2.3. Protein extract preparation

For total protein extract preparation, parasites were resuspended in lysis buffer (5% NP40, 2 M $MgCl_2$, 500 mM EDTA, 10 mM DTT, 250 mM Tris–HCl (pH 7.6), 100% glycerol, 0.25 M sucrose) supplemented with protease inhibitors as described in Ref. [23].

2.4. RNA silencing and transfection of T. brucei

The constructs were generated following protocols described in Ref. [16] and cloned into the pZJM and pLew100 vector. To generate the *T. brucei* transgenic cell lines, strain 29–13 was used for transfection and clonal population of the transgenic parasites were obtained as described [16]. Northern analysis and primer extension to monitor the silencing and splicing defects were as previously described [16]. A list of all the oligonucleotides used in these assays is provided as Supplemental Material.

TAP-tag purification of T. brucei U2AF35, U2AF65, SF1. To generate the TAP-tagged versions, the genes were amplified with primers specified in Supplemental Material.

The fragments were cloned into the pLew79-MHTAP (carrying also Myc-His tags between the protein and the TAP-tag, kindly provided by M. Parsons and B. Jensen, Seattle Biomedical Institute, WA, USA) as described in Ref. [23]. After verifying the sequence, the plasmids were transfected *to T. brucei* 29–13 as described above. Expression of the tagged proteins was induced for 60 h with tetracycline (10 μg/ml). Cells (500 ml ~10^7 cells/ml) were harvested and washed with PBS. Extracts were prepared essentially as described in Ref. [23], except that extraction was at 50 mM KCl to preserve large intact splicesomal complexes. The extracts were fractionated on a Superdex 200 column as described in Ref. [25]. To examine the association of SF1, with U2AF65, extracts were prepared as descried previously [23], except that 0.1% NP40 was added to the cells, they were lysed by douncing 20 times with a type A pestle. The TAP-tagged SF1 and U2AF35 were purified using IgG-agarose beads as in Ref. [23]. The proteins bound to the beads were separated on a 10% SDS-PAGE gel, blotted and reacted with anti-TcU2AF65 antibodies (this study).

Immunofluorescence cells of T. brucei expressing the TAP-tagged proteins. Cell were washed with PBS, mounted on poly-L-lysine coated slides, and fixed with 4% formaldehyde in PBS at room temperature for 30 min. Cells were incubated with PBS containing 10% FCS at room temperature for 30 min, and then with IgG antibodies. Later, the cells were washed with PBS, and reacted with anti-goat

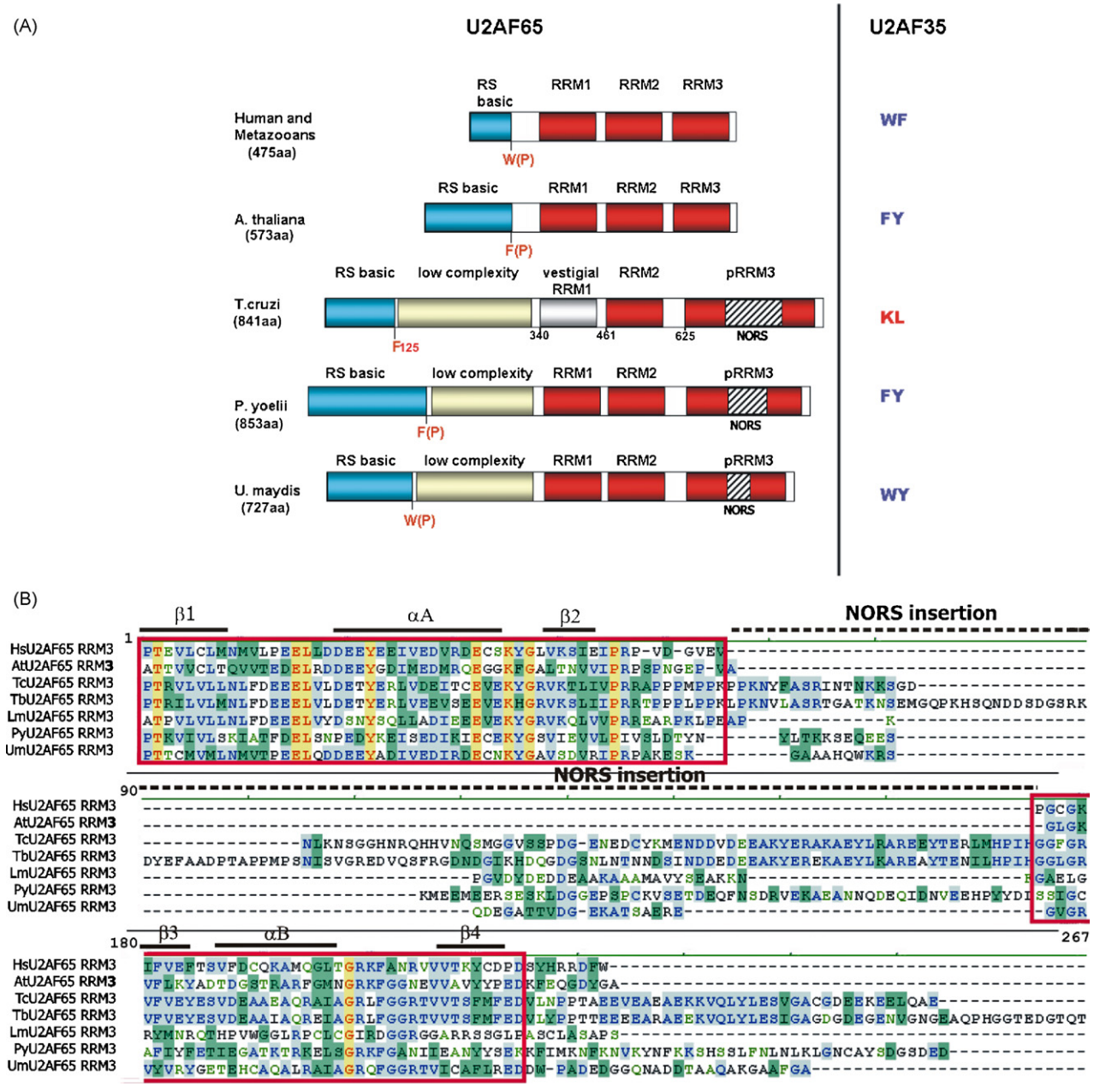

Fig. 1. Analysis of the divergent trypanosome U2AF65. (A) Schematic representation of U2AF65 splicing factors from different organisms. Domains are depicted and the aromatic (W or F) residues in a proline environment that are necessary for interaction with U2AF35 are also indicated. On the right, aromatic residues present in the different U2AF35 proteins are indicated. Note that *Trypanosoma cruzi*, lost both aromatic amino acids in those positions. (B) Sequence alignment of RRM III domains. *T. cruzi* pRRM III domain sequence from region 625 to 841 was aligned to RRM III domains from Human (HsU2AF65 RRM3), *Arabidopsis thaliana* (AtU2AF65 RRM3), *Trypanosoma brucei* (TbU2AF65 RRM3), *Leishmania major* (LmU2AF65 RRM3), *Plasmodiun yoelli* (PyU2AF65 RRM3) and *Ustilago maydis* (UmU2AF65 RRM3). The two halves of RRM III structure are red enclosed and NORS insertions indicated by a dashed line.

anti-rabbit conjugated to fluorescein (FITC). The cells were imaged by the Zeiss LSM 510 META inverted microscope as previously described [24].

3. Results

3.1. Trypanosome U2AF65 splicing factor

BLAST searches probing the Tri-tryp database (http://www.genedb.org/) with the 470 amino acid long human U2AF65 protein, as well as profile based searches with RNA recognition motif (RRM) were both unable to identify any trypanosome orthologue. This implied that if the parasite U2AF65 protein existed, it deviated strongly from its orthologues. Using the first 50 amino acids of the human U2AF65 RRMIII domain as query in PSI-BLAST, a *T. cruzi* gene containing this domain was identified (E_{value} = 1.4e−06). The encoded protein was composed of 841 amino acids. The homologous protein in *T. brucei* was 878, and in *L. major* 758 amino acids long. Trypanosome U2AF65 conserved the N-terminal RS basic domain, followed by a low complexity region (Fig. 1A). The C-terminal portion contained three RRM domains (I, II and III) arranged in tandem, but two of them differed from the mammalian ones. In RRMI, only the RNP-1 signature was evident, termed vestigial RRM1, vRRM1 in Fig. 1A. RRMII was conserved, whereas the RRMIII domain (pRRMIII in Fig. 1A) included the insertion of a hydrophilic region without secondary structure between the second and the third beta strands, classified as NORS, NOn-Regular secondary Structure, by the NORSp software, Fig. 1B and C, [25].

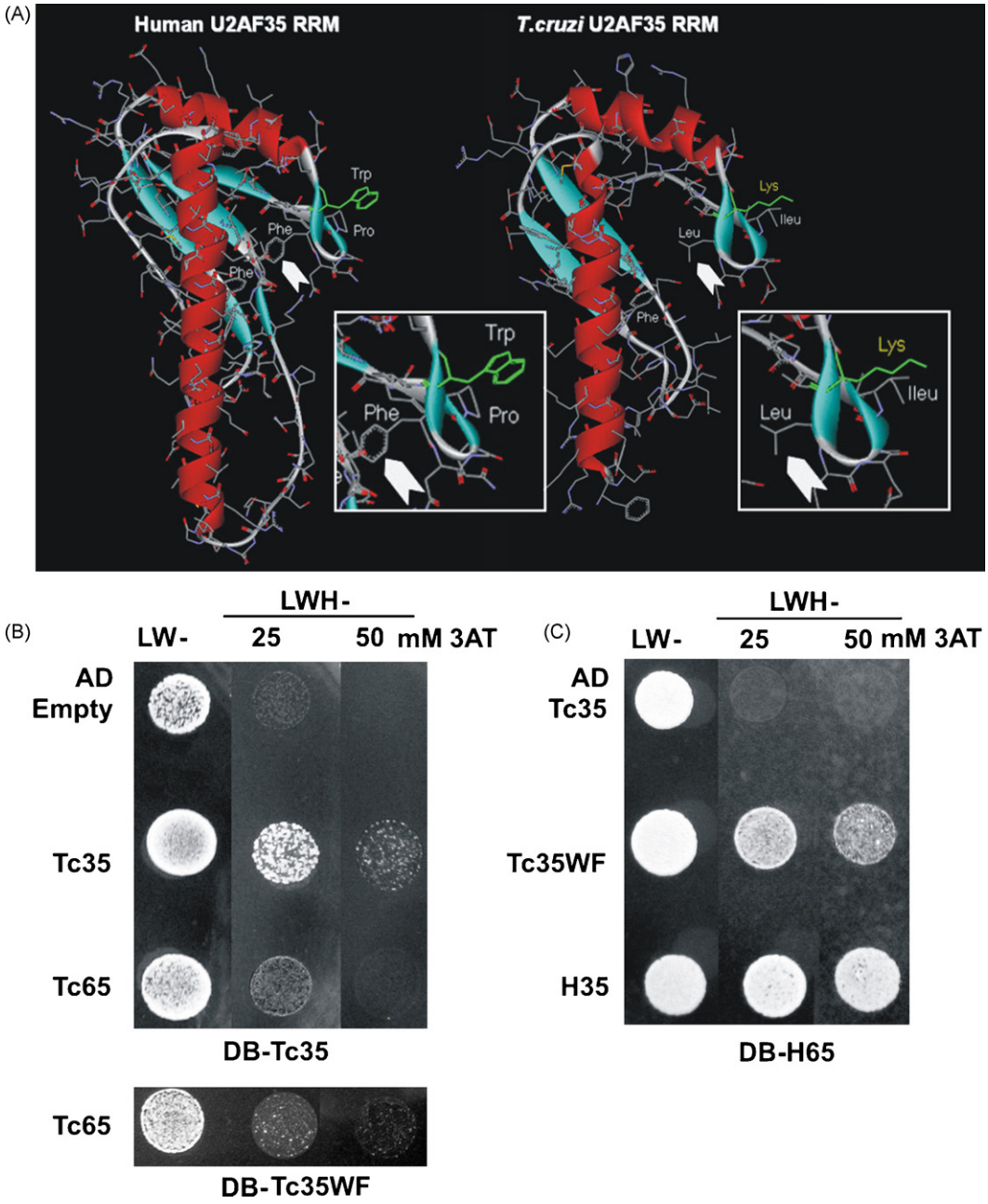

Fig. 2. Trypanosome U2AF35 interaction assays. (A) Molecular 3D modeling of human and *T. cruzi* U2AF35 RRMs. In green, the essential aromatic residues are indicated, aromatic residue Trp134 is replaced in *T. cruzi* by Lys122 at a homologous position. Arrowhead points to the essential Phe135 inside the hydrophobic pocket replaced in *T. cruzi* by Leu123. (B) Yeast two-hybrid assays. Tc35, TcU2AF35; Tc65, TcU2AF65; H35, human U2AF35; H65, human U2AF65; Tc35WF, mutant TcU2AF35 (Lys122 changed to Trp and Leu123 changed to Phe). AD, activaction domain. DB, DNA binding domain. LW-, non-selective plate. LWH-, selective plates. Yeast two hybrid assays were always performed in duplicates with independent clones. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

This region was 88, 120 and 32 amino acids long for *T. cruzi*, *T. brucei* and *L. major*, respectively. For both, American and African trypanosomes, the RRM structure was regenerated by deletion of the NORS sequence (Fig. 1B). This was not the case for *Leishmania major* because, in addition to the short intervening sequence, the fourth β strand of the RRMIII was replaced by an extra alpha helix (Fig. 1B and C).

Two other unicellular pathogens, *Plasmodium yoelii* and *Ustilago maydis*, have U2AF65 proteins with features that resemble those of trypanosomatids, Fig. 1A, low complexity regions between the RS domain and RRMI and a modified RRMIII, pRRM3 in Fig. 1A. The NORS in *P. yoelli* and *U. maydis* were 70 and 29 amino acids long, respectively. All NORS showed several conserved residues Fig. 1B.

In *P. yoelli* and *A. thaliana* both U2AF65 and U2AF35 had Phe residues for the reciprocal "tongue in grove" interaction (Fig. 1A) [8,26].

In trypanosomes, U2AF65 presented a Phe in position 125 (Fig. 1A).

Fig. 2A shows that human and trypanosome U2AF35 RRMs superimpose albeit with two main differences, the replacement of human Trp 134 and Phe 135 by Lys and Leu in trypanosomes (white arrowhead in Fig. 2A). The same was found for U2AF35 of *T. brucei* and *L. major*. Because trypanosome U2AF65 had a Phe in an environment that did not favor U2AF heterodimeric interaction, and U2AF35 lacked key Trp and Phe, it was predicted that they would not interact [15].

To test this hypothesis, yeast two hybrid pair wise interactions were examined. The strong interaction of the human U2AF65–U2AF35 heterodimer served as control (Fig. 2C). Results in Fig. 2B, showed that the interaction between TcU2AF65 and TcU2AF35 was very weak or not detectable, as was the case for the *T. brucei* pair (not shown), in the presence of 25 mM 3-aminotriazole, 3AT, which is the threshold level of detection for this system. This was also confirmed by screening a yeast two hybrid *T. brucei* genomic library with TbU2AF35 as a bait. *T. cruzi* U2AF35 did not interact with human U2AF65 (Fig. 2C). However, a "humanized" version of TcU2AF35, in which the Lys-Leu dipeptide was substituted by Trp-Phe, Tc35WF, did interact with human U2AF65 (Fig. 2C), suggesting reestablishment of the "tongue in groove" interaction. However, the Trp-Phe substitution did not seem to restore the interaction with TcU2AF65, or if it did, the interaction was very weak, below the threshold detection level (Fig. 2B, bottom panel). A moderate self-interaction for TcU2AF35 and Tc35WF was also detected (only the self-interaction of TcU2AF35 is shown in Fig. 2B).

3.2. Trypanosome SF1 and features of the trypanosome SF1–U2AF65 interaction

Using the 640 amino acids long human SF1, [27] to probe the Tritryp database allowed the identification of the *T. cruzi*, *T. brucei* and *L. major* SF1 versions that were 267, 271 and 289 amino acids long, respectively. They lacked the C-terminal Pro rich region and the CCHC Zinc finger motif, Fig. 3A, but conserved the KH RNA binding domain and the N-terminal Ser-Arg-Trp (SRW) motif, essential for interaction with the RRMIII of U2AF65, Fig. 3A [28]. The aromatic Trp residue of SF1 establishes an unidirectional interaction with the hydrophobic pocket of the RRMIII of U2AF65. In the case of human SF1, the Trp was accompanied by a run of basic amino acids that stabilized the SF1–U2AF65 interaction [28]. Trypanosome SF1 lacked this basic run. In *T. cruzi*, two Pro preceded the Trp (red circles in Fig. 3A) while in *T. brucei* and *L. major* only one Pro before the Trp was observed (Fig. 3A).

We tested the interaction of TcU2AF65 and TcSF1, in the two-hybrid assay. The interaction between human SF1 and the human U2AF65 RRMIII domain served as positive control (Fig. 3B).

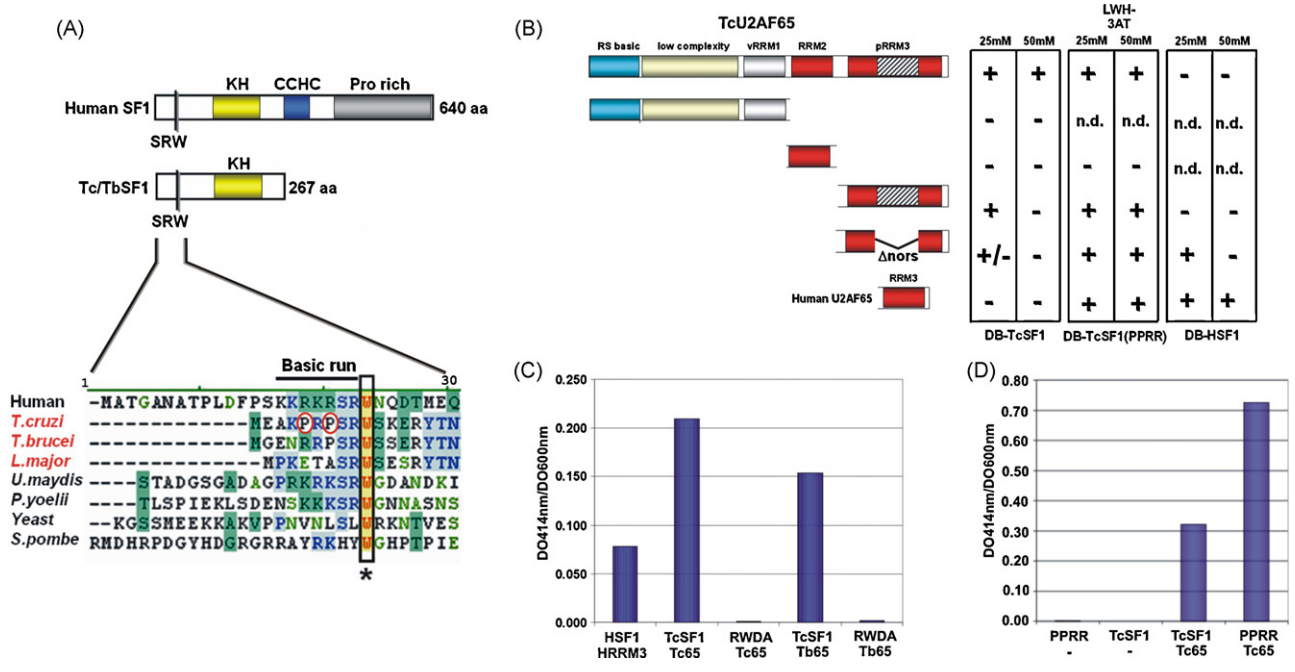

Fig. 3. Trypanosome SF1 interaction assays. (A) Trypanosomatid SF1 compared with eukaryotic counterparts. Schematic domain representation of human and trypanosomatid SF1. The SRW amino acids signature for interaction with U2AF65 is indicated. The amino acids surrounding the N-terminal SRW signature are compared for several organisms, where aromatic W is highlighted and the preceding basic run of residues is also indicated. Prolines interrupting the basic run in *T. cruzi* are highlighted in circles. (B) Interaction of SF1 with U2AF65. Deletion constructs of *T. cruzi* U2AF65 are shown on the left. Interactions with different SF1 constructs are shown on the right: (+) strong interaction, (−) negative interaction, (±) weak interaction, n.d. not determined. TcSF1, *T. cruzi*; HSF1, Human; TcSF1(PPRR), *T. cruzi* modified protein, the Pro (P) in circle were changed to Arg (R). LWH-, medium lacking Leu, Trp and His. Interactions were probed at two different concentrations of 3AT, 25 and 50 mM. (C) Mutation analysis of the SRW sequence. Yeast two-hybrid interactions were tested by activation of LacZ reporter gene in liquid ONPG assays. HRRM3, human RRM3 domain; Tc65, *T. cruzi* U2AF65; RWDA, *T. cruzi* SF1 modified protein amino acids RW were changed to DA; Tb65, *T. brucei* U2AF65. (D) Quantitative two-hybrid interaction assay for PPRR SF1 modified protein.

Remarkably, TcSF1 interacted strongly with TcU2AF65 (Fig. 3B). A similar result was obtained for the *T. brucei* SF1 and U2AF65 proteins (not shown) and confirmed by screening the yeast two hybrid *T. brucei* genomic library with TbU2AF65 as bait. The interaction between TcSF1 and TbU2AF65 was also tested (Fig. 3C). Deletion of the NORS from U2AF65 pRRMIII decreased the strength of the interaction (ΔNORS in Fig. 3B). No interaction of TcSF1 with the human RRMIII was observed (Fig. 3B). In contrast, human SF1 interacted only with ΔNORS pRRMIII, but not with the complete pRRMIII (Fig. 3B).

To test the SRW signature of TcSF1 as to its role in interaction with U2AF65, and confirm the specificity of the interaction, the Arg-Trp dipeptide (RW) was replaced by Glu-Ala, (DA) [28]. The modified TcSF1, named RWDA in Fig. 3C, did not recognize TcU2AF65, indicating that this motif was involved in the interaction.

Since the region preceding the Trp residue in TcSF1 was interrupted by Pro residues, we tested the effect of replacing them by Arg. This modified factor, named TcSF1PPRR, showed a twofold increase in the strength of its interaction with TcU2AF65 (Fig. 3D). Interestingly, SF1PPRR interacted strongly with both ΔNORS pRRMIII and the human RRMIII domain (Fig. 3B).

3.3. Cellular fractionation and affinity selection confirm yeast two hybrid results

To confirm the nature of the association among these factors, transgenic *T. brucei* cell lines expressing the corresponding tagged protein under control of the inducible EP promoter were generated. The transgenic parasites produced polypeptides of the expected molecular mass. To examine possible co-fractionation of these proteins in large complexes, whole cell extracts were prepared from each transgenic cell line and fractionated on Superdex 200 sizing column. The fractions were analyzed by Western blotting with antibodies against U2AF65 or the TAP for extracts prepared from cells expressing the TAP-tagged version of the proteins (TbU2AF35, TbU2AF65 and TbSF1). In Fig. 4A, we demonstrated co-fractionation of TbU2AF65 with TbSF1 but not with TbU2AF35. Next, we examined the ability to affinity select TbU2AF65 together with TbSF1. To this end, TAP tagged TbSF1 was affinity selected and the beads were examined for the co-selection of TbU2AF65 using anti-TcU2AF65 antibodies. The results (Fig. 4B, upper panel) showed that TbU2AF65 was co-selected with TbSF1. The specificity of the antibodies (two right lanes) was verified by demonstrating the reduction of

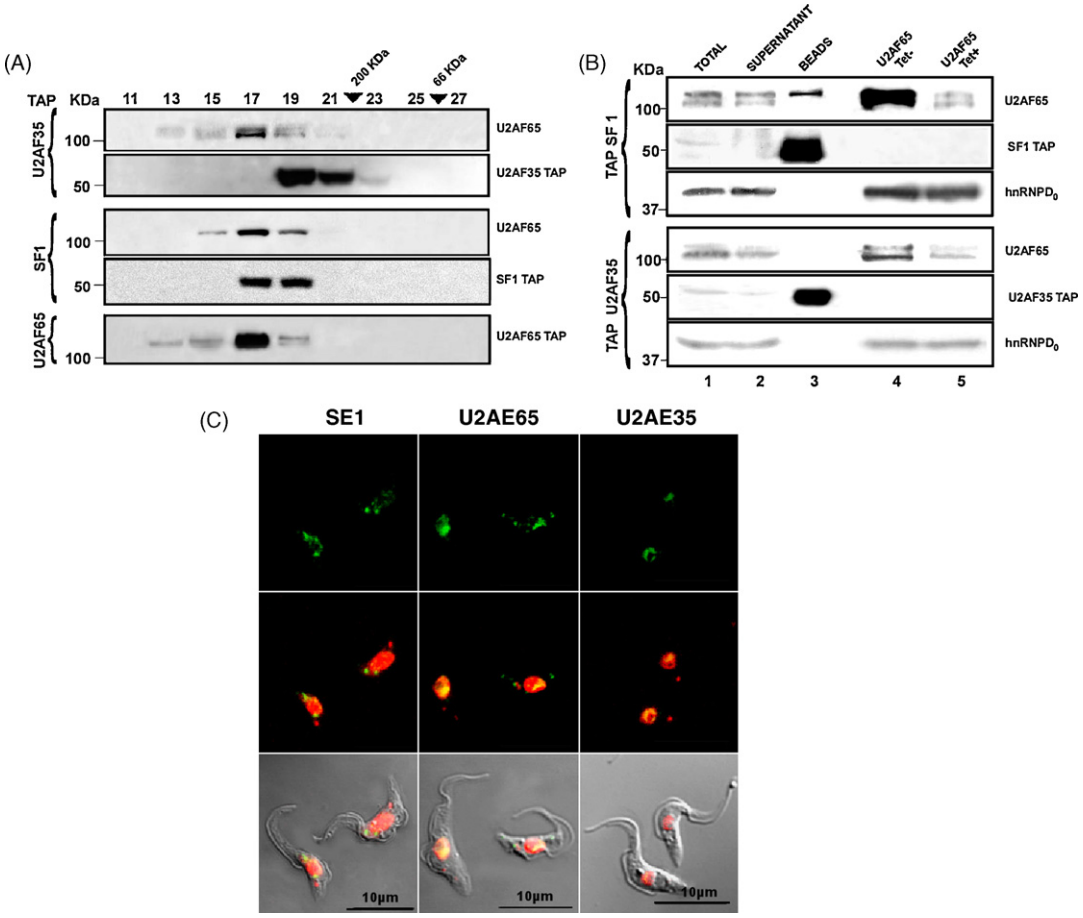

Fig. 4. (A) Fractionation of extracts from cells expressing TAP-tagged U2AF35, U2AF65 and SF1. Whole cell extracts were prepared from (500 ml ~10^7 cells/ml) expressing the fusions as described in Section 2. Proteins were subjected to Western analysis using anti-U2AF65 antibodies. Twenty percent of each fraction was analyzed. The identity of the protein examined is indicated. The identity of the cell line is indicated near the bracket. The elution positions of marker proteins BSA (66 kDa) and β-amylase (200 kDa) are indicated with arrows. (B) Affinity selection of U2AF65 with TAP-tagged SF1 and TAP-tagged U2AF35. Extracts were prepared from cell lines carrying the TAP-tagged proteins. After affinity selection, lane 1, total proteins from the extracts (5%); lane 2, Supernatant (5%) and lane 3, proteins from the whole beads. Lanes 4 and 5, proteins from cell line carrying the U2AF65 silencing construct, before induction (−Tet) and after 3 days of induction (+Tet), respectively. The proteins were subjected to Western analysis with anti-U2AF65 and anti-hnRNPD$_0$ antibodies. (29) (C) Immunofluoresence of TAP-tagged U2AF35, U2AF65 and SF1. Cells expressing the different tagged proteins were induced for 24 h and the tagged protein was visualized. Cells were fixed with 4% formaldehyde for 20 min and permeablized with 0.1% Nonidet P-40. The cells were incubated with IgG antibodies and nuclei stained with propidium iodide (PI). The images were obtained using confocal microscopy. Upper panel, FITC-conjugated anti-rabbit; middle panel, merge image with PI (red); lower panel, merged images with differential interference contrast (DIC). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

TbU2AF65 in the RNAi silenced cells after induction of silencing with tetracycline as in Fig. 6C. To demonstrate the specificity of selection, the presence of hnRNPD$_0$ was followed [29]. Indeed, hnRNPD$_0$ was not co-selected with TbSF1. When the same affinity selection was performed with TAP-tagged TbU2AF35, no reactivity with TbU2AF65 was detected (Fig. 4B, lower panel). These results were in complete agreement with the observations in yeast two-hybrid assays. On the contrary, in mammalian cells, U2AF35 and U2AF65 co-elute from a polyU-Sepharose column in 6 M urea after washing the column with 2 M KCl [30]. The size of the complex that included trypanosome U2AF65/SF1 is larger than 400 kDa, and may carry additional proteins. Indeed, in other eukaryotes, the RRMIII motif of U2AF65 is known to directly interact with the SAP155 component of the SF3a complex [31].

3.4. U2AF35, U2AF65 and SF1 were localized in the nucleus of trypanosomes

Antibodies against the epitope tagged versions of *T. brucei* U2AF65, U2AF35 and SF1 showed that the proteins were all localized in the nucleus as expected for their splicing factor activities (Fig. 4C). A peri-nuclear localization was also detected for SF1 and U2AF65, but not for U2AF35. This differential localization of SF1 and U2AF65 is in agreement with results that show the strong SF1–U2AF65 interaction in yeast two hybrid assays, density gradient fractionation and affinity-selection.

Antibodies against recombinant *T. cruzi* U2AF65, U2AF35 and SF1 showed the same localization pattern as in *T. brucei* (data not shown, and Ref. [15]).

3.5. Silencing of U2AF35, U2AF65 and SF1 in T. brucei suggest their involvement in the initial steps of trans-splicing

Using RNA interference (RNAi), we evaluated the functional relevance of U2AF35, U2AF65 and SF1 in *trans*-splicing.

First, we silenced the TbU2AF35 by producing dsRNA from two T7 opposing promoters. The construct was linearized for integration into the non-transcribed rRNA spacer. A clonal population was obtained as previously described [16]. The growth curve of the U2AF35 cell line showed that the cells depleted in U2AF35 cannot survive after Tet induction (Fig. 5A). No apparent morphological changes were observed in these cells. Northern analysis confirmed depletion of U2AF35 mRNA (Fig. 5B). Western blots showed the reduction in the protein levels (Fig. 5C).

As expected, TbU2AF35 depletion affected *trans*-splicing. As observed in Fig. 5D and E, around 171.33 ± 37.52% (based on three experiments) accumulation of the capped SL RNA, and 60.33 ± 15.56% (based on three independent experiments) reduction in the levels of the Y structure intermediate, with respect to the non-induced cells, were evident (Fig. 5D and E). These results suggest that silencing of U2AF35 inhibited the first step of splicing as observed in humans [11].

Initial experiments to silence TbU2AF65 and TbSF1 using T7 opposing promoter constructs failed. However, both genes were silenced using stem-loop constructs. The expression of the silencing products in this construct is achieved by the tetracycline inducible EP promoter [16,17,23]. After transfection with the corresponding linear stem loop constructs (described in Section 2), clonal cells were selected for further analysis. The growth curves for these cell lines showed inhibition of growth as a result of silencing

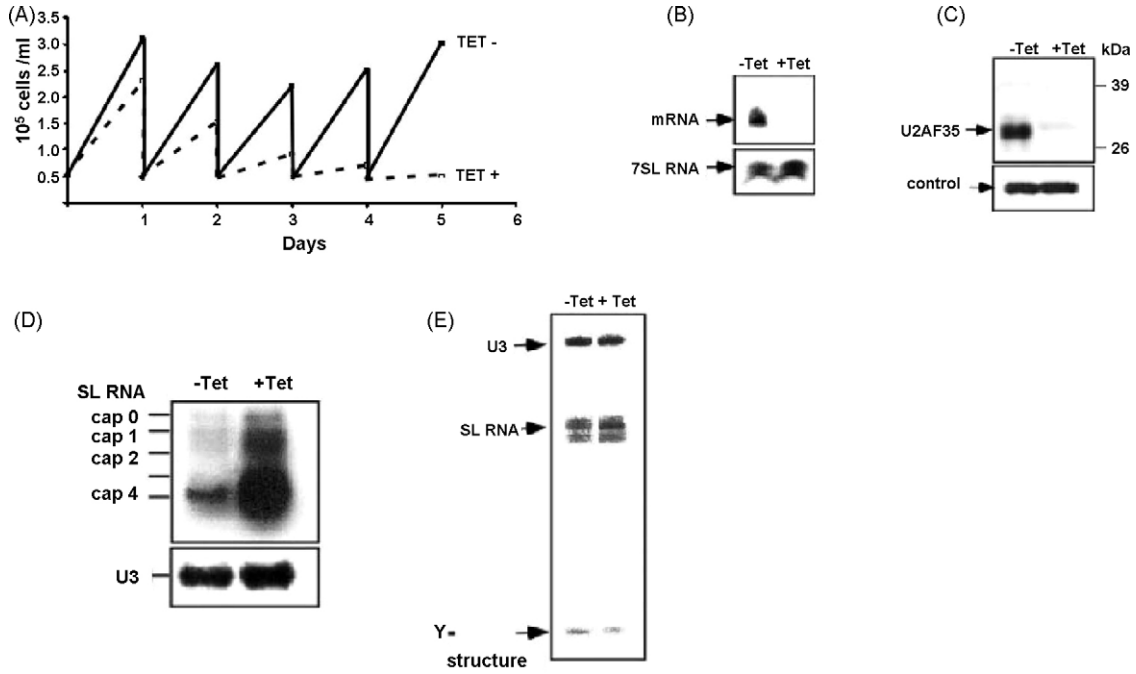

Fig. 5. RNAi silencing of TbU2AF35. (A) Growth curves of procyclic trypanosome cell line containing inducible U2AF35 RNAi constructs were obtained after RNAi induction with tetracycline or uninduced as control. Both induced and uninduced cultures were diluted daily to 5×10^4 cells per ml. The growth of uninduced cells (−Tet) was compared with cells induced for double-stranded RNA production (+Tet). (B) Northern analysis of U2AF35mRNA upon RNAi silencing. RNA was prepared from cells carrying the RNAi construct, uninduced (−Tet) and after 3 days of induction (+Tet). Total RNA (30 μg) was separated on 1.2% agarose, 2.2 M formaldehyde gel. The RNA was blotted and hybridized with a randomly labeled probe specific for the U2AF35 gene. The level of 7SL RNA (used as a control for equal loading) was determined. (C) Western analysis of U2AF35 protein. Whole cell extract (10^6 per lane) was prepared from cells carrying the RNAi construct, uninduced (−Tet) and after 3 days of induction (+Tet). The extract was fractioned on a 12% SDS-polyacrylamide gel and subjected to western analysis with the anti-U2AF35 antibody. Reactivity with antibodies to hnRNPDo was used as a control for the amount of protein loaded. (D) Effect of U2AF35 silencing on the level of SL RNA. Total RNA was prepared from cells carrying the RNAi construct without induction (−Tet) or after 3 days of induction (+Tet). Total RNA (10 μg) was subjected to primer extension with radiolabeled oligonucleotides complementary to SL RNA and U3 snoRNAs. The positions of the cap nucleotides are indicated. (E) The level of the Y structure intermediate. Total RNA from uninduced cells (−Tet) or after 3 days of induction was subjected to primer extension with an oligonucleotide situated in the intron region (specified in Supplementary Material). The products were separated on a denaturing gel. The positions of SL RNA and Y structure intermediate are marked with arrows. U3 snoRNA extension was used to demonstrate that equal amount of RNA was used in the experiment.

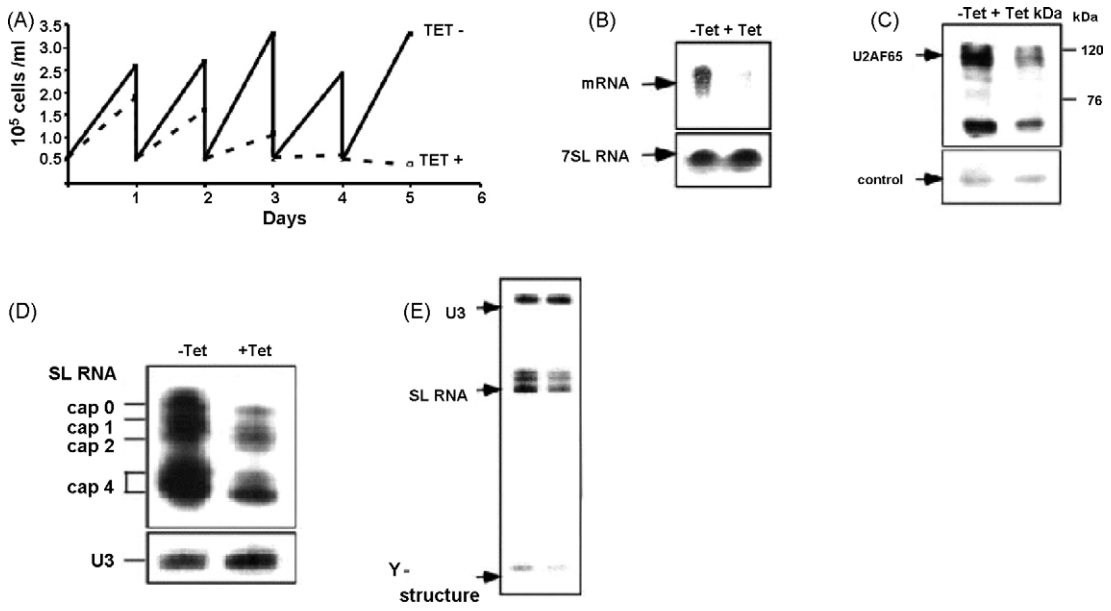

Fig. 6. RNAi silencing of TbU2AF65. (A) Growth curves of cells carrying the U2AF65 RNAi construct were prepared as explained in Fig. 5. The growth of uninduced cells (−Tet) was compared with cells induced for double-stranded RNA production (+Tet). (B) Northern analysis upon RNAi silencing was performed as in Fig. 5. (C) Western analysis of U2AF65 protein. Whole cell extract (10^6 per lane) was prepared from cells carrying the RNAi construct and the procedure was as described in Fig. 5. (D) Effect of U2AF65 silencing on the level of SL RNA. Total RNA was prepared from cells grown at 26 °C carrying the RNAi construct and the procedure was as described in Fig. 5. (E) Effect of U2AF65 silencing on the level of the Y structure intermediate in cells silenced. The procedure was as described in Fig. 5.

(Figs. 6A and 7A), suggesting that these factors were also essential for parasite viability. Degradation of U2AF65 mRNA upon TET induction was almost complete (Fig. 6B) but the protein decrease was only slight, an effect that may be explained by protein recycling (Fig. 6C). To assess if growth inhibition in the silenced cells was caused by deficiencies in splicing, the levels of Y structure and SL RNA were followed upon induction. In the case of U2AF65 cells, the levels of SL RNA were reduced by 60.33 ± 23.71% (based on three experiments) with respect to the non-induced cells (Figs. 6D), whereas Y structure formation decreased dramatically, around 74.25 ± 14.29% (based on three independent experiments) (Figs. 6E), suggesting that *trans*-splicing was inhibited by TbU2AF65 silencing. The SF1 silenced cells

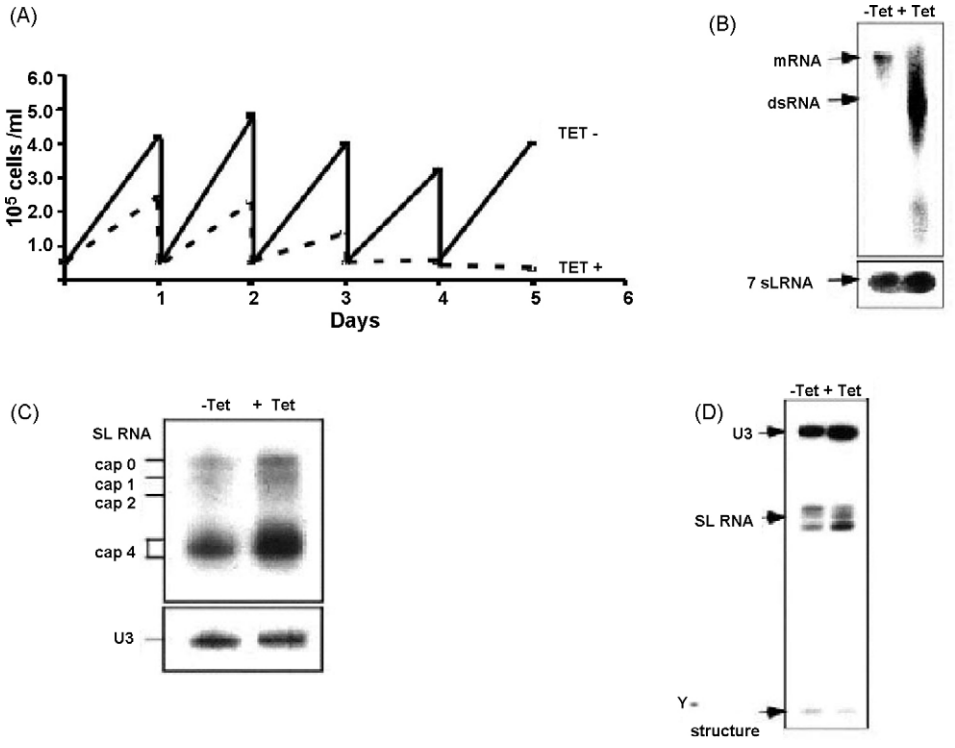

Fig. 7. RNAi silencing of TbSF1. (A) Growth curves of cells carrying the SF1 RNAi construct were prepared as explained in Fig. 5. The growth of uninduced cells (−Tet) was compared with cells induced for double-stranded RNA production (+Tet). (B) Northern analysis of SF1 mRNA upon RNAi silencing was performed as described in Fig. 5. (C) Effect of SF1 silencing on the level of SL RNA. Total RNA was prepared from cells grown at 26 °C carrying the RNAi construct and the procedure was as described in Fig. 5. (D) Effect of SF1 silencing on the level of the Y structure intermediate in cells silenced. The procedure was as described in Fig. 5.

showed an increase of 142 ± 21.21% in the levels of SL upon tetracycline induction (Figs. 7C), and a decrease in the levels of Y structure intermediate of 58.66 ± 24.54% (based on three independent experiments) (Fig. 7D). All together, these results strongly suggest that both trypanosome U2AF65 and SF1 proteins are *bona fide* splicing factors that affect the first step of splicing. As can be seen, although the mRNA was almost completely eliminated by the silencing, the levels of U2AF65 protein were not completely abolished, even after three days of silencing. In contrast, after U2AF35 RNAi induction, an impressive reduction in the protein level of this factor was noted. Since splicing factors are recycling, no growth phenotype can be observed until complete factor deprivation ensues.

4. Discussion

In this study, we characterized splicing factors U2AF35, U2AF65 and SF1 from trypanosomes. All three showed significant differences when compared to the mammalian orthologues. At the functional level, their involvement in the *trans*-splicing reaction was demonstrated by RNAi experiments. In the three cases (Figs. 5–7) their RNAi-induced knock down affected the initial step of the *trans*-splicing reaction, as indicated by the sharp decrease in Y structure determined immediately after TET induction (Figs. 5–7).

Trypanosomes presented both U2AF35 and U2AF65 splicing factors, but they did not form the typical U2AF heterodimer [8]. Using yeast two hybrid assays, in vivo co-fractionation on superdex columns, or TAP-affinity selections in trypanosome extracts we were unable to detect interaction between them. Alternatively, it is also possible to postulate that the interaction was very weak and undetectable with the methods used in this study. Whichever the case may be, this was in agreement with our comparative sequence analysis showing the lack of Trp residues in positions that would allow the establishment of the typical strong U2AF heterodimeric, tongue-in-groove interaction.

Indeed, trypanosome U2AF65 diverged from the eukaryotic consensus presenting a unique type of RRMIII domain interrupted by an amino acid sequence that was structurally characterized as a NORS, pRRMIII (Fig. 3). This variation was not only present in trypanosome U2AF65 proteins, but also in *P. yoelii,* and *U. maydis* (Fig. 2). Remarkably, the elimination of the NORS in pRRMIII decreased the strength of interaction of pRRMIII to parasite SF1 and allowed the interaction of ΔNORS pRRMIII with human SF1 (Fig. 3). These results confirmed that pRRMIII was the trypanosome version of the mammalian U2AF65 RRM III domain, containing an intervening sequence, NORS, that functioned in optimizing the interaction between trypanosome factors SF1 and U2AF65. *In vivo*, this interaction was confirmed by fractionation and affinity-selection experiments, in which both SF1 and U2AF65 co-fractionated in a complex of approximately 400 kDa (Fig. 4A), and U2AF65 was affinity-selected with TAP tagged SF1, but not with U2AF35 (Fig. 4 B). Immuno-localization experiments also pointed to a differential cellular distribution (Fig. 4C), whereas U2AF35 was only detected in the nucleus, both SF1 and U2AF65 were not only detected in the nucleus, but also in perinuclear regions (Fig. 4C).

In mammalian cells, the size constrains of BBP-Py tract to AG 3′ splice site determine the need of U2AF35, U2AF65 and SF1 to interact in order to be functional. In *S. cerevisiae*, recognition of branch site needs SF1, which is essential but UA2F65, Mud2p, is not [14] and U2AF35 does not exist. In yeast, branch-point recognition is based mainly on base-pair interaction between the U2 RNA sequence, GUGUUA, and the pre-mRNA, UCUAAC [32].

Trypanosomes differed from both mammals and yeast. The U2 snRNA lacked the sequence to interact with the branch consensus, suggesting that RNA–RNA interactions were not used for branch point recognition [33]. Moreover, the distance between the BBP and the AG splice site was 10–80 nt, not as in mammals, in which the adenosine used as the branch point reaction needs to be within −18 and −37 upstream the 3′ SS. In 30% of trypanosome pre-mRNAs this distance was longer than 30 nt [33,34]. Moreover, in *T. cruzi,* shifting the position of the 3′ SS several bases downstream from the original one did not decrease the rate of *trans*-splicing, nor did distances greater than 40 nt from the 3′ AG to the closest branch point [19], whereas in *T. brucei* increasing the distance between the 3′ splice site and branch point did increase *trans*-splicing efficiency [35]. Because in trypanosomes the distance between the branch site and the 3′ AG splice site was longer, a larger U2AF65 may be needed.

It was of interest for this study that in eukaryotes, differences in Py lengths seemed to be in agreement with the features of the different U2AF65 proteins involved. Human U2AF65 had two RRM domains with high affinity for poly U which, in turn, renders unnecessary large Py tracts. Trypanosome U2AF65 had only one canonical RRM domain with key amino acid deviations from the RNP-1 and RNP-2 consensus that may need longer Py tracts for binding [36].

Surprisingly, U2AF65 is the only trypanosome splicing factor so far, that when silenced, provoked a decrease in the level of SL RNA of knock-down cells. Indeed, all the splicing factors that were knocked down by RNAi including Lsm proteins [17], Prp31 and Prp43 [18] and snRNA-specific Sm proteins that bind to U2 and U4 [24], caused an increase in the level of SL RNA as a consequence of defects in spliceosome assembly or disassembly. Accordingly, silencing of U2AF35 and SF1 increased the level of SL RNA as was observed for all other splicing factors. The decrease in SL RNA in U2AF65 silenced cells suggests that U2AF65 may interact directly with SL RNP during the recruitment of the SL RNP to the spliceosome. It is therefore tempting to speculate that, in trypanosomes, U2AF65 may mediate the interaction with SL RNP and not SF1 as in nematodes [37].

Acknowledgements

This work was mainly supported by grants from World Health Organization/Special Program for Research and Training in Tropical Diseases (WHO/TDR)- South South Initiative (SSI-WHO/TDR), a Mini-Grant of the Howard Hughes Medical Institute (Chevy Chase, MD, USA), the Chaire Internationale de Recherche Blaise Pascale (Region Ile de France, France), the SMMAD 40402 Project of the European Community, the FONCYT-PICT 01-06803 project to MJL, and FONCYT–PICT REDES 2003-00300, UBACyT X-153 (University of Buenos Aires) and PIP-CONICET 5492 to MV. MV and MJL are investigators of CONICET (Argentinean National Research Council). We acknowledge technical support from technicians Sonia Lafon and Maria de los Angeles Curto, both from CONICET.

S.M is an HHMI International Scholar in Molecular Parasitology and this study was also supported by a grant from United States-Israel Binational Science foundation. S.M. holds the David and Inez Myers Chair in RNA silencing of diseases.

MJL has been International Research Scholar from the Howard Hughes Medical Institute, Chevy Chase, MD, USA (2002–2006) and Professor of a Blaise Pascal International Chair of Research, Fondation ENS-Region Ile de France, France (2005–2006). In addition, MJL was European Community Marie Curie International Fellow at Institut Cochin, Paris, France during the period September 2007–March 2008.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2008.12.009.

References

[1] Martinez-Calvillo S, Yan Y, Niguyen D, Fox M, Stuart K, Myler PJ. Transcription of leishmania major Friedlin chromosome 1 initiates in both directions within a single region. Cell 2003;11:1291–9.

[2] Liang XH, Haritan A, Uliel S, Michaeli S. Trans and cis splicing in Trypanosomatids: mechanism, factors, and regulation. Eukaryot Cell 2003;2:830–40.
[3] Reed R. Mechanisms of fidelity in pre-mRNA splicing. Curr Opin Cell Biol 2000;12:340–5.
[4] Ruskin B, Zamore PD, Green MR. A factor, U2AF, is required for U2 snRNP binding and splicing complex assembly. Cell 1988;52:207–19.
[5] Zamore PD, Patton JG, Green MR. Cloning and domain structure of the mammalian splicing factor U2AF. Nature 1992;355:609–14.
[6] Zhang M, Zamore PD, Carmo-Fonseca M, Lamond AI, Green MR. Cloning and intracellular localization of the U2 small nuclear ribonucleoprotein auxiliary factor small subunit. Proc Natl Acad Sci USA 1992;89:8769–73.
[7] Wu S, Romfo C, Nilsen T, Green M. Functional recognition of the 3′- splice site AG by the splicing factor U2AF35. Nature 1999;402:832–5.
[8] Kielkopf PF, Radionova NA, Green MR, Burley SK. A novel peptide recognition mode revealed by the X-ray structure of a core U2AF35/U2AF65 heterodimer. Cell 2001;106:595–602.
[9] Valcárcel J, Gaur RK, Singh R, Green MR. Interaction of U2AF65 RS region with pre-mRNA branch-point and promotion of base pairing with U2 snRNA. Science 1996;273:1706–9.
[10] Ito T, Muto Y, Green MR, Yokoyama S. Solution structures of the first and second RNA-binding domains of human U2 small nuclear ribonucleoprotein particle auxiliary factor (U2AF65). EMBO J 1999;18:4523–34.
[11] Potashkin J, Naik K, Wentz-Hunter K. U2AF homolog required for splicing in vivo. Science 1993;262:573–5.
[12] Zorio DA, Blumenthal T. Both subunits of U2AF recognize the 3' splice site in *Caenorhabditis elegans*. Nature 1999;402:835–83.
[13] Rudner DZ, Kanaar R, Breger KS, Rio DC. Mutations in the small subunit of the Drosophila U2AF splicing factor cause lethality and developmental defects. Proc Natl Acad Sci 1996;93:10333–7.
[14] Abovich N, Rosbash M. Cross-intron bridging interactions in the yeast commitment complex are conserved in mammals. Cell 1997;89:403–12.
[15] Vázquez M, Atorrasagasti C, Bercovich N, Volcovich R, Levin MJ. Unique features of the *Trypanosoma cruzi* U2AF35 splicing factor. Mol Biochem Parasitol 2003;128:77–81.
[16] Mandelboim M, Barth S, Biton M, Liang XH, Michaeli S. Silencing of Sm proteins in *Trypanosoma brucei* by RNA interference captured a novel cytoplasmic intermediate in spliced leader RNA biogenesis. J Biol Chem 2003;278:51469–78.
[17] Liu Q, Liang XH, Uliel S, Belahcen M, Unger R, Michaeli S. Identification and functional characterization of Lsm proteins in *Trypanosoma brucei*. J Biol Chem 2004;279:18210–9.
[18] Biton M, Mandelboim M, Arvatz G, Michaeli S. RNAI interference of XPO1 and Sm genes and their effect on the spliced leader RNA in *Trypanosoma brucei*. Mol Biochem Parasitol 2006;150:132–43.
[19] Ben-Dov CP, Levin MJ, Vázquez MP. Analysis of the highly efficient pre-mRNA processing region HX1 of *Trypanosoma cruzi*. Mol Biochem Parasitol 2005;140:97–105.
[20] Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR. Site directed mutagenesis by overlap extension using polymerase chain reaction. Gene 1989;77:51–9.
[21] Avila ML, Bercovich N, Westergaard G, Levin MJ, Vazquez MP. Mapping of the protein-binding interface between splicing factors SF3b155 and p14 of *Trypanosoma cruzi*. Biochem Biophys Res Commun 2007;364:26–32.
[22] Rain JC, Selig L, De Reuse H, et al. The protein–protein interaction map of *Helicobacter pylori*. Nature 2001;409:211–5.
[23] Lustig Y, Goldshmidt H, Uliel S, Michaeli S. The *Trypanosoma brucei* signal recognition particle lacks the Alu-domain-binding proteins: purification and functional analysis of its binding proteins by RNAi. J Cell Sci 2005;118:4551–62.
[24] Tkacz ID, Lustig Y, Michael ZS, et al. Identification of novel snRNA-specific Sm proteins that bind selectively to U2 and U4 snRNAs in *Trypanosoma brucei*. RNA 2007;13:30–43.
[25] Liu J, Rost B. NORSp: predictions of long regions without regular secondary structure. Nucleic Acids Res 2003;31:3833–5.
[26] Kielkopf CL, Lucke S, Green MR. U2AF homology motifs: protein recognition in the RRM world. Genes Dev 2004;18:1513–26.
[27] Arning S, Grüter P, Bilbe G, Krämer A. Mammalian splicing factor SF1 is encoded by variant cDNAs and binds to RNA. RNA 1996;2:794–810.
[28] Selenko P, Gregorovic G, Sprangers R, et al. Structural basis for the molecular recognition between human splicing factors U2AF(65) and SF1/mBBP. Mol Cell 2003;11:965–76.
[29] Michaeli S, Goldring A, Agobian N. Identification of mRNA-binding proteins of the lower trypanosomatid Leptomas collosoma. Exp Parasitol 1993;76:308–13.
[30] Krämer A, Utans U. Three protein factors (SF1, SF3 and U2AF) function in pre-splicing complex formation in addition to snRNPs. EMBO J 1991;10:1503–9.
[31] Gozani O, Potashkin J, Reed R. A potential role for U2AF-SAP 155 interactions in recruiting U2 snRNP to the branch site. Mol Cell Biol 1998;18:4752–60.
[32] Parker R, Siliciano PG, Guthrie C. Recognition of the TACTAAC box during mRNA splicing in yeast involves base pairing to the U2-like snRNA. Cell 1987;49:229–39.
[33] Lucke S, Jurchott K, Hung LH, Bindereif A. mRNA splicing in *Trypanosoma brucei*: branch-point mapping reveals differences from the canonical U2 snRNA-mediated recognition. Mol Biochem Parasitol 2005;142:248–51.
[34] Mottram JC, Murphy WJ, Agabian N. A transcriptional analysis of the *Trypanosoma brucei* hsp83 gene cluster. Mol Biochem Parasitol 1989;37:115–27.
[35] Benz C, Nilsson D, Andersson B, Clayton C, Lys Guilbride D. Messenger RNA processing sites in *Trypanosoma brucei*. Mol Biochem Parasitol 2005;143:125–34.
[36] Siegel TN, Tan KSW, Cross GAM. Systematic study of sequence motifs for RNA trans splicing in *Trypanosoma brucei*. Mol Cell Biol 2005;25:9586–94.
[37] Denker JA, Zuckerman DM, Maroney PA, Nilsen TW. New components of the spliced leader RNP required for nematode trans-splicing. Nature 2002;417:667–70.