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Tools for *Trans-Splicing* Drug Interference Evaluation in Kinetoplastid

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1. Introduction

The scientific progress of the last century has led man to understand every day the intricate mechanisms involved in the transfer of genetic material and how it is interpreted to trigger the most important biochemical processes of the cells for their survival.

It should be noted that the biochemical cell repertoire is conserved in all living organisms on the planet. Since 1990, the scientists observed that eukaryotes have excessively large genomes with hundreds of thousands of introns which were inserted into genes. Most protein-coding genes in human genome are composed of multiple exons interrupted by introns. Advances in the knowledge of genomics and proteomics of mammals confirmed the need for reprogramming of mRNA used to obtain the products of genes and production of proteins and the significance of pre-mRNA splicing, which is an essential step of gene expression by removing noncoding sequences (introns) to ligate together coding sequences (exons). The exons are usually relatively short about 50–250 base pairs, whereas introns are much larger and can be up to several thousands of base pairs. Splicing is a complex nuclear RNA processing event, where different exons from pre-mRNA molecules are joined together. The intron sequences are removed from the pre-mRNA and the exons fused together resulting in the formation of the mature messenger RNA (mRNA), which is subsequently capped at its 5′ end and polyadenylated at its 3′ end, and transported out of the nucleus to be translated into protein in the cytoplasm.

In general, most genes give rise to multiple spliced transcripts by alternative splicing, leading to different mRNA variants and the synthesis of alternative proteins. Utilizing different alternative 5' or 3' splice sites, multiple protein isoforms can be generated from a commom primary transcript (van Santen & Spritz, 1986; Adami & Babiss, 1991; Gattoni et al., 1991; Guo et al., 1991; reviewed by Horowitz & Krainer, 1994; Sharp, 1994, as cited in Eul et al., 1995).

The simple existence of alternatively spliced products greatly increases cellular and organism complexity and may have allowed for evolution by producing additional regulation and diversification of gene function. It is obvious that splicing and alternative splicing must be tightly regulated and executed in time and space not to interfere with the normal cellular and organism physiology. The spliceosome, an intracellular complex of multiple proteins and ribonucleoproteins, is the main cellular machinery guiding splicing. Recently, two natural compounds interfering with the spliceosome were found to display anti-tumour activity *in vitro* and *in vivo*. Therefore, it is conceivable that inhibiting the spliceosome could serve as a novel target for anticancer drug development (Kaida et al., 2007; Kotake et al., 2007, as cited in van Alphen et al., 2009).

Cis-splicing occurs when two exons are initially part of a contiguous RNA, and trans-splicing, where the two exons are initially on two separate RNAs. In the latter case, the partial intron sequences flanking the two exons pair through sequence complementarity to form a catalytic structure joining the associated exons (Will & Lurhmann, 2006).

To direct correct processing of pre-mRNA, the intron sequences contain a number of core splicing signals, notably the conserved splice-site sequences at their extreme 5′ and 3′ ends and a conserved branch point region. The branch point sequence and the branch point itself, usually an adenosine, are located about 20–40 nucleotides upstream of the 3′ splice site. The critical process of recognizing splice sites and the removal of introns and/or exons is the task of the spliceosome (Staley & Guthrie, 1998; Will & Luhrmann, 2001, as cited in van Alphen et al., 2009). The spliceosome consists of five non-coding uridine-rich small nuclear ribonucleoproteins (U snRNPs) and a multitude of associated proteins, creating a network of RNA–RNA, RNA–protein and protein–protein interactions (Zhou et al., 2002; Jurica & Moore, 2003, as cited in van Alphen et al., 2009). See figure 1 for details.

In fact, well over 200 different splicing factors interacting with the spliceosome have been identified, and it is clear that much more research is needed before we fully understand all the intricacies of RNA splicing (Jurica & Moore, 2003; Wang & Burge, 2008, as cited in van Alphen et al., 2009). The various spliceosome components, in particular, the small nuclear RNPs, are sequentially recruited to the splice sites. Next, they are assembled into the spliceosome after which splicing is initiated by a series of two transesterification reactions producing two ligated exons and a liberated intron (Staley & Guthrie, 1998; Wang and Burge, 2008, as cited in van Alphen et al., 2009). See figure 1 for additional details.

Through interactions with various proteins that recognise specific splice site features, the spliceosome components, that is, small nuclear ribonucleoproteins (snRNPs) designated with U1, U2, U4, U5 and U6 are sequentially recruited to the splice site and assembled into the spliceosome. Once completed, splicing is catalysed in two consecutive transesterification reactions. In the initial step, the 2′ OH group of the branch point adenosine upstream of the 3′ end of the intron reacts with the 5′ splice junction, forming a novel 2′, 5′ phosphodiester bond between the branch point and the 5′ terminal nucleotide of the intron, giving rise to a lariat structure. In the second reaction, the 3′ OH of the 5′ exon attacks the 3′ splice junction producing linked 5′ and 3′ exons and liberating the intron. Subsequently, the snRNPs involved are released and recycled in the splicing process. In the top right-hand corner, a detailed view of U2 snRNP with subcomplexes, SF3a and SF3b, is shown. The figure and legend were transcribed from van Alphen et al. (2009).

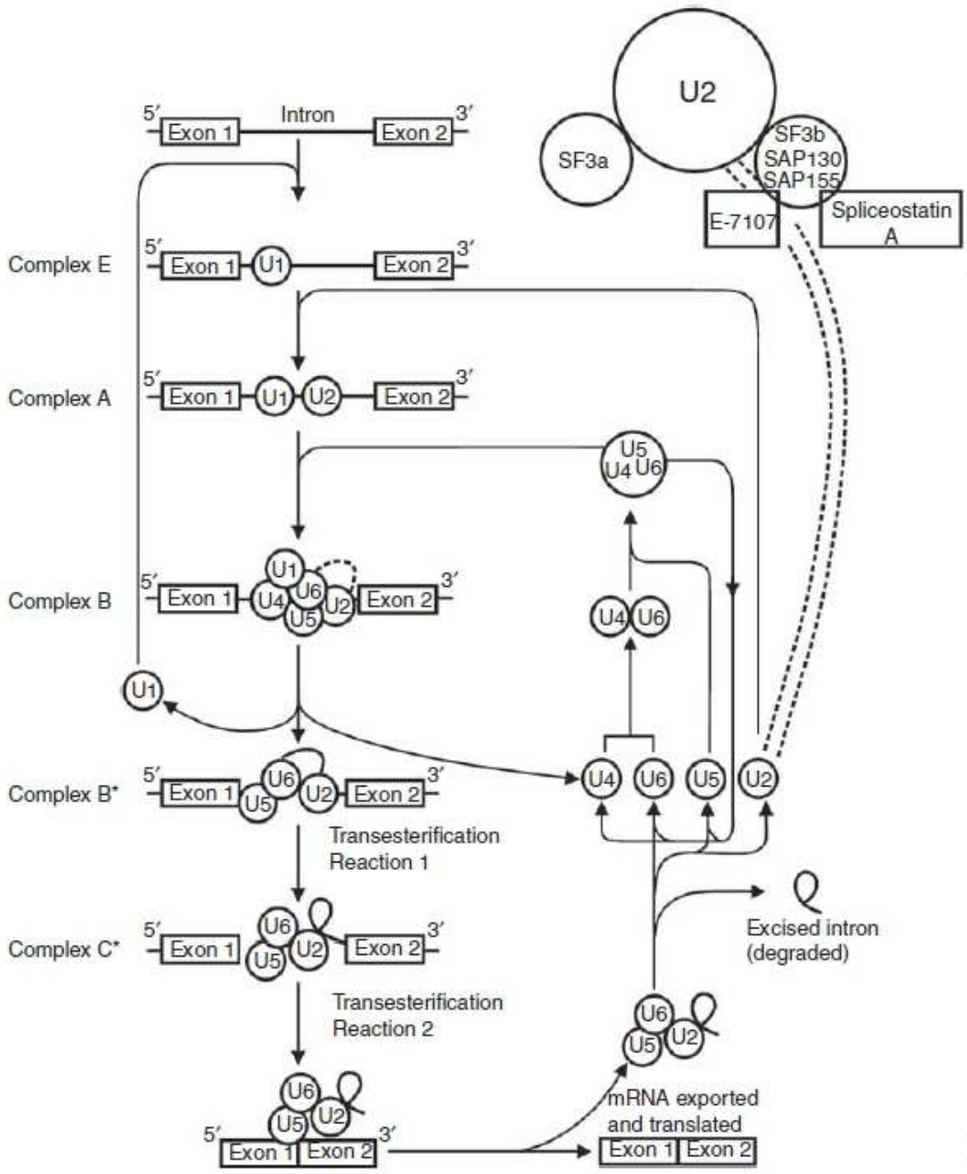


Fig. 1. The spliceosome assembly cycle.

Splicing at different locations in the same pre-mRNA transcript, alternative splicing, enables a gene to produce variant mature mRNAs, and consequently functionally different proteins (House & Lynch, 2008, as cited in van Alphen et al., 2009). The precise mechanisms of and/or triggers for alternative splicing are yet unknown. In higher eukaryotes, like humans, the accuracy of splicing is not solely dictated by base-pairing interactions of the U snRNPs with the pre-mRNA. Owing to degeneracy and poor definition of small splice sites at the end of long introns, splice-site recognition is also influenced by (a) the coupling of splicing with other processes such as transcription, (b) the velocity of the splicing reaction, (c) external stimuli, like the presence of growth factors or oxidative stress and (d) the three-dimensional structure of pre-mRNA itself

(Tazi et al., 2005b; Disher & Skandalis, 2007; House & Kristen, 2008, as cited in van Alphen et al., 2009). In the latter case, the three-dimensional folding of pre-mRNA determines whether a (in pre-mRNA embedded) splice regulator will be located in the vicinity of a splice site (for details see van Alphen et al., 2009).

In trans-splicing, two pre-mRNAs are processed to produce a mature transcript that contains exons from both precursors. This process also involves two trans-esterification steps that result in the linking of the two exons by a normal 3'-5' phosphodiester bond. This process has been described mostly in trypanosomes, nematodes, chloroplasts, and plant mitochondria (Bonen, 1993, as cited in Caudevilla et al., 1998).

Kinetoplastids are a remarkable group of protists. They contain a range of ubiquitous free-living species – pathogens of invertebrates, vertebrates and even some plants. *Trypanosoma* species cause sleeping sickness and Chagas disease, whereas the leishmaniases kill and debilitate hundreds of thousands of people worldwide each year. Furthermore, these morphologically rather simple unicellular organisms are masters at finding unorthodox solutions to the problems of being a eukaryotic cell. Kinetoplastid peculiarities include: (1) complex and energy-consuming mitochondrial RNA editing; (2) a unique mitochondrial DNA architecture; (3) trans-splicing of all mRNA transcripts; (4) the arrangement of genes into giant polycistronic clusters; (5) unprecedented modifications of nucleotides; (vi) the compartmentalization of glycolysis; (vii) evasion of the host immune response using a variable surface coat; and (viii) the ability to escape destruction by migrating out of phagocytic (McConville et al., 2002; Sacks & Sher, 2002).

Trypanosomes are protozoan which intriguing the scientists since a long time for various reasons. Primarily by the pathogenicity followed by the biology and evolution on the phylogenetic tree.

To our studies, *Trypanosoma cruzi* always is a very important model, because it is the etiologic agent of Chagas disease, an endemic parasitic disease in Latin America, discovered by Carlos Chagas in 1909, which still remains without an effective treatment. *T. cruzi* is transmitted by triatomine insects and can also be transmitted by blood transfusion or the placenta, during breastfeeding, through organ transplantation and by processed food contaminated with the insect vector (Lana & Tafuri, 2000; Rey, 2001).

Currently, between 12 and 14 million people are infected and more than 100 million live in endemic areas. Chagas disease is a major neglected diseases considered in the world and affects mainly low-income people. Treatment of infected people in endemic areas has been recommended by the National Health Foundation of Brazil for acute or recent chronic cases as well as for congenital and accidental infections and for children with positive serology (Rey, 2001; WHO, 2005).

The pathogenicity pattern of the disease may be influenced by the characteristics of both human host and the lineage of the *T. cruzi* strain. Thus, the course of infection in susceptible vertebrates is influenced not only by the environmental features or genetic constitution of the human host, but mainly by morphological characteristics of the infecting strains versus host immune response, as well as of therapeutic agents and susceptibility of the *T. cruzi* strain. These are the most challenge limiting the successful treatment and cure of the disease (Teixeira et al., 2006).

In late 1960 and early 70s, two drugs were first used in the treatment of Chagas disease: nifurtimox (NFX - Bayer) and benznidazole (BZ - Roche), sold, respectively, by the trademarks, Lampit® and Rochagan®. Both are more effective to eliminate parasites in the acute phase, reducing the course of infection, since the disease had not affected the cardiovascular system, leading to cure in 78% of patients in acute phase. However, the effectiveness of both drugs in treatment of chronic disease is limited to 8% cure (Coura & de Castro, 2002; Teixeira et al., 2006).

The low efficiency of these drugs with undesirable side-effects imposed limitations on their use. Furthermore, different strains of *T. cruzi* present different levels of susceptibility to benznidazole and nifurtimox, which may explain in part the observed differences in the effectiveness of chemotherapy. In Brazil, nifurtimox was withdrawn of the market due to severe side effects. Thus, the benznidazole became the only alternative for treatment, despite widespread criticism due also to its adverse side effects and low effectiveness in the chronic phase. To date, despite several studies, there is no available vaccine for Chagas disease. Therefore, the identification of new chemotherapeutic and vaccine methods is the biggest challenge for the control of Chagas disease (Issa & Bocchi, 2010).

Several strains of *T. cruzi* were isolated from different countries and geographical areas. Major differences in resistance or susceptibility to the benznidazole in experimental or clinical studies were reported between different strains of the parasite. These findings further complicate the search for new anti-*T. cruzi* drugs. The same approach would be evaluated for vaccine.

Either in Chagas disease as well as other parasitic diseases, cell biology and immunology studies help to understand the mechanisms involved in the ability of certain stages of the parasites (trypomastigotes) invade cells, transforming into replicative forms (amastigotes) which escapes the immune system. Furthermore, the development of new chemotherapeutic attack points takes into account the mechanisms of drug resistance. Thus, the development of work in this field of research may eventually corroborate to minimize or destroy the parasitism, searching for new drugs targeting only the parasites and not cause damage in human cells (Coura & de Castro, 2002). The study of new trypanocidal drugs involves specific objectives related to the structure and RNA processing in *T. cruzi*, which should be first studied on *in vitro* experiments.

Trypanosomatids are early-diverged, protozoan parasites some of them cause severe and lethal diseases in humans, as already described before in *T. cruzi*. To better combat these parasites, their molecular biology has been a research focus for more than 3 decades. Messenger RNA (mRNA) maturation in trypanosomes differs from the process in most eukaryotes mainly because protein-coding genes are transcribed into polycistronic RNAs and processed by trans-splicing in which a common spliced leader sequence (SL) is acquired at the 5'-end to yield mature transcripts. SL trans-splicing has been characterized mainly in trypanosomes and nematodes and requires, in addition to the SL RNP, the small nuclear ribonucleoproteins (U snRNPs) U2, U4/U6, and U5.

Such addition of short, nontranslated leaders to coding transcripts has been observed in representatives from both the protists and animal kingdoms and the phenomenon has been intensively studied particularly in trypanosomes and nematodes, which about 15% of the

genes are transcribed by trans-splicing mechanism. To date, the *in vitro* trans-splicing reaction was better study in nematode pre-messenger RNA (Nielsen, 1993).

Until recently, only two snRNP-specific proteins had been studied in trypanosomes, namely, the orthologs of human U2A' (originally termed U2-40K) and the U5-specific PRP8. While the latter was identified by sequence homology, U2A' was copurified with the U2 snRNA in high-stringency U snRNP purifications which typically left only the core structures intact. For a more comprehensive biochemical characterization of U snRNPs and/or of the spliceosome, it was therefore essential to purify the RNA-protein complexes under conditions of lower stringency. A method well-suited for this purpose is tandem affinity purification (TAP), which is based on expressing a known protein factor fused to a composite TAP tag. TAP comprises two consecutive high-affinity chromatography steps which are carried out under nearly physiological conditions. Since the advent of this technology, the TAP tag and the TAP method have been modified in various ways to accommodate different systems, extracts, or protein complexes. For the purification of nuclear protein complexes in trypanosomes, the PTP (protein C epitope-TEV protease cleavage site-protein A domains) modification of TAP has proven to be very useful. One of the first applications of the PTP tag was the purification of the trypanosome U1 snRNP (Günzl 2010). The figure 2 illustrates the different spliceosomal factors of humans and trypanosomes.

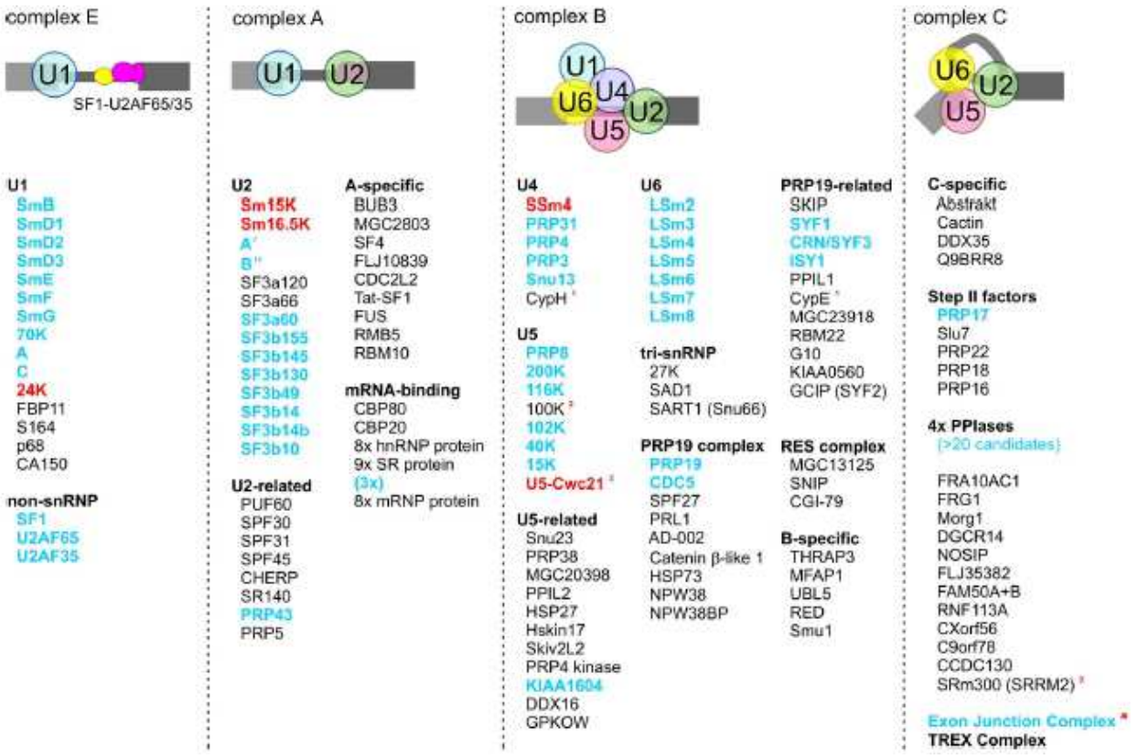


Fig. 2. Comparison of known spliceosomal factors of humans and trypanosomes. For more details the figure was transcribed from Günzl (2010).

2. Trans-splicing and alternative splicing in mammalian cells

Trans-splicing of artificial pre-mRNAs in mammalian cells *in vitro* has been reported but with some limitations. In addition, nematodes or Simian virus 40 spliced leader RNAs can be accurately trans-spliced in transfected COS cells, which revealed functional conservation in the splicing machinery between lower eukaryotes and mammals and demonstrated the potential occurrence of the trans-splicing in mammalian cells (Bruzik & Maniatis, 1992). Studies *in vivo* also have shown that a synthetic pre-mRNA substrate containing an exon and a 5' donor splice site can be efficiently trans-spliced to another synthetic pre-mRNA (3' trans-splicing substrate) if it contains either exonic enhancers or a 5' downstream splice site. Several examples of possible natural trans-splicing in mammalian cells have been reported, but none of these trans-splicing have been demonstrated *in vitro* (Caudevilla et al., 1998).

The hypothesis that trans-splicing is a regular event in mammalian cells was first suggested by Dandekar & Sibbald (1990). Later, the capability of mammalian cells to perform trans-splicing reaction with appropriate foreign RNAs also was demonstrated *in vivo* (Eul et al., 1995). In this work the authors have demonstrated the potential of mammalian cells to generated functional mRNA molecules by trans-splicing. However, there is a competition between cis- and trans-splicing in these cells, which was also demonstrated in nematodes. These worms perform both RNA splice patterns simultaneously (Blumental & Thomas, 1988; Conrad et al., 1993a, b; Nielsen, 1993). In these cells, cis-splicing is dominant when a protein coding precursor RNA contains a valid 5′ and 3′ splice site at its most proximal part. When, however, the 5′ splice site is deleted, cis-splicing is prevented and the remaining 3′ splice site combines with the 5′ splice site of an SL RNA molecule to generate a new mRNA molecule by trans-splicing (Conrad et al., 1991, 1993a). Maybe this is a precursor mechanism of alternative splicing in mammals. Since trypanosomes are protozoan and therefore more primitive eukaryotes, the trans-splicing mechanism occurs more frequently than cis-splicing, although the latter is also present in this cell type as mentioned before.

For trans-splicing in plant mitochondria and plastids, extensive base pairing within the introns of the two pre-mRNAs seems to be essential (Chapdelaine & Bonen, 1991; Knoop et al., 1991; Sharp, 1991, as cited in Eul et al., 1995). These organelles do not contain U snRNAs ans RNPs (Cech, 1986; Saldanha et al., 1993) and the functions of the different snRNAs are replaced by a highly conserved RNA secondary structure specific for all group II introns (Sharp, 1991; Suchy & Schmelzer, 1991; Saldanha et al., 1993).

Caudevilla et al. (1998) have shown that the rat-liver carnitine octanoyltransferase (COT) is a single-copy gene, which is able to be processed in several mature transcripts involving cis- and trans-splicing events. The authors showed the occurrence of three purine rich exonic-splicing enhancers (ESE) in the sequence of exon 2 of the COT gene, which are detected at positions 49 (GAAGAACGAA), 106 (GAAGAA), and 117 (GAAGAAG). This is showed in the figure 3.

The exons are represented by boxes and introns by dotted lines. Sequences 5 '- and 3'-intronic splicing sites are indicated in lowercase. The ESE (exonic-splicing enhancers) is represented in exon 2 in capital letters. Simultaneous cis- and trans-splicing of hepatic mRNA COT are produced. Trans-splicing can be promoted by ESE sequences. Three different transcripts can be produced: one by cis-splicing and two by trans-splicing where

exon 2 or exon 2 and exon 3 are repeated. The organization of mature mRNAs is represented at the foot of the figure. The figure was transcribed from Caudevilla et al. (1998).

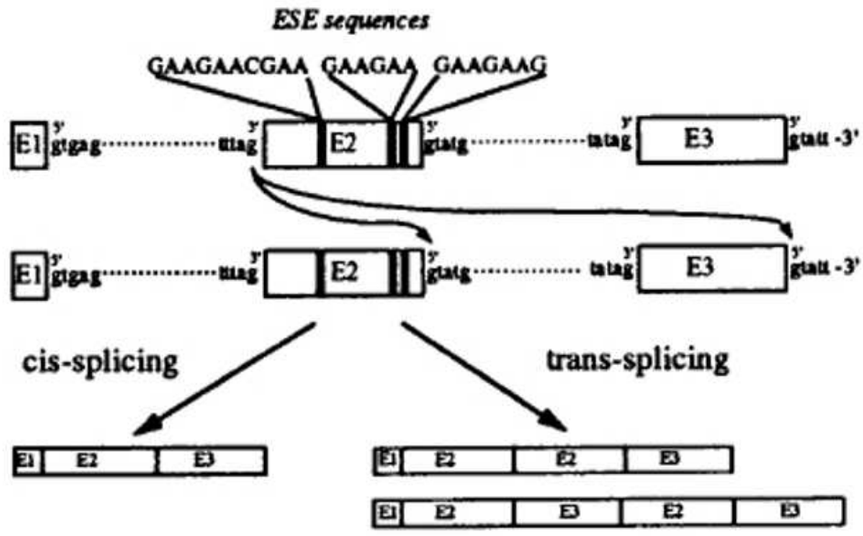


Fig. 3. Model of cis and trans-splicing in mammalian cells (COT pre-mRNA).

Splicing enhancers facilitate the assembly of protein complexes on mRNAs containing a 3′ splice site, and these complexes are sufficiently stable to interact with 5′ splice site located on separate mRNAs.

Conrad et al. (1991, 1993b) demonstrated that a cis-splicing acceptor can act as a trans-splice acceptor if it is moved into a proper context, referred to an outron, which is an intron without a well defined 5' donor splice site. So, in this context, trans-splicing in mammals can be used to reprogram or interfere with specific pre-mRNAs, thus modifying or completely changing the gene expression (Puttaraju et al., 1999; Mansfield et al., 2000, as cited in Codony et al., 2001). In the reprogramming of the target pre-mRNA, it is first necessary to establish which exons of the target pre-mRNA are susceptible to involvement in a trans-splicing reaction. The same principle seems to occur with genes in trypanosomes as well as poly A polymerase, which are cis-spliced, instead of trans-splicing.

Intervening sequences have been described in the Poly A Polymerase (PAP) gene of *Trypanosoma brucei* and *Trypanosoma cruzi*, and a U1 snRNA sequence in *T. brucei*, demonstrating that both cis- and trans-splicing can occur in these organisms, with a prevalence of trans (Mair et al., 2000).

RNA splicing is carried out by the spliceosome, which consists of the five small nuclear ribonucleoprotein particles (snRNPs) U1, U2, U4, U5 and U6, as well as non-snRNP proteins. The ribonucleoproteins are complexes consisting of small uridine-rich RNAs (UsnRNAs), interacting with common Sm proteins and specific proteins for each snRNP. Although well characterized in humans and yeast, little is known about the specific proteins involved in trans-splicing in trypanosomatids (Mayer & Floeter-Winter, 2005). It would be

interesting to speculate that the cis-splicing of the poly A polymerase genes can be used as a protozoan mechanism to maintain the viability of their most mRNAs newly trans-spliced.

Many external trans-RNA, like trans-donor or a trans-acceptor that will participate in a trans-splicing reaction, can be synthesized to interfere with other pre-mRNAs (Bruzik & Maniatis, 1995; Chiara & Reed, 1995).

Because of trans-splicing is a post-transcriptional processing event, which was first demonstrated in T*rypanosoma brucei* (Murphy et al., 1986; Sutton & Boothroyd, 1986), in these cells, a 39 nucleotides (nt), "mini-exon", derived from a 140 nt small leader (SL) RNAs is trans-spliced to all pre-mRNA molecules (reviewed by Bonen, 1993; Ullu et al., 1993, as cited in Eul et al., 1995). It was demonstrated that in mammals the cells can utilize a cryptic 5' splice site within the second exon and the conventional 3' splice site, even the cis-splicing is dominant in these cells (Eul et al., 1995). Competition between trans- and cis-splicing has also been demonstrated in nematodes, which perform both RNA splice patterns simultaneously (Blumenthal & Thomas, 1988; Conrad et al., 1993a, b; Nielsen, 1993). In these cells, cis-splicing is dominant when a protein coding precursor RNA contains a valid 5' and 3' splice site at its proximal part. When, however, the 5' splice site is deleted, cis-splicing is prevented and the remaining 3' splice site combines with the 5' splice site of an SL RNA molecule to generate a new RNA molecule by trans-splicing (Conrad et al., 1991, 1993a; Eul et al., 1995).

A diagram comparing the two mechanisms is shown in Figure 4.

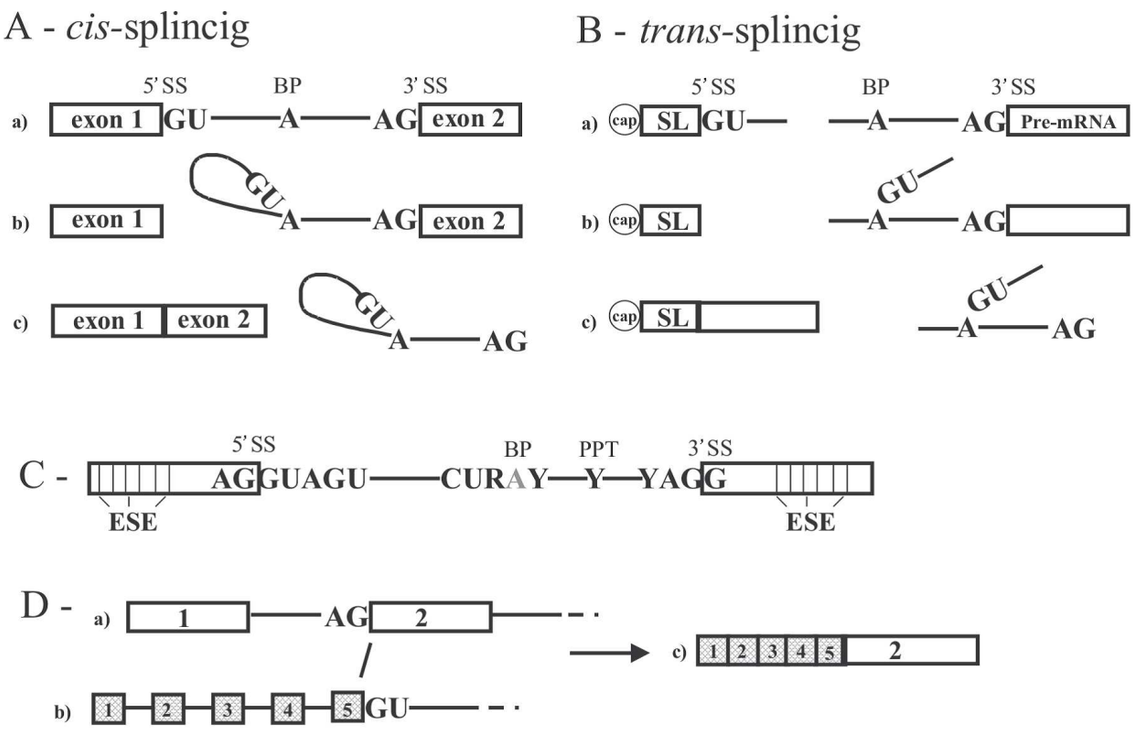


Fig. 4. Schematic representation of cis- and trans-splicing pathways.

A: cis-splicing occurs through a two-step mechanism, each step consisting of a transesterification reaction (Moore et al. 1993, as cited in Mayer & Floeter-Winter, 2005). In the first transesterification (a to b), a nucleophilic attack of a 2'-OH group from an adenosine residue within the intron (branch point, BP) to the phosphorous atom at the 5' splice site (5' SS) generates two intermediates: exon 1 with a free 3'-OH terminus and an intron-exon 2 in a lariat configuration. In the second transesterification (b to c), the 3'-OH group form the exon 1 intermediate acts as a nucleophile and attacks the phosphorous atom at 3' splice site (3' SS), resulting in the displacement of the 3' end of the intron and joining of the 5' and 3' exons. B: SL-addition trans-splicing is very similar to the canonical cis-splicing, except for the use of two substrate molecules, which is the reason for the generation of a Y branched intermediate instead of a lariat. C: cis regulatory elements involved in pre-mRNA splicing. 5' SS, 3' SS, and BP consensus sequences are shown. Polypyrimidine tract (PPT) and exonic splicing enhancers (ESE) are indicated. D: schematic representation of trans-splicing between (a) ABP (androgen-carrier protein produced in the testicular Sertoli cell) and (b) HDC (histidine decarboxylase) pre-mRNAs; (c) represent the mature chimeric mRNA. This figure was transcribed from Mayer & Floeter-Winter, (2005).

Recent research has revealed that alternatively spliced products can be linked to various (genetic) diseases and also may play a role in cancer development (Wang & Burge, 2008, as cited in van Alphen et al., 2009). A systematic approach using large-scale sequencing and splicing-sensitive microarrays gave for the first time a view of the vast spectrum of alternative transcripts (Blencowe, 2006; Ben-Dov et al., 2008, as cited in van Alphen et al., 2009). One or more alternatively spliced exons can be found in transcripts of two-thirds of human genes. In general, the function of the encoded protein remains unchanged. However, some of the alternative protein products may display a malignant phenotype (Blencowe, 2006, as cited in van Alphen et al., 2009). Whether alternative splicing is the cause or result of malignant behavior in cancer cells remains unclear.

However, as alternative splicing affects most genes, it is likely that cell cycle control, signal transduction, angiogenesis, motility and invasion and the metastasis and apoptosis processes that are often impaired in cancer will be affected (Skotheim & Nees, 2007, as cited in van Alphen et al., 2009).

The delineation of splice sites used in alternative splicing or the functional characterization of aberrant proteins expressed as a result of alternative splicing may provide the necessary insight for therapeutic interference. Specific splice-site mutations or proteins might (a) prove to be of predictive value and (b) become potential prognostic markers (Yu et al., 2007, as cited in van Alphen et al., 2009), (c) prove to be targets of future (anticancer) antibody-guided drugs (Orban & Olah, 2003; Heider et al., 2004; Xiang et al., 2007, as cited in van Alphen et al., 2009) or (d) become a target for peptide receptor radionucleotide therapy. An example of the latter is a splice variant of the cholecystokinin receptor found in colorectal and pancreatic cancer that can be targeted by radionuclide therapy (Laverman et al., 2008, as cited in van Alphen et al., 2009). A disease in which an understanding of the alternative splicing process is used for therapeutic purposes is Duchenne's muscular dystrophy (DMD), which is a neuromuscular disease caused by deletions/duplications or point mutations in the 2.4Mb DMD gene, encoding dystrophin, causing disruption of the open reading frame (ORF). The induction of exon skipping, circumventing the mutated exon through intramuscular injection of carefully designed antisense oligonucleotides, corrects the ORF of

dystrophin in *in vitro* cell lines, animal models and humans (van Deutekom et al., 2007, as cited in van Alphen et al., 2009). Another example concerns spinal muscular atrophy (SMA) caused by the deletion of SMA1 gene and the inability of the remaining SMA2 gene, which is virtually identical to SMA1, to compensate for the SMA1 loss, as its transcript lacks exon 7 (for review see van Alphen et al., 2009). Maybe a similar approach would help to evaluate the study of drug target in trans-splicing mechanism in parasites.

3. Trans-splicing in mammalian cells versus methodology for detection

Although trans-splicing has been suggested as being a widespread RNA processing system among eukaryotic cells (see references in Eul et al., 1995), no clear experimental evidence has been obtained so far that authentic mRNA molecules and functional proteins are generated in mammalian cells by trans-splicing. However, some results from Eul et al. (1995) supported the hypothesis that trans-splicing is a regular RNA processing mechanism in mammalian cells, since using molecular biology tools in rat cells were able to generate the T1 antigens (for SV-40 virus) by means of trans-splicing. This is not so hard to understanding nowadays, since trypanosomes also use cis-splicing as an alternative mRNA processing.

The best candidates for the trans-splice reaction are those donor pre-mRNA molecules, where 5' splice donor site is not followed by a functional 3' splice site, which would favour a cis-splice reaction. Any candidate for an RNA acceptor molecule must still contain a functional 3' splice acceptor site. In the case of T1 mRNA, donor and acceptor RNA molecules are the same primary transcripts; but it may also be that independent transcripts, derived from different loci, are joined together to generate hybrid protein molecules splice site through trans-splicing (Eul et al., 1995).

The features of an RNA molecule that lead to trans-splicing are still not clear. Caudevilla et al. (2001) in their work assumed that RNA molecules which contain cryptic splice sites that cannot be used for cis-splicing, are good candidates for heterologous trans-splicing. Cryptic splice sites can be a genuine feature of a gene (e. g. HIV-nef) or they can be generated and activated by point mutation. It is noteworthy that splice site mutations are frequently associated with human genetic diseases. One interesting question in heterologous trans-splicing is how RNA molecules that are transcribed from different genes find each other, differently on the trypanosomes trans-splicing mRNAs which have always the 39 nucleotides in 5' end. The model in mammals proposes that the splice sites from stable complexes with ribonucleoproteins and with splice factors such as SR proteins (Fu & Maniatis, 1992; Staknis & Reed, 1994, as cited in Caudevilla et al., 2001) that mediate the association of the two RNA molecules by protein-protein interaction (Eul et al., 1995). SR proteins have both exon-dependent and independent functions in pre-mRNA splicing (Hertel & Maniatis, 1999, as cited in Caudevilla et al., 2001). These proteins bind to purine-rich sequences called exon splicing enhancer (ESE), promoting both the assembly of the pre-spliceosomal complex (Staknis & Reed, 1994, as cited in Caudevilla et al., 2001) and the subsequent splicing steps (Chew et al., 1999, as cited in Caudevilla et al., 2001). The importance of these purine-rich sequences for trans-splicing *in vivo* was further confirmed by analyzing COT mRNA processing in Cos7 monkey cells. In contrast to the rat COT gene, the second exon of the monkey COT gene lacks an ESE and the cells do not perform homologous COT trans-splicing. Moreover, direct RNA-RNA association via

complementary sequences may additionally facilitate heterologous trans-splice efficiency as it was shown for ribozyme trans-splicing (Koehler et al., 1999; Puttaraju et al., 1999, as cited in Caudevilla et al., 2001) and for homologous SV40 T-antigen trans-splicing (Eul et al., 1995).

The mechanism of the trans-splicing process presumably is more similar to cis-splicing than the trans-splicing process in trypanosomes, nematodes and plant cell organelles, requiring the formation of an equivalent pre-spliceosomal complex. Formation of the pre-spliceosomal complex may be facilitated by a direct base pairing between the two precursor mRNA molecules (Eul et al., 1995). In analogy to cis-splicing, the U1 snRNP could bind via its snRNA to the 5′ cryptic splice site on the first pre-mRNA (molecule A) and the U2 snRNP to the branch site of the second pre-mRNA (molecule B) The donor 5′ splice site and the branch site of the two RNA molecules are brought together by a U1/U2 snRNP interaction/association (Mattaj et al., 1986; Chabot & Steitz, 1987; Lutz & Alwine, 1994, as cited in Eul et al., 1995).

Although trans-splicing has been suggested as being widespread RNA processing system among eukaryotic cells (Konarska et al., 1985; Solnick, 1985; Sharp, 1987; Dandekar & Sibbald, 1990; Joseph et al., 1991; Nigro et al., 1991; Shimizu et al., 1991; Sullivan et al., 1991, as cited in Eul et al., 1995), no clear experimental evidence has been obtained that authentic mRNA molecules and functional proteins are generated in mammals cells by trans-splicing. But the results obtained by Eul et al. (1995) supported the hypothesis that trans-splicing is a regular RNA processing mechanism in mammalian cells, since they showed that the best candidates for the trans-splice reaction are those donor pre-mRNA molecules, where the 5′ splice donor site is not followed by a functional 3′ splice site, which would favour a cis-splice reaction. Any candidate for an RNA acceptor molecule must still contain a functional 3′ splice acceptor site (Eul et al., 1995).

Excision of intronic sequences from pre-mRNAs (pre-mRNA splicing) is an obligatory step for the expression of the majority of higher eukaryotic genes and requires the function of a complex molecular machinery, the spliceosome, which is composed of five small nuclear ribonucleoprotein (snRNP) complexes and more than 200 proteins (for review, see Wahl et al., 2009, as cited in Corrionero et al., 2011). The function of the spliceosome relies on an intricate network of protein–protein, protein–RNA, and RNA–RNA interactions that undergo significant conformational and compositional rearrangements to facilitate intron excision (for review, see Smith et al., 2008; Wahl et al., 2009, as cited in Corrionero et al., 2011). These interactions can be modulated to allow the generation of alternative patterns of splicing, a phenomenon reported to occur in the majority of human genes (for review, see Chen & Manley, 2009; Nilsen & Graveley, 2010, as cited in Corrionero et al., 2011). Alternative splicing plays important roles in the development of multicellular organisms and in numerous pathologies, including cancer (for review, see Cooper et al., 2009; David & Manley, 2010, as cited in Corrionero et al., 2011).

The efficiency and precision required for intron removal is in contrast to the limited sequence consensus at intron/exon boundaries, a feature likely connected to the establishment of mechanisms of splice site regulation mediated by a diverse array of auxiliary sequences and cognate factors (for review, see Wang & Burge, 2008; Barash et al., 2010, as cited in Corrionero et al., 2011).

In higher eukaryotes, the 39 end of the intron is characterized by a polypyrimidine-rich sequence (the pY-tract) followed by a conserved AG as the intron 39-most dinucleotide. Upstream of the pY-tract is the branch point, which functions optimally when it coincides with the invariant yeast sequence UACUAAC (Zhuang et al., 1989, as cited in Corrionero et al., 2011), but which corresponds to the loose consensus YUNAY in higher eukaryotes (Y representing pyrimidines, R purines, N any nucleotide, and the A represents the invariant adenosine that engages in a 29–59 phosphodiester bond with the 59 end of the intron after the first catalytic step of the splicing reaction) (Gao et al., 2008; for review, see Wahl et al., 2009, as cited in Corrionero et al., 2011). Initial recognition of the 39 splice site sequences involves cooperative binding of the branch point-binding protein (BBP/SF1) to the branch point, and of the U2 snRNP auxiliary factor (U2AF) 65-kDa and 35-kDa subunits to the pY-tract and the AG dinucleotide, respectively (Ruskin et al., 1988; Zamore et al., 1992; Berglund et al., 1997; Merendino et al., 1999; Wu et al., 1999; Zorio & Blumenthal, 1999; Liu et al., 2001; Selenko et al., 2003, as cited in Corrionero et al., 2011). RNA–protein and protein–protein contacts involving these factors are believed to enforce a unique RNA structure that triggers subsequent events in 39 splice site recognition (Kent et al., 2003, as cited in Corrionero et al., 2011). These interactions, together with recognition of the 59 splice site by U1 snRNP and possibly other contacts across the intron, establish the earliest ATP-independent (E) complex that commits the pre-mRNA to the splicing pathway (Michaud & Reed, 1991, 1993, as cited in Corrionero et al., 2011).

The next step in 39 splice site recognition is the recruitment of U2 snRNP, which is composed of U2 snRNA and several polypeptides, including two protein subcomplexes (SF3a and SF3b), which are part of the 17S U2 snRNP complex (for review, see Wahl et al., 2009, as cited in Corrionero et al., 2011). SF3a is composed of three subunits of 60, 66, and 120 kDa, while SF3b is composed of at least eight subunits of 10, 14a, 14b, 49, 125, 130, 140, and 155 kDa (for review, see Wahl et al., 2009, as cited in Corrionero et al., 2011). U2 snRNP recruitment involves at least two separable steps. U2 snRNP has been detected in E complexes, but U2 binding in this complex is not stable and does not require the presence of a branch point (Hong et al., 1997; Das et al., 2000; Donmez et al., 2004, 2007, as cited in Corrionero et al., 2011). Stable binding of U2 snRNP to form pre-spliceosomal complex A requires ATP and depends on base-pairing interactions between the branch point and a region of U2 snRNA known as the branch point recognition sequence (bprs) (Parker et al., 1987; Nelson & Green, 1989; Wu & Manley, 1989; Zhuang & Weiner, 1989, as cited in Corrionero et al., 2011). Base-pairing between the branch point and the bprs bulges out the branch point adenosine, which facilitates the first catalytic step (Query et al., 1994; Smith et al., 2009, as cited in Corrionero et al., 2011). It has been proposed recently that initial interaction of the bprs with the branch point requires a stem–loop structure in U2 snRNA, which is subsequently disrupted to facilitate stable base-pairing between the branch point and the bprs (Perriman & Ares, 2010, as cited in Corrionero et al., 2011). This rearrangement of RNA–RNA interactions could be mediated by Prp5, a member of the RNA-dependent DExH/D-box family of ATPases/helicases involved in fidelity of splice site recognition and other key spliceosomal transitions (for review, see Konarska et al., 2006, as cited in Corrionero et al., 2011), because the ATPase activity of Prp5 has been linked to kinetic proofreading of branch point/bprs base-pairing interactions (Xu & Query, 2007, as cited in Corrionero et al., 2011).

U2 snRNP binding serves as a platform for additional RNA–RNA and protein interactions, leading to the recruitment of the tri-snRNP U4/U5/U6 and formation of mature spliceosome complexes, within which numerous RNA rearrangements and changes in protein composition facilitate splicing catalysis (for review, see Smith et al., 2008; Wahl et al., 2009, as cited in Corrionero et al., 2011). A recent report by Roybal and Jurica (2010) concluded that spliceostatin A (SSA) inhibits spliceosome assembly subsequent to pre-spliceosome formation, impeding the transition between complex A and B, implying a role for SF3b in maturation steps of the spliceosome after its established function in early U2 snRNP recruitment.

4. Drug targeting aspects for parasite infection and trans-splicing in human cells could be target by similar therapies

Since all trypanosomatid mRNAs are trans spliced and trypanosomatid genes typically do not harbor introns, it was long thought that these organisms use RNA splicing exclusively for SL transfer, and accordingly, trypanosome-specific deviations of splicing factors were hypothesized to be trans splicing specific. It therefore came as a surprise when the *T. brucei* PAP gene (TriTryp database [TriTrypDB] accession no. Tb927.3.3160), encoding poly (A) polymerase, was shown to harbor a single intron that was removed by conventional cis-splicing. The search for further introns revealed only one more gene in *T. brucei* (Tb927.8.1510), encoding a putative RNA helicase, whose pre-mRNA was shown to be cis spliced. Interestingly, a recent characterization of the *T. brucei* transcriptome by high-throughput RNA sequencing strongly indicates that there are no other introns disrupting protein-coding genes (Mair et al., 2000).

Since differences were observed in the machinery of cis- and trans-splicing, the latter mechanism would represent an alternative to development of new drugs more potent and with fewer side effects. Because of this, several techniques have been developed to detect possible interference in the process of trans-splicing. The first studies involving the union of distinct sequences in transcripts were done in 1985, where by using the machinery of HeLa cells was possible to combine two independent exons transcribed into molecules (Konarska et al., 1985, Solnick, 1985).

Only a few years later, Ullu and Tschudi (1990) established the reaction using permeable cells, aiming to study transcription and trans-splicing in *Trypanosoma brucei* cells. These cells can be treated with lysolecithin that makes the parasites permeable to triphosphate and radioactive nucleotides, as well as other molecules, and were able to synthesize the mature mRNAs. The detergent treatment is relatively mild and the overall cellular morphology and integrity of the plasma membrane are retained. Nevertheless, after exposure to lysolecithin, trypanosome cells become permeable not only to nucleotide triphosphates but also to other macromolecules like DNAse I, which readily penetrates the nuclei and digests the nuclear DNA and DNA to oligomers up to 23 nt long. These observations suggest that the intercalation of lysolecithin in the lipid bilayer can cause profound effects on the permeability characteristics of the cellular membranes.

Using permeable parasites was possible to incorporate other elements on the reaction as well as the radioactive nucleotide, usually [α-^{32}P] UTP and ATP, GTP, CTP, creatine kinase and creatine phosphate. After this step, followed by electrophoresis in 6% acrylamide gel with urea, it can be revealed the abundance of newly synthesized mRNA during the

incubation time. This is an important parameter because it indicates RNA is degraded (Figure 4). It was demonstrated by permeable cell system that the efficiency synthesis and accumulation of various cellular RNAs is severely affected by the concentrations of monovalent ion and, to a lesser extent, of divalent ions. Potassium concentrations greater than 20 mM were strongly inhibitory for the synthesis of SL, U4, and 7SL RNAs and magnesium ions have less dramatic effects than potassium ions on the overall synthesis of SL, 7SL, U4 and tubulin mRNAs. However, above 3 mM [Mg++] occured accumulation of a 3' end shortened form of the SL RNA (SL RNA 130) (Ullu & Tschudi, 1990).

To determine if trans-splicing is taking place in permeable cell system, was necessary to determine the structure of newly synthesized SL RNA sequences by RNAse protection experiments, using a transcript containing antisense sequence to SL RNA as a probe, followed by enzymatic treatment with RNases A and T1, which degraded non-hybrid mRNAs and allowed the localization of SL exon and intron, respectively. The probe used in this kind of experiment was an unlabelled antisense SL RNA, which is complementary to nucleotides 7 to 128 of the SL RNA. It was possible detected four fragments with approximate motilities of 129, 123, 90, and 39 nt. The 123 nt RNA species had the correct size expected for protection of SL RNA which had not been cleaved at the 5' splice site. The 129 nt RNA was longer than the fragment expected for full protection of the probe (123 nt) and may be derived from incomplete cleavage of intact SL RNA by ribonuclease A and TI. This would be the case if the 129 nt SL RNA fragment has a fully methylated cap structure (in which the first four nucleotides adjacent to the cap are methylated), and therefore the ribonucleases would not be able to cleave the 5' end sequence. Therefore, both the 123 and 129 SL RNA fragments represented SL RNA which had not been cleaved at the 5' splice site (Ullu & Tchudi, 1990). On the other hand, the 90 nt and 39 nt long SL RNA fragments had the sizes consistent with their identity as the SL intron and the SL exon, respectively (Figure 5).

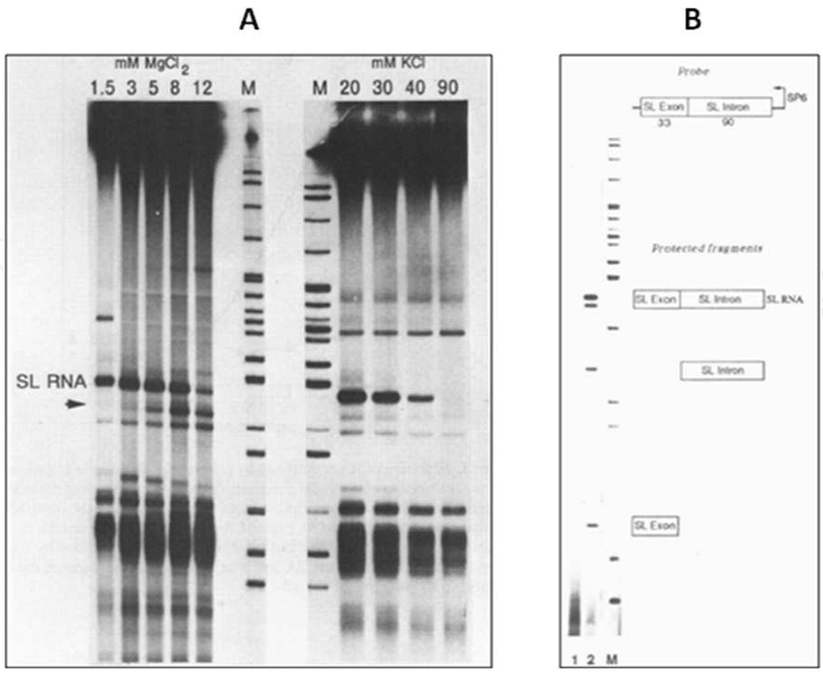


Fig. 5. Trans-splicing in *T. brucei* permeable cells and RNase protection.

A: RNA synthesis. Radiolabelled RNA was synthesized for 8 min. in permeable cells using variable amounts of $MgCl_2$ and KCl, as indicated above each lane (mM). Aliquots from each reaction were fractionated by electrophoresis on a 6% polyacrylamide-7 M urea gel and ^{32}P-RNA was detected by authoradiography. The position of the SL RNA is indicated and a solid arrowhead indicates the position of the SL RNA 130 (see text for details). M, end labelled MspI fragments of pBR322 as molecular weight markers. B, Fractionation of radiolabelled RNA fragments protected from RNAse digestion by the antisense SL RNA probe. Radiolabelled RNA from 6x 10^6 cells was analyzed by RNAse mapping in the absence (lane 1) or presence (lane 2) of 200 ng of unlabelled SL antisense RNA essentially as described (3) except that 40% formamide was included in the hybridization solution and hybridization was carried out at 37°C. The products of digestion were analyzed by electrophoresis as described in Figure 1. M, molecular weight marker. The structures of the antisense SL RNA probe and of the expected products of RNAse digestion are shown on the right. This figure was transcribed from Ullu & Tschudi, (1990).

Experiments using S-adenosyl-L-homocysteine (Ado-Hcy) were pioneers in demonstrating the use of permeable cells as a model to study substances capable of interfering with the reaction of trans-splicing (Ullu & Tschudi, 1990). The use of Ado-Hcy inhibited trans-splicing reaction by competitive inhibition of S-adenosyl-L-methionine (Ado-Met) mediated by 5'end methylation reactions and it allowed the analysis of the methylation as a rule for trans-splicing reaction. Its addition in the transcription/trans-splicing reaction mix, following by RNase protection as described before, allowed the localization of methylated-SL exon + SL intron (figure not showed).

Ambrósio and co-workers (2004) demonstrated that standardization of trans-splicing reaction with *T. cruzi* epimastigote forms (Y strain) permeable cells facilitates the study of RNA processing in these trypanosomatids and can be used as a model of study drug activity on trans splicing mechanism. The authors also demonstrated that pro-drug NFOH-121, derived from nitrofurans (Chung et al., 2003), was able to interfere with RNA processing this parasite, although the reaction of trans-splicing has not been affected (Ambrósio et al., 2004). The nitrofurans are compounds with the capacity to inhibit trypanotiona reductase, an important enzyme involved in the antioxidant metabolism of *T. cruzi*. To develop more selective drugs with low toxicity and more specific targets, a reciprocal prodrug of nitrofurazone, the hydroxymethylnitrofurazone (NFOH-121), was developed and showed higher trypanocidal activity than nitrofurazone and benznidazol in all stages (Chung et al., 2003). In order to analyze the drug activity in *T. cruzi* RNA processing, NFOH-121 was added to the mixture of reagents in different final concentrations. The bands which were revealed in electrophoresis acrylamide gel (Figure 6) decreased when higher concentrations of the drug were used suggesting that the drug would affect the RNA processing in these parasites. However, the presence of double band (SL RNA methylation) shows that even if the drug interferes in mRNA processing, the trans-splicing reaction still occurred, even the protein would not be active. This would mean that the drug activity was prominent at level of the mRNA processing in these parasites and maybe by interfering in the transcription rate (Figure 6, panel A).

Recent data also show that the technique of permeable cells/RNAse protection can be used in assessing the activity of natural compounds on the mRNA processing machinery/trans-splicing (Figure 6, panels B and C). (−)-Cubebin, a compound extracted from the dry seeds

of the Asian pepper (*Piper cubeba* L.) and leaves of *Zanthoxylum naranjillo*, seems to provide treatments with little collateral effect as compared to benznidazole, evaluated by *in vitro* (cells) and *in vivo* (mice) tests (Bastos et al., 1999). Partial synthesis of (−)-cubebin resulted in (−)-6,6′- Dinitrohinokinin (DNH) and (−)-hinokinin (HQ) which were also tested in *T. cruzi* parasites and HQ showed to be a potential candidate for the development of a new drug to treat Chagas' disease (Saraiva et al., 2007, 2010). It showed higher trypanocidal activity than benznidazole against epimastigote forms and similar activity against amastigote forms (De Souza et al., 2005; Saraiva et al., 2007). Furthermore, *in vivo* assays showed significant parasitemic reduction after administration of HQ in mice infected, and it was observed that the groups treated with HQ displayed better survival rates than that treated with benznidazole. However, DNH presented less activity than benznidazole (Saraiva et al., 2007, 2010). It was also demonstrated that HQ and DNH were not capable to cause strong interference in the RNA processing by trans-splicing in *T. cruzi*, as observed by the RNase protection reaction. HQ, mainly, leads to an increase dose-dependent of mRNA synthesis which demonstrates the parasites attempt to remaining viable. This result was also observed in Bolivia strain but at lower concentration, which seems to reinforce the higher Bolivia resistance than Y strain and would indicate a strain-dependent activity of the substances. In both, Y and BOL strains, after the addition of the substances, there was no blocked of the trans-splicing processing, therefore it was continued observing the methylated SL RNA bands in gel, which were related to the capping reaction, as well as the exon and intron bands, referring to trans-splicing reaction. These results showed that the substances might have interfered in any step of the RNA transcription, promoting alterations in the RNA synthesis, even though the RNA processing mechanism still occurs (Andrade e Silva et al., 2011).

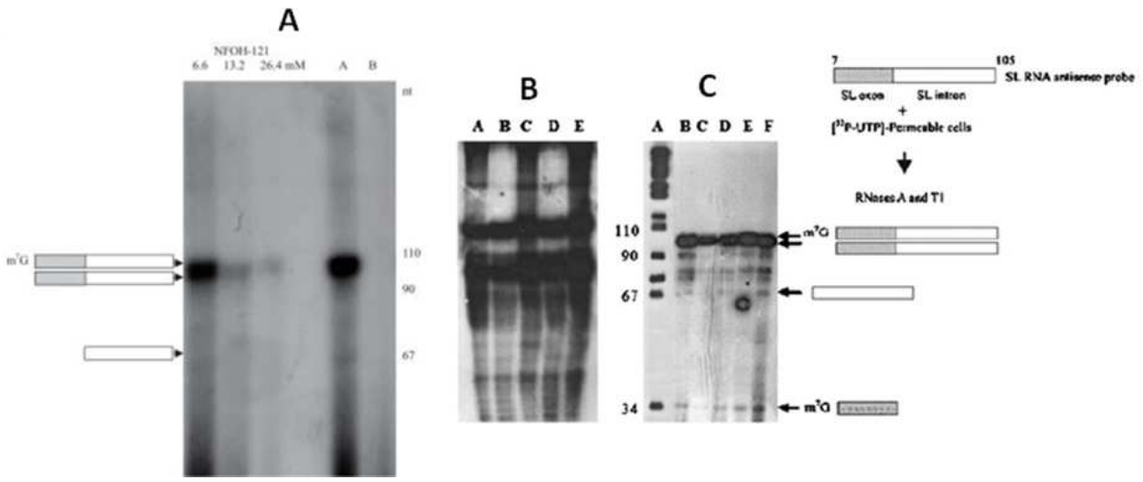


Fig. 6. Trans-splicing in *T. cruzi* permeable cells and RNase protection.

Panel A: RNase protection reaction with Trypanosoma cruzi Y strain using NFOH-121 and electrophoresed on a 10% polyacrylamide - 7M urea gel and detected by autoradiography. The concentrations of NFOH-121 are indicated in the figure. The bands correspondent to SL exon + SL intron methylated, non-methylated and SL intron are indicated in the figure by the symbols and arrows; control with no NFOH-121 (A) and RNase control in the absence of SL antisense probe (B) are indicated. The molecular weight in nucleotides (nt) is indicated in

the figure; m7G: 7-methylguanosine. Transcribed from Ambrosio et al. (2004). Panel B: Permeable cells experiments with *T. cruzi* Y strain, electrophoresed on a 10% polyacrylamide–7 M urea gel and detected by autoradiography. A, control (without substances); B, 1 mM DNH; C, 2 mM DNH; D, 1 mM HQ; E, 2 mM HQ. Panel C: RNase protection experiments with *T. cruzi* Y strain permeable celss, electrophoresed on a 10%polyacrylamide–7 M urea gel and detected by autoradiography. A, pBR322 digested with MspI labeled with [32P]-dCTP; B, control (without substances); C, 1 mM DNH; D, 2 mM DNH; E, 1 mM HQ; F, 2 mM HQ. The bands correspondent to SL exon+SL intron methylated, non-methylated, SL intron, and SL exon are indicated in the figure by the symbols and arrows, and the bands non-indicated are different sizes of intron structures. m7G 7-methylguanosine. Transcribed from Andrade e Silva et al. (2011).

Experiments using permeable cells have to use radioactive material, making more difficult to use the technique, because besides the high cost of material, it requires specific license to obtain the material and trained personnel to establish the method in the laboratory. Based on this view, Barbosa et al. (2007) developed a rapid test to detect interference from molecules in the reaction of trans-splicing using the permeable cell model and traditional silver staining. They replaced the isotope by a nonradioactive nucleotide. It was possible to observe reduction of specific bands on polyacrylamide gels stained with silver staining method. Figure 7 shows the gel and snRNAs bands. Reducing costs and simplifying the method, it was possible to observe the interference of trypanocida molecules in the processing of messenger RNA in different strains of *T. cruzi*, taking into account the phenotypic and genotypic diversity of this species. Tests made using the prodrug NFOH-121 (Figure 6, panel A) demonstrated a reduction of the band U2, U1, U4, U5, U6 snRNAs in different strains of *T. cruzi* (Y, NCS and Bolivia strains). All six bands analyzed in the gel showed decreased when higher concentrations of NFOH-121 were used, suggesting that the drug did affect RNA processing, mainly in the Y and NCS parasite strains. However, the drug showed no effect on the Bolivia strain. The activity of NFOH-121 compared with the parental drug NF (nitrofurazone, Andrade & Brener, 1969) was evaluated either by direct biological activity on the parasites or reducing RNA processing, showing that NFOH-121 drug's activity is dose-dependent (Barbosa et al., 2007).

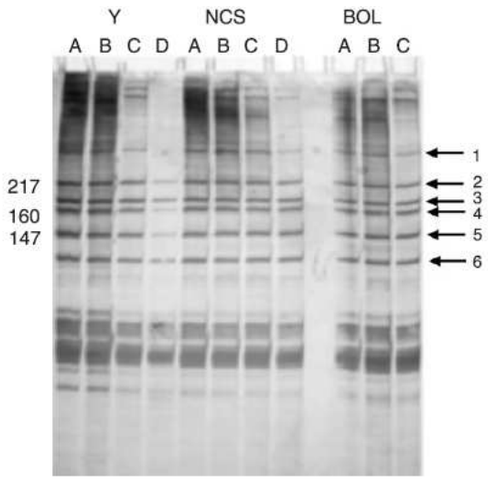


Fig. 7. RNAs (1 trough 6) from *T. cruzi* permeable epimastigote cells from Y, NCS, and BOL strains.

Cells were treated with the hydroxymethylnitrofuran (NFOH-121) and electrophoresed on 10% polyacrylamide-7 M urea gel. A, Control without NFOH-121; B, 2 µM NFOH-121; C, 5 µM NFOH-121; D, 12.5 µM NFOH-121. 1, Unrelated small nuclear RNA; 2, U2; 3, U1; 4, U4; 5, U5; 6, U6 small nuclear RNAs. Molecular weights in base pairs are given on the left. Silver-stained gel. This figure was transcribed from Barbosa et al. (2007).

More recent data, using the combination of RNA interference (RNAi) of some proteins involved in splicing reactions and reverse transcriptase-polymerase chain reaction (RT-PCR) demonstrate the possibility of a relatively simple tests being used in evaluation of the splicing reaction in trypanosomatids. The discovery of RNAi in *T. brucei* occurred unexpectedly and simultaneously with the some findings made in *Caernorhabditis elegans*, in which the parasites transfected with expression vectors for proteins showed multiple morphological phenotypes, including multiple nucleous and kinetoplasts. These observations showed that a hairpin similar to RNA 5'-UTR of alpha-tubulin (α-tubulin) was the element responsible for the new phenotypes. Transfections with double-stranded RNA (dsRNA) constructed with T7 RNA polymerase generated parasites with the same characteristics mentioned earlier (Ketting et al., 2003).

Currently, the mechanism of RNAi is a powerful tool to studying the molecular biology of parasitic trypanosomatids, allowing the specific interference of endogenous genes. Due to the nature of this type of silencing (post-transcriptional and dominant), this mechanism of interference constitutes a particularly useful technique for silencing gene expression in diploid organisms, where the traditional knockout of target genes would be highly costly and, in the case of essential genes, virtually impossible (Ullu & Tschudi, 2003). By the other hand, studies using this mechanism in *T. brucei*, allowed to extrapolate the results obtained for other species of trypanosomatids in which the machinery of RNAi silencing is not competent, for example, in *T. cruzi*.

Having in account that the RNAi approaches described above were transient, vectors were constructed to achieve long-lasting RNAi response expressing dsRNAs under control of tetracycline-inducible promoters. At present, two types of vectors are available that afford regulated expression of either hairpin RNA or dsRNA transcribed from opposing T7 RNA polymerase promoters. Both vectors types are stably integrated in the genome by homologous recombination at the nontranscribed spacer of the ribosomal DNA (rDNA) locus and require a recipient trypanosome cells expressing T7 RNA polymerase ant *tet* repressor. In the hairpin-type vector, dsRNA is produced from a sense and antisense fragment of the gene of interest cloned upstream and downstream from a stuffer fragment, respectively. The purpose of the stuffer fragment is to stabilize the plasmid for replication in bacteria. The double T7 vector contains two opposing T7 polymerase promoters, each of them followed by two T7 terminators arranged in tandem. A portion of the gene of interest is cloned between the two promoters (Ullu & Tschudi, 2003).

After the discovery of RNAi in trypanosomes, different authors have used this tool to check whether different genes and their protein products are potentially involved in splicing reaction. The first information was obtained about the essentiality of the gene for the parasite viability (Shi et al. 2000). In most cases, gene silencing proved to be essential for survival, as demonstrated by growth curves of induced RNAi cells compared to the non-induced controls.

To check the interference of the splicing reaction of a set of RT-PCR can be designed, using primers directed to the processed and unprocessed gene as Poly-A polymerase (cis-splicing) and alpha-tubulin (trans-splicing). The primers synthesized for specific regions of PAP (Figure 8) are capable of binding to the exons flanking the single intron found in this gene. During the induction time with appropriate antibiotic it was observed accumulation of unprocessed transcripts of PAP (band around 780 bp), and reduced level of mature transcripts (band of approximately 130 bp), demonstrating the interference in cis-splicing in these trypanosomatids (Figure 8). Similarly, a semi-quantitative reaction of RT-PCR, using oligos targeted to specific regions of alpha-tubulin was able to detect reduction of non-processed transcript (band of approximately 750 bp) and accumulation of non-throughput (band of about 150 bp), as shown in Figure 8.

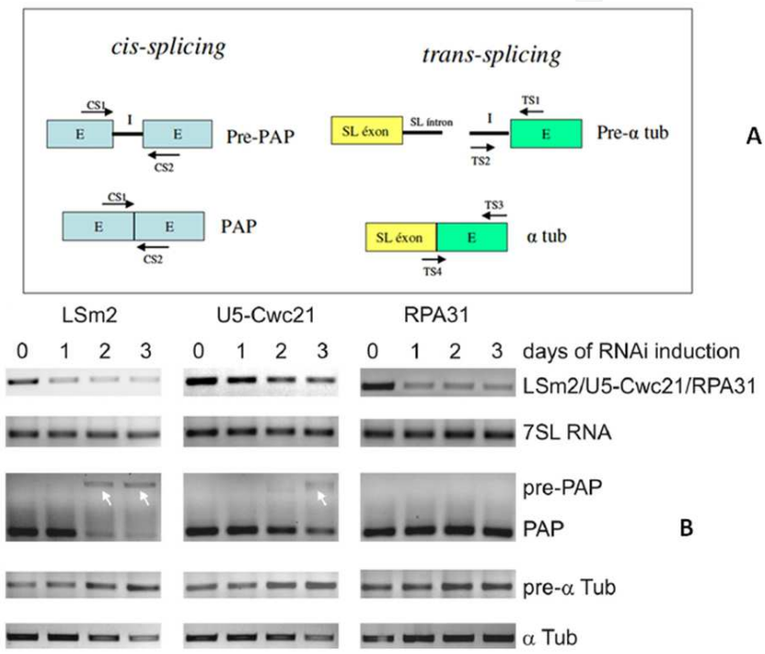


Fig. 8. *In vitro* evaluation of cis- and trans-splicing reaction in *T. brucei*.

A. Schematic diagram of the annealing regions of primers used in functional analysis of cis- and trans-splicing reactions. CS1 and CS2 anneal to the exon transcribed from the poly A polymerase unprocessed (pre-PAP) and processed (PAP); TS1 and TS2 anneal to the exon and intron of the transcript not processed for α-tubulin (Pre-α-tub). To evaluate the processing of α-tubulin, TS3 binds to another region of the exon of α-tubulin, while TS4 anneals to both the SL exon and α-tubulin exon (α-tub). E, exon ; I, intron. (B) Semi-quantitative RT-PCR analysis of RNA abundance in total RNA preparations from cells in which dsRNA synthesis was induced for 0, 1, 2, or 3 days. As a control, the knockdown of the RNA polymerase I subunit RPA31 was co-analyzed. The first panels show the knockdown of the respective mRNAs and the second panels the analysis of 7SL RNA which is not sensitive to splicing defects. The third panels show a competitive PCR of the PAP pre-mRNA/mRNA. The white arrows point to the pre-mRNA amplification products. Pre-mRNA (pre-α Tub) and mature mRNA of α-tubulin (α-Tub) were amplified in the fourth and fifth panels, respectively. The figure 8 B was transcribed from Ambrósio et al. (2009).

Newly discovered genes have been characterized for their participation in the splicing using the tests described above. Cwc21 protein, which was described being exclusive of trypanosomatids, proved to be essential for survival of the parasite and its silencing caused inference in both cis- and trans-splicing (Ambrósio et al, 2009). Several other examples are presented in the literature as highly conserved protein of U5 snRNP, U5-15K (da Silva et al., 2011) and recently described SMN (Palfi et al., 2009) from *T. brucei*, whose participation in splicing was detected using semi-quantitative RT-PCR.

Although different molecules are still to be tested for interference on the reaction of trans-splicing, in theory, it can be possible to employ semi-quantitative RT-PCR for this purpose. In this case, it would require molecules with features for passage through cell membranes to reach the splicing machinery.

After two decades of unsuccessful attempts in establishing an *in vitro* trans-splicing system, such a system was recently established by Shaked et al. (2010). The failure to date to establish an *in vitro* system in trypanosomes lies in the use of radiolabeled pre-mRNA substrates that were extensively degraded, and the deficiency in essential factor(s) in the extract. The degradation could not be eliminated even if the mRNA was capped at the 50-end or at the 30-end with pCp. The success in detecting the trans-spliced product in lied in three parameters: (i) the extract used was crude but contain all the factors necessary for the reaction. It is prepared very rapidly to eliminate the potential inactivation of essential factors. However, because the extract is not purified, it contains enzymes that may inactivate essential factors, thus reducing the reaction efficiency. (ii) A large amount of pre-mRNA is added to the reaction, leaving sufficient amounts of substrate (after its immediate degradation) to assemble into an active complex. The amount of pre-mRNA (4 pmol) is 50-fold higher than the amount of SL RNA in the extract (~80 fmol). Indeed, it was demonstrated that increasing the amount of pre-mRNA increases the reaction efficiency. (iii) The assays were the most critical parameter that made it possible to detect the trans-spliced product. Three different assays were used. In all the assays, only the end-product but none of the intermediates are monitored. The reaction efficiency, roughly calculated by the RNase protection assay, is ~1–2% of the input pre-mRNA utilizing almost 50% of the SL RNA present in the extract. Thus, the limiting factor in this system is the SL RNA (Shaked et al., (2010).

Although the traditional assays to monitor splicing so far are based on the use of radiolabeled substrate, the real-time PCR assay represents a much desired substitute. It avoids the use of radiolabeled materials; it is quantitative, and is able to quantitatively detect the amount of trans-spliced product even at levels less than a few fentomoles. In addition, the assay is convenient relative to the RNase protection assay.

An additional parameter that was essential for establishing the *in vitro* system was the choice of pre-mRNA substrate. The results indicated that the TIR pre-mRNA (pNS21-TIR carrying the beta-tubulin intergenic region) was a better substrate than the PIR pre-mRNA (EP-2-3, (Tb927.6.520), positions 226182-227114 on chromosome 6, in fusion with the luciferase gene). The poor performance of the PIR substrate *in vitro* might stem from its size. The larger substrate appeared more vulnerable to degradation. In other splicing systems, it was demonstrated that shortening both the exons and the introns resulted in a substrate that is spliced more efficiently (Shaked et al., 2010; for review and more details also see Michaeli, 2011).

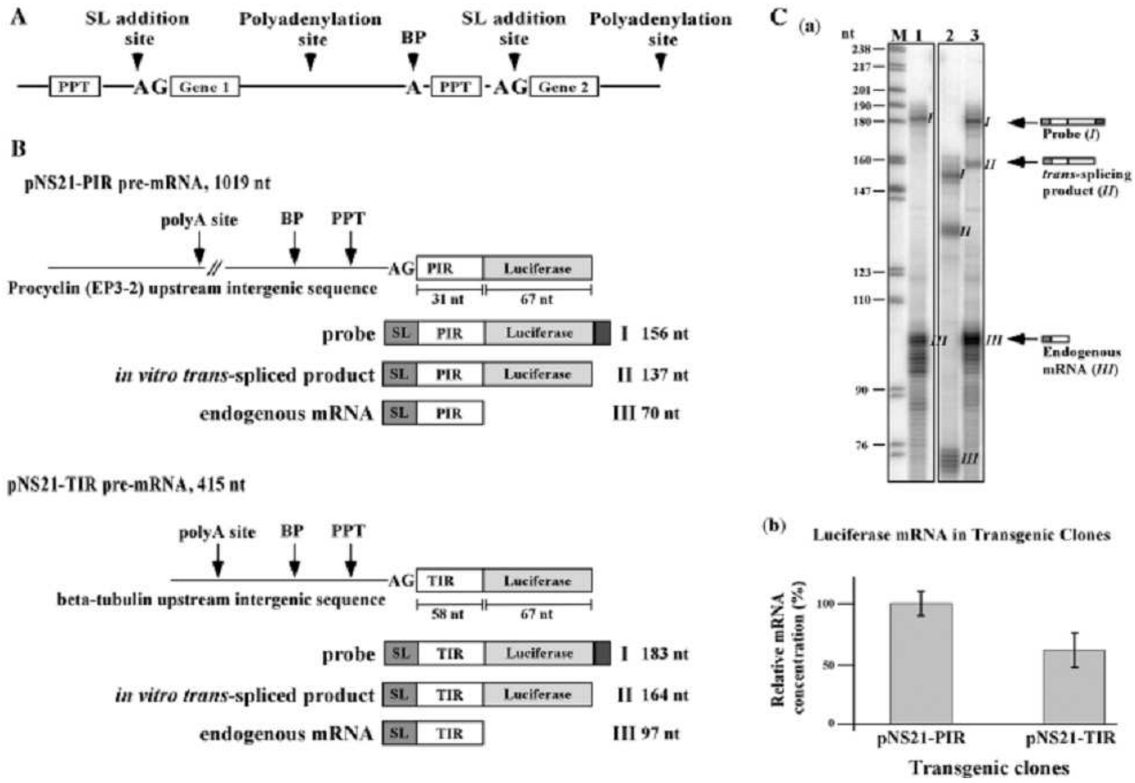


Fig. 9. *In vitro* trans-splicing reaction using RNase protection assay.

(A) Schematic representation of a trypanosome polycistronic pre-mRNA. The significant sequence elements essential for trans-splicing are indicated. (B) Pre-mRNA substrates used in trans-splicing establishment. Schematic representation of the pre-mRNA substrates highlighting the significant sequence elements: polyadenylation site (polyA site), polypyrimidine tract (PPT) and AG splice site. The lengths of the PIR and TIR UTRs as well as the Luciferase ORF are indicated. Underneath each pre-mRNA, the respective probe, in vitro trans-spliced product and endogenous mRNA are shown, along with the expected size of the protected fragments after RNase protection. (C) RNase protection assay to monitor the expression of the TIR and PIR constructs. (a) Thirty micrograms of total RNA was hybridized with labeled antisense probe, complementary to the trans-spliced product. Protected RNA products were separated on a 6% sequencing gel. M- DNA marker, labeled pBR322 DNA MspI digest. The size of the marker is indicated on the left. Lane contents are as follows: 1, RNA from parental strain; 2, RNA from transgenic parasites expressing the pNS21-PIR construct; 3, RNA from transgenic parasites expressing the pNS21-TIR construct. The scheme on the right hand side of the gel indicates the structure of the probe and the protected fragments. (b) Relative expression of the tubulin-luciferase or the EP-luciferase to the endogenous tubulin and EP transcripts. The figure was transcribed from Shaked et al. (2010).

5. Conclusions

From the data presented above, it appears that there are still many difficulties to overcome in the sense that scientists can adequately and favorably control the trans-splicing reaction either in mammals or parasites.

Curiously, although the trypanosomes process virtually all their mRNAs by trans-splicing, the establishment of this reaction *in vitro* using cell-free extracts still presents some methodological obstacles, unlike the reaction in mammalian cells, which was relatively rapidly set up *in vitro* a few years ago.

Thus, efforts should be made to obtain a very similar *in vitro* methodology in parasite trypanosomatids as in mammalian cells to better understand the reaction itself and study all the requirements similarly to evaluation already done in mammals, which would help in the study of interesting and more specific therapeutic targets for parasites. One of the advantages of specific targets to block the trans-splicing reaction in these parasites would be that the drug action it is independent on the parasite strain and also its life stage.

However, based on current studies, some of them reported in this chapter, there are some interesting points in the trans-splicing reaction that could be targets for drug action and could be different from mammalian cells and parasites. For the latter, drugs that interfere or prevent the binding of the SL RNA/SL RNP in the pre-mRNA, ie, which act in the first transesterification reaction, it would become very attractive because they probably block all mRNA processing in the parasite. This type of drug would be highly selective for the parasites and possibly will not affect the host cell.

Regarding to potential drugs that interfere with the trans-splicing reaction in mammals, in this chapter we mentioned that spliceostatin A, an anti-tumor drug, interfered in the fidelity of the recognition of U2 snRNP to the pre-mRNA, leading to interference in the formation of the cis-spliceosome. Although these results referred to the alternative splicing studies and, we may question the extent to which alternative splicing occurring in mammalian cells would not be compared to the trans-splicing in the same cells? In this particular case, as described before, the SR proteins appear to play an important role in this reaction *in vivo* as well as ESE (exonic-splicing enhancers), which bind to SR proteins and probably helps the cryptic 5′ splice site becomes the donor site. A drug which interfering in SR-ESE binding probably would destabilize the complex and the trans-splicing reaction in mammals.

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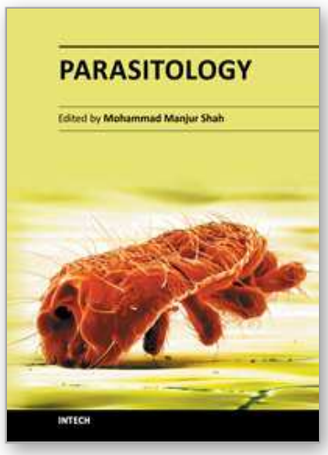
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Parasitology

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Parasitology is an established discipline that covers a wide area of subjects, ranging from the basics (study of life cycle, ecology, epidemiology, taxonomy, biodiversity, etc) to the advanced and applied aspects (human and animal related, although control aspect remains the most important task). There is a great scarcity in the amount of available literature that is freely accessible to anyone interested in the subject. This book was conceptualized with this in mind. The entire book is based on the findings of various studies performed by different authors, comprising reviews and original scientific papers. I hope this book will be helpful to diverse audiences like biologists, zoologists, nematologists, parasitologists, microbiologists, medical doctors, pathologists as well as the molecular biologists, by providing them with a better understanding of the subject.

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