Basal Splicing Factors Regulate the Stability of Mature mRNAs in Trypanosomes*

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Background: Trypanosome trans-splicing depends on basal splicing factors such as U2AF35, U2FA65, and SF1. Results: Transcriptome analyses of RNAi-silenced cells of basal splicing factors reveal differential reliance on factors for trans-splicing and a role for the splicing factors in mRNA stability.

Conclusion: Basal splicing factors regulate trans-splicing and mRNA stability.

Significance: This is the first study to suggest that basal splicing factors regulate mRNA stability.

Gene expression in trypanosomes is mainly regulated post-transcriptionally. Genes are transcribed as polycistronic mRNAs that are dissected by the concerted action of trans-splicing and polyadenylation. In trans-splicing, a common exon, the spliced leader, is added to all mRNAs from a small RNA. In this study, we examined by microarray analysis the transcriptome following RNAi silencing of the basal splicing factors U2AF65, SF1, and U2AF35. The transcriptome data revealed correlations between the affected genes and their splicing and polyadenylation signaling properties, suggesting that differential binding of these factors to pre-mRNA regulates trans-splicing and hence expression of specific genes. Surprisingly, all these factors were shown to affect not only splicing but also mRNA stability. Affinity purification of SF1 and U2AF35 complexes supported their role in mRNA stability. U2AF35 but not SF1 was shown to bind to ribosomes. To examine the role of splicing factors in mRNA stability, mutations were introduced into the polypyrimidine tract located in the 3’ UTR of a mini-gene, and the results demonstrate that U2AF65 binds to such a site and controls the mRNA stability. We propose that transcripts carrying splicing signals in their 3’ UTR bind the splicing factors and control their stability.

Trypanosomes are unicellular parasites that cause devastating diseases. Trypanosoma brucei is the causative agent of sleeping sickness in humans. The parasite cycles between the insect host (procyclic form) and the mammalian host (bloodstream form). mRNA processing in trypanosomes differs from this process in other eukaryotes. All mRNAs are trans-spliced, although two cis-introns have been identified. No distinct promoters for protein-coding genes exist, and thus, all mRNAs are transcribed as long polycistronic units. trans-Splicing, in conjunction with polyadenylation, functions to dissect the polycistronic transcripts into individual mRNAs. In trans-splicing, a small exon, the spliced leader (SL), is added to all mRNAs from a small RNA, the SL RNA (1–3).

Several recent studies shed light on the contribution of trans-splicing and polyadenylation to global gene expression in these parasites. Together, these studies identified alternative processing of transcripts at either their 5’ or, in a larger number of cases, at their 3’ end (4, 5). The sequences that determine the choice of splice site are the AG splice site and the polypyrimidine tract (PPT). Heterogeneity in the composition of the PPT and its distance from the AG splice site might regulate trans-splicing events (4–6). Despite these recent studies, the most robust mechanism that was shown so far to regulate the trypanosome transcriptome is mRNA stability (7, 8).

Relatively little is known regarding the contribution of splicing factors to regulating trans-splicing in trypanosomes. The most highly conserved basal factors that bind to the pre-mRNA are essential for both cis- and trans-splicing (2, 3, 9). The trypanosome U2AF65, which binds to PPT, differs from its homologues in metazoa. The protein contains three RNA recognition motif (RRM) domains, but the RRMIII includes an insertion of a hydrophobic region lacking secondary structure that was classified as a nonregular secondary structure. This nonregular secondary structure sequence was shown to be essential for the interaction of U2AF65 and SF1 in a yeast two-hybrid assay, and the proteins were shown to interact in vivo (10). The trypanosome U2AF35, which is believed to interact with the 3’ splice site (AG), lacks the residues that mediate the interaction with U2AF65 to form a heterodimeric complex like in metazoa (11). Trypophan 134 needed for this reciprocal “tongue in groove” heterodimerization is mutated to lysine in the trypanosome U2AF65 (11), and thus, these trypanosome proteins do not interact in vivo (10). Silencing of U2AF35, U2AF65, and SF1 compromises trans- and cis-splicing, but

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3 The abbreviations used are: SL, spliced leader; PPT, polypyrimidine tract; RNP, ribonucleoprotein; hnRNP, heterogeneous nuclear RNP; PTB, polypyrimidine tract binding; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; nt, nucleotide; RRN, RNA recognition motif; TAP, tandem affinity purification.
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interestingly, silencing of U2AF35 and SF1 leads to accumulation of SL RNA, whereas SL RNA level is reduced in U2AF65-silenced cells. U2AF65 may therefore have a very special role in trans-splicing and may participate in the recruitment of SL RNP to the spliceosome (3). The obligate interaction between SF1-U2AF35-U2AF65 in mammals forces a very strict spatial distance between the 3’ splice site, PPT, and the branch site (12, 13). However, in trypanosomes, this is not the case, and flexibility exists in the distance between the 3’ splice site and the PPT tract (4, 5). This spatial flexibility may provide room for binding of protein(s) that regulate trans-splicing.

Several RNA-binding proteins that were shown to participate in splicing regulation in metazoan also exist in trypanosomes. Among these are heterogeneous nuclear ribonucleoprotein (hnRNP) and proteins carrying a serine-arginine motif (SR) (2, 3, 9). Three SR proteins were described in trypanosomes as follows: TSR1, TSR1IP, and TRRM1 (14–16). However, their exact role in trans-splicing is not currently known. Polypyrimidine tract-binding proteins (PTB) or hnRNP homologues were shown to be required for trans-splicing of mRNAs carrying a C-rich polypyrimidine (17, 18). The PTB proteins were also shown to regulate mRNA stability (17, 18).

In this study, the role of the T. brucei basal splicing factors U2AF35, U2AF65, and SF1 on the transcriptome was examined in cells depleted of these factors by RNAi. The basal splicing factors were shown to differentially affect the transcriptome, suggesting that each of these factors controls different groups of genes. Bioinformatic studies were conducted to identify a correlation between the affected genes and the properties of their SL and poly(A) addition sites and spatial organization of their splicing signals. Indeed, U2AF65 and SF1 target genes were shown to undergo differential polyadenylation and to possess a short PPT poor in pyrimidines. Over a third of the transcripts previously shown to undergo differential polyadenylation (4) were regulated by the splicing factors mentioned above. Although each of these factors globally affected trans-splicing, the major effect on mRNA levels resulted from the role of these splicing factors in regulating mRNA stability. Biochemical purification of these basal splicing factors identified their association with RNA-binding proteins that are implicated in functions other than splicing. We propose that the binding of splicing factors to the 3’ UTR affects the stability of mRNAs, and we demonstrate this concept using a mini-gene assay, showing that U2AF65 binds a PPT site present in the 3’ UTR.

We propose that trans-splicing, known to be coupled to polyadenylation (19–22), determines which poly(A) site will be chosen and thus the size of the 3’ UTR and its ability to interact with basal splicing factors and other binding proteins that dictate mRNA stability.

EXPERIMENTAL PROCEDURES

Cell Growth and Transfection—Procyclic T. brucei strain 29-13, which carries integrated genes for the T7 polymerase and the tetracycline repressor, was grown in SDM-79 medium supplemented with 10% fetal calf serum in the presence of 50 μg/ml hygromycin and 15 μg/ml G418. Cells were transfected as described previously (23).

Construction of RNAi Constructs—The stem-loop constructs for silencing of U2AF65, SF1, and U2AF35 were described previously (10). The expression of dsRNA was induced using 8 μg/ml tetracycline.

Tagging of the Splicing Factors in Leishmania tarentolae and T. brucei—For generation of the tagged constructs, genes were amplified using the primers listed in supplemental S-1. The fragments were cloned into the pSNSAP1 vector (kindly provided to us by Dr. Larry Simpson, UCLA). The cloned vector (20 μg) was transfected into L. tarentolae and selected using neomycin resistance (24). To generate in T. brucei the PTP-tagged constructs that encode a triple tag composed of the ProtC-binding site, tobacco etch virus protease recognition site, and protein A, the gene of interest was amplified with primers listed in supplemental S-1 and cloned into the PTP vector (25).

Preparation of Nuclear and Cytoplasmic Extracts—T. brucei procyclins (10^9) were harvested and washed with phosphate-buffered saline. The cell pellet was resuspended in hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, and 5 μg/ml leupeptin). Next, the cells were broken by 20 strokes in a Dounce homogenizer in the presence of 0.1% Nonidet P-40, and the extract was loaded on the top of a 3-ml sucrose cushion (0.8 M sucrose, 0.5 M MgCl2). The nuclei were collected at 8000 × g for 10 min. The pellet and the cytoplasmic fractions were analyzed by Western blotting.

MTT Assay—T. brucei procyclins (10^7) were harvested, washed, and suspended in 50 μl of PBS. The cells were then treated with 100 μl of 5 mg/ml MTT solution for 20 min at 27 °C. The cells were then centrifuged and lysed into 50 μl of DMSO. The cell debris was pelletted by centrifugation, and absorbance was measured at 570 nm (26).

Purification of the Complexes Associated with the Splicing Factors—Tandem affinity purification was performed from whole L. tarentolae cell extracts, as described previously (27). The proteins were analyzed by mass spectrometry as follows. The samples were digested by trypsin, analyzed by LC-MS/MS on LTQ-Orbitrap (Thermo), and identified by Sequest 3.31 software against the GeneDB Leishmania major-specific database.

Northern and Primer Extension Analyses—Primer extension was performed as described previously (28–30). The extension products were analyzed on 6% acrylamide denaturing gels. Primers are listed in supplemental S-1. For Northern analysis, total RNA was extracted, separated on agarose-formaldehyde gel, and analyzed using a DNA probe that was prepared by random labeling (23). Primers are listed in supplemental S-1.

mRNA Stability Analysis—Uninduced cells and cells 3 days after induction (1.5 × 10^6 cells) were concentrated and resuspended into 25 ml of the SDM-79 medium. Cells were aliquoted into five batches and incubated at 27 °C for 30 min. Cells were pretreated with 2 μg/ml sinefungin (Sigma) for 10 min and then with 30 μg/ml actinomycin D (Sigma). The RNA was subjected to Northern analysis as described previously (18). Each experiment was repeated three times. The RNA level was normalized to 1 at t = 0, and the decay was fitted to exp(−Rt). R was calculated by regressing −ln(normalized RNA level) on t (in minutes), using ordinary least squares without the intercept term. The half-life was then calculated as ln(2)/R.
RT-PCR—Total RNA was extensively treated with a DNase-based DNA inactivation reagent (DNA-free; Ambion) to remove the DNA contamination. Reverse transcription was performed using 1 μg of total RNA with 0.2 μg of random hexamer primer (Fermentas revert aid first strand cDNA synthesis kit) as described previously (18, 31). Next, the cDNA was used for PCR amplification using the primers listed in supplemental S-1.

Microarray Analysis—Total RNA was isolated from uninduced cells and from silenced cells after 3 days of induction. Total RNA was labeled using the Ambion Amino Allyl MessageAmp II aRNA kit (Ambion). DNA microarrays were obtained through Pathogen Functional Genomics Resource Center (managed and funded by the Division of Microbiology and Infectious Diseases, NIAID, National Institutes of Health, Department of Health and Human Services, and operated by the J. Craig Venter Institute), hybridized using the Gene Expression hybridization kit (Agilent Technologies), and processed as described previously in detail (18). The data from all arrays were first subjected to background correction and analyzed using GeneSpring GX software (Agilent Technologies). Array normalization was performed using the intensity-dependent Lowess normalization method (32). Normalized data from two to four biological replicates were analyzed to identify genes whose expression was up- or down-regulated by an arbitrary cutoff of at least 1.5-fold and had $p$ value $< 0.05$ in all replicates when testing for differential expression ($t$ test). Heat maps were generated using the Matlab function clustergram (distance metric, Euclidean; linkage method, average). Pie diagrams were prepared after annotating regulated genes manually, assisted by GeneDB.

Fractionation of Extracts on Sucrose Gradients—*T. brucei* cells ($5 \times 10^9$) were harvested and washed, and extracts were produced using extraction buffer A (33). The cells were broken using a Dounce homogenizer in the presence of 0.1% Nonidet P-40 and extracted with 150 mM KCl. The lysate was cleared at 50,000 $g$ for 30 min and centrifuged through a 10–30% (w/v) sucrose gradient at 4°C for 3 h at 35,000 rpm in a Beckman SW41 rotor.

Construction of the Minigene—The regulatory elements present in pNS21b, carrying a luciferase reporter gene, were used, as described previously (18, 34). To fuse the asparagine synthetase 3′ UTR regulatory sequence to the luciferase reporter in pNS21b, a fragment of 802 nt was generated by PCR using primers listed in supplemental S-1 and was cloned into the BamHI and XhoI sites, replacing the pre-existing 3′ UTR of pNS21b (18).

Statistics of Splicing and Poly(A) Signals—Table 1 compares statistics of splicing and poly(A) signals between regulated and nonregulated genes. The signals were calculated using the list of splice sites and poly(A) sites published by Kolev et al. (4). The only genes for which the major splice site or the major poly(A) site had at least 10 reads were considered. For each row in Table 1, the “regulated genes (mean)” column presents the mean value calculated over all genes regulated in a given experiment and in a given direction (e.g. U2AF65 up). The “other genes (mean)” column gives the mean value calculated over all other *T. brucei* genes (i.e. genes not regulated in the given experiment and direction) whose fold-change was reported in at least one of the microarrays. The $p$ value reported was calculated using the nonparametric Mann-Whitney $U$ test against the hypothesis that the two distributions (in regulated genes and in the other genes) have an equal median. The statistics were calculated as follows. SL dispersion is the weighted average of the distance of all minor splice sites from the major site. The weight of each splice site is its relative number of reads (number of reads for the site/total number of reads for the gene, including the major site). SL diversity is a measure of the utilization of the different splice sites. Formally, if the relative number of reads of site $i$ is $p_i$, the diversity is defined as $-\sum p_i \log p_i$, where the sum is over all sites of the given gene. For PPT length, the PPT was calculated from the sequence of the 120 nts upstream of the major splice site. It represents the maximal (and most downstream) sequence of pyrimidines, separated by no more than one purine. The PPT-AG distance was from the major splice site to the (3′) end of the PPT, where the PPT was calculated as described above. The pyrimidine fraction upstream of splice site is the fraction of pyrimidines in the 120 nts upstream of the major splice site. The purine fraction downstream of splice site is the fraction of purines in the 120 nts downstream of the major splice site. Poly(A) dispersion was the same as SL dispersion, but for the poly(A) sites. The distance from poly(A) site to downstream splice site was between the major poly(A) site of the given gene and the major splice site of the downstream gene. Genes were ordered as in the transcriptome reported in Ref. 4.

RESULTS

Microarray Analysis of U2AF65-, U2AF35-, and SF1-silenced Cells Identifies Transcripts Whose Level Is Controlled by These Factors—We previously provided evidence that silencing of U2AF35, U2AF65, and SF1 results in reduction of the Y structure intermediate generated in the first step of the trans-splicing reaction, suggesting that these factors are essential for the splicing of many transcripts (10). To follow the kinetics of the trans-splicing defects and its correlation with the depletion of the splicing factors, we transfected cells with TAP-PTP-tagged constructs that integrate into the authentic site of the genes (35). The levels of the SL RNA and the Y structure intermediate were examined in cells carrying the silencing construct and the tagged splicing factors. Silencing of these factors resulted in growth arrest (data not shown), and MTT assay demonstrated that despite the growth arrest the parasites remained metabolically active until the 5th day of silencing (data not shown). The results demonstrate that in all cases, the tagged protein was almost completely depleted by the 2nd day of silencing (Fig. 1A). SL RNA started to accumulate after the 2nd day of silencing in U2AF35- and SF1-silenced cells but decreased in U2AF65-silenced cells, as described previously (10). These results suggest that U2AF65 may have a special function in SL RNP biogenesis, which, in its absence, leads to degradation of the SL RNA because it cannot be incorporated into the spliceosome (3).

The variation and heterogeneity in the splice sites, PPT, and the distance between these sites (4, 5) may affect the avidity of the factors to their cognate sites, thus affecting their ability to trans-splice. It is expected that the splicing of transcripts with
low affinity to the factors will be severely influenced as a result of the RNAi silencing. To examine this possibility, microarray analysis was performed using RNA extracted from cells silenced for 3 days. This time point was chosen for analysis because the depletion of the factor was maximal, and trans-splicing defects were observed (Fig. 1A), and the cells were still metabolically active. Previously, the 3rd day of silencing was used as the preferred time point to investigate the phenotype associated with depletion of splicing factors by RNAi (36–41). Each experiment was repeated between two and four times (biological replicates), and two technical replicates were performed for each experiment. Normalized data were used to identify genes whose expression was significantly up- or down-regulated (p value <0.05 (t test)) with magnitude exceeding a cutoff of 1.5-fold. The cutoff was heuristically selected as being large enough to enable an experimental validation and eliminate false positives and yet small enough to capture as many truly regulated genes as possible and enable the statistical analysis of their properties. A list of the regulated genes is provided in supplemental S-2. The depletion of U2AF65 and SF1 resulted in both up- and down-regulated transcripts. In U2AF65-silenced cells, 404 genes were down-regulated, and 360 were up-regulated (supplemental S-2). For SF1, 395 genes were down-regulated, and 386 were up-regulated (supplemental S-2). Because these two factors interact (10), significant overlap was expected between the down-regulated and the up-regulated genes of U2AF65 and SF1. Indeed, a high degree of overlap is visually evident from a heat map (Fig. 1B), as well as from a
Venn diagram (Fig. 1D, panel i). The overlap was maximal for a fold-change of 1.5 (data not shown), further supporting our choice of the cutoff.

Microarray analysis performed on cells silenced for U2AF35 (Fig. 1B) suggests that hundreds of genes were also significantly down-regulated (336 genes (supplemental S-2)) or up-regulated (281 genes (supplemental S-2)). As opposed to the hundreds of transcripts that were co-regulated in U2AF65-SF1-silenced cells, only 90 transcripts were co-regulated in U2AF35-SF1 (Fig. 1D, panel ii), supporting the finding that these factors do not interact in vivo (10). The microarray data were verified by semi-quantitative RT-PCR (data not shown) and Northern analysis (Fig. 2).

The sets of regulated genes seem to be enriched with distinct biological functions. Because conventional programs such as DAVID (42) provide a very vague annotation of gene families, we manually curated the genes and divided them into distinct functional groups that are significant for the parasite homeostasis as follows: transporters, chaperones, or genes involved in protein degradation, metabolism, RNA processing, ribosome biogenesis, and others. A pie diagram demonstrating enrichment of such gene families is presented in Fig. 1C. The co-regulation of families of genes resembles previous findings demonstrating the presence of RNA operons, which constitute genes involved in various aspects of cell division and macromolecular biosynthesis (43).

Next, we sought to determine whether the changes in expression during splicing factor depletion reflect a specific effect on the transcriptome or a general effect due to growth inhibition. To this end, we compared the genes found to be regulated in this study to genes whose expression is changed either during S phase (44) or in high density growth-arrested bloodstream parasites (supplemental S-3) (43). However, no significant correlation was found. Among the 52 genes regulated in S phase (44), 10 were regulated in splicing factor depletion, only slightly less than expected ($p$ value 0.3, Fisher's exact test). Among the 29 transcripts under the “ribosomal” category whose expression changed under growth cessation (43), none was detected among the equivalent group reported in our study. Thus, the changes in ribosomal transcripts observed in our study are not
related to growth arrest as in Ref. 43 but reflect a specific effect on subgroups of transcripts.

Transcript Are Regulated by Binding of Splicing Factors to the Mature mRNA.—The most surprising result in our dataset was the findings that the number of up-regulated genes was similar to the number of down-regulated genes, suggesting that the effect on the transcriptome may reflect other processes beyond splicing. To examine this possibility, pairs of mature mRNA and its corresponding precursor were analyzed by Northern analyses (Fig. 2). Each Northern contained as a control the changes in the level of the transcript and its precursor in SmD1-silenced cells that are severely compromised for both splicing and polyadenylation signals. A table was prepared for 6056 genes that were detected in the microarray experiments, and we found that from the 360 genes that were up-regulated on the 3rd day of silencing, 278 were already up-regulated at the 2nd day of silencing and from the 404 genes that were down-regulated on the 3rd day of silencing, 278 were already reduced on the 2nd day of silencing. The data demonstrate that the typical changes to the transcriptome could already be observed on the 2nd day but became stronger on the 3rd day of silencing.

Next, the localization of the precursor and mature mRNAs was examined to validate that there was no change in localization of the RNAs during the depletion. The nuclear and cytoplasmic extracts were prepared as described under “Experimental Procedures,” and the quality of the fractionation was examined by Northern analysis using an RNA probe (7SL RNA) and nuclear RNA (U3). The results show only minor contamination of nuclear RNA in the cytoplasm (Fig. 2D, left panel). RNA was then subjected to Northern analysis with a probe to Tb927.2.3460, and the results indicate that that mature mRNA was enriched almost exclusively in the cytoplasm, demonstrating no change in localization of these RNAs, especially demonstrating that accumulated precursor is confined to the nucleus (Fig. 2D, right panel). Next, we examined if the splicing factors associate with mature mRNAs. Binding of U2AF65 and SF1 to mature mRNA was examined by affinity selecting TAP-tagged proteins and looking for the presence of the regulated transcripts. The cell lines expressing the tagged proteins described above were used. To stabilize the mRNA-protein interactions by cross-linking, the cells were exposed to UV irradiation. Extracts were prepared from the cells, and RNA was extracted from the affinity-selected RNPs. The extracted RNA was then subjected to RT-PCR using a gene-specific antisense primer and an SL sense primer, which is present on all mature trypanosome mRNAs. The results demonstrate binding of U2AF65 (Fig. 3A) and SF1 (Fig. 3B) to transcripts whose levels were either up- or down-regulated during silencing. No binding was observed for substrates whose level was unaffected in the silenced cells. These results demonstrate the specific association of U2AF65 and SF1 with the mature mRNAs of the transcripts whose levels they regulate.

Splicing and Poly(A) Signals in Transcripts Regulated by U2AF65, U2AF65, and SF1—Because trans-splicing is globally perturbed in the silenced cells, we searched for correlations between the affected genes and properties of their splicing and polyadenylation signals. A table was prepared for 6056 T. brucei genes that were detected in the microarray experiments, and...
either their SL or poly(A) sites were mapped (4). Information regarding the SL and poly(A) site heterogeneity, the distance between these sites, and the properties of the splicing signals was compiled using the RNA-Seq data published by Kolev et al. (4) (supplemental S-3).

Next, a statistical analysis was performed comparing the genes affected under splicing depletion versus the rest of the genes present in the list (Table 1). The first group of statistics describes splicing/polyadenylation heterogeneity. These include SL/poly(A) dispersion, which is the average distance of the minor splice/poly(A) sites from the major site, and SL/poly(A) diversity, which is a measure of the utilization of the different splice sites. Then, the properties of the PPT and its distance to the AG dinucleotide were compared, as well as the distance between splice site to upstream poly(A) sites. A full description of these statistics is presented under “Experimental Procedures.”

Inspection of Table 1 suggests the existence of several statistically significant correlations. For genes up- and down-regulated under U2AF65 and SF1 depletion, polyadenylation is almost always more heterogeneous than in the rest of the transcriptome, suggesting that these genes are enriched in differential polyadenylation. Interestingly, and as expected, genes down-regulated under U2AF65 depletion had significantly fewer pyrimidines in the region upstream of their splice site and consequently had shorter PPTs. These genes most likely possess a weaker affinity to U2AF65, and hence are affected more strongly upon depletion of this factor. The genes up-regulated under U2AF65 and SF1 depletion were found to be enriched with purine-rich exons. This may suggest that these transcripts are also regulated by other factors such as hnRNP proteins, which are known to bind purine-rich motifs (2). In addition, U2AF35 regulated genes had less diversity in the 3′splice site. This may suggest that these U2AF35-regulated transcripts lack alternative 3′AG sites, and when the major site cannot be selected because it depends on binding of U2AF35, no other option for trans-splicing exists; thus trans-splicing is severely affected.

Interestingly, from the 1412 genes that were regulated by splicing factors, 264 genes were shown to be regulated during differentiation, based on published data (6, 43, 45, 46). This is significantly more than expected (198 expected, p value 1.3 × 10^-8, Fisher’s exact test), suggesting that the set of splicing-regulated genes is significantly enriched with genes that are developmentally regulated.

Note, that we listed only 1412 genes whose levels were significantly changed under splicing factor depletion. We anticipated an even larger list, because these splicing factors are basal and are expected to affect splicing of the ~9000 trans-spliced transcripts. However, the observed changes in the transcriptome are also governed by other processes, such as mRNA stability (see below), and hence the effect resulting from splicing may be masked. However, despite some masking of the splicing effects on the transcriptome, the data in Table 1 suggest that the changes observed in the transcriptome correlate with the properties of the splicing signals, which dictate the efficiency and specificity of trans-splicing.

**U2AF65, U2AF35, and SF1 Regulate mRNA Stability**—The presence of up-regulated transcripts under splicing factor perturbation was unexpected. The most likely mechanism that can explain such findings is if these factors modulate mRNA stability. If this is indeed the case, the half-lives of regulated mRNAs should change as a result of silencing of these factors. To examine this possibility, the half-lives of regulated mRNAs were compared between silenced and uninduced cells. Up-regulated (Fig. 4A) and down-regulated (Fig. 4B) mRNAs following silencing of the three splicing factors were selected. To measure half-lives, cells were treated with sinefungin and actinomycin D to completely inactivate mRNA production (47). Northern analysis was performed; mRNA levels were measured by densitometry; the values were normalized using the level of 7SL RNA, and the decay of the mRNAs in uninduced cells was compared with the decay in silenced cells. For most mRNAs, half-lives were significantly different between uninduced and silenced cells (p value < 0.005 (t test)), suggesting that changes in the level of mRNAs are correlated with changes in mRNA stability.

One may suggest that the regulation observed under splicing factor silencing is a reflection of the intrinsic mRNA half-life, *i.e.* our up- and down-regulated transcripts are nothing but long lived and short lived transcripts, respectively. To examine this possibility, we calculated, for each splicing factor separately, the Pearson correlation between the fold-changes during the splicing factor depletion and the half-life time based on data presented previously (48). Although the correlation was negative for U2AF35, there was significant positive correlation for U2AF65 (0.09) and for SF1 (0.14). However, this correlation, while significant, is small and explains only 0.75% (U2AF65) or 1.9% (SF1) of the variance in the fold-changes. Also, we found several intrinsically long lived transcripts that were down-regulated and short lived transcripts that were up-regulated. Thus, we conclude that most of the effect on stability we observed is specific and does not reflect the intrinsic half-life of the mRNA.

The role of the splicing factors in mRNA stability can be corroborated by exploring the cellular distribution and the subset of proteins associated with these factors. Because mRNA stability regulation takes place primarily in the cytoplasm, the cell line expressing TAP-PTP-tagged proteins in *T. brucei* was used to examine the level of these factors in the nucleus and cytoplasm. In addition, we also used antibodies that were raised against U2AF35, PTB1, and hnRNP F (prepared by our group). To evaluate the quality of the separation, we used a cell line expressing tagged tSNAP42, which is the SL RNA-specific transcription factor (35). The results in Fig. 5A demonstrate that in the extracts prepared, tSNAP42 was found exclusively in the nucleus; PTB1, which is implicated to function in both nucleus and cytoplasm (18), was found in both compartments. The splicing factors U2AF65, U2AF35, and SF1 were found mostly in the nucleus but also in the cytoplasmic fraction.

Next, the proteins associated with these factors *in vivo* were examined. The *L. tarentolae* system was used for purifying the proteins associated with the splicing factors. We demonstrated in the past the successful purification of Sm, Lsm, and U1A complexes using this system (27). The *L. tarentolae* system enables purification of a large amount of complexes, suitable for
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#### TABLE 1
Genes affected by splicing factors and their splicing and poly(A) signals

We used the splice sites and poly(A) sites published in Ref. 4 to compare splicing and poly(A) signals between regulated and nonregulated genes. Each row corresponds to one property of the signals, with the "regulated genes (mean)" column presenting the mean value of the property over all genes regulated in a given experiment and in a given direction (e.g., U2AF65 up), and with the "other genes (mean)" column presenting the mean value of the statistic over all other *T. brucei* genes. We then used the Mann-Whitney *U* test (MWU) to compute the significance of the difference. We highlight in boldface the rows with *p* < 0.001 and in italics the rows with *p* < 0.01. Complete details on the calculation of the splicing and poly(A) signals appear under “Experimental Procedures.” Briefly, the SL/poly(A) dispersion is a weighted average of the distance of all minor sites from the major sites (where the weight of each splice site corresponds to its utilization). This is a measure of the spatial dispersion of the splicing/poly(A) sites. The SL/poly(A) diversity is a similar measure that summarizes the heterogeneity of splicing/poly(A) site selection, which does not take into account the distances between the sites but only their relative utilization. For all other statistics, only the major site was considered. The PPT was predicted computationally as the longest stretch of pyrimidines (separated by at most one purine) in a window upstream of the major splice site.

| Property                                      | U2AF65 down-regulated genes | U2AF65 up-regulated genes |
|-----------------------------------------------|-----------------------------|---------------------------|
|                                               | Regulated genes (mean)      | Other genes (mean)        | P-value (MWU) | Regulated genes (mean) | Other genes (mean) | P-value (MWU) |
| SL dispersion                                 | 18.7053                     | 14.4757                   | 0.390497      | 14.0959                  | 14.7558                  | 0.844714      |
| SL diversity                                  | 0.584285                    | 0.588941                  | 0.55444       | 0.548569                 | 0.590835                 | 0.206619      |
| PPT length                                    | 17.3217                     | 18.2121                   | 0.001772      | 18.2993                  | 18.1523                  | 0.210399      |
| PPT-AG distance                               | 36.7307                     | 37.6786                   | 0.317399      | 38.8762                  | 37.5558                  | 0.345687      |
| Pyrimidine fraction upstream of splice site   | **0.622564**                | **0.637159**              | **2.86E-05**  | **0.645603**             | **0.635807**             | **0.002916**  |
| Purine fraction downstream of splice site     | 0.536891                    | 0.545495                  | 0.015532      | **0.560722**             | **0.544145**             | **0.00182**   |
| PolyA dispersion                              | **141.733**                 | **70.3383**               | **0.008235**  | **100.767**              | **72.8548**              | **0.007276**  |
| PolyA diversity                               | 2.0603                      | 1.88919                   | 0.188601      | 2.10312                  | 1.88879                  | 0.068491      |
| Distance from polyA site to downstream splice site | 309.319                  | 216.822                   | 0.286505      | 312.783                  | 217.278                  | 0.036365      |

| Property                                      | SF1 down-regulated genes    | SF1 up-regulated genes    |
|-----------------------------------------------|-----------------------------|---------------------------|
|                                               | Regulated genes (mean)      | Other genes (mean)        | P-value (MWU) | Regulated genes (mean) | Other genes (mean) | P-value (MWU) |
| SL dispersion                                 | 20.3242                     | 14.385                    | 0.100607      | 8.95714                  | 15.0322                  | 0.013722      |
| SL diversity                                  | 0.602038                    | 0.587866                  | 0.308541      | 0.528526                 | 0.591906                 | 0.022768      |
| PPT length                                    | **17.5536**                 | **18.1966**               | **0.009508**  | **18.2483**              | **18.1551**              | **0.306812**  |
| PPT-AG distance                               | 37.8088                     | 37.6122                   | 0.905193      | 35.6046                  | 37.732                   | 0.161013      |
| Pyrimidine fraction upstream of splice site   | **0.624583**                | **0.637014**              | **0.000504**  | **0.638317**             | **0.636201**             | **0.386982**  |
| Purine fraction downstream of splice site     | 0.531765                    | 0.54579                   | 0.001435      | **0.561029**             | **0.544131**             | **0.000173**  |
| PolyA dispersion                              | **85.7916**                 | **73.5316**               | **0.28442**   | **143.851**              | **69.301**               | **1.49E-07**  |
| PolyA diversity                               | **1.72321**                 | **1.90628**               | **0.083936**  | **2.30613**              | **1.87025**              | **2.56E-06**  |
| Distance from polyA site to downstream splice site | **181.018**                | **222.926**               | **0.538294**  | **317.188**              | **215.225**              | **0.041497**  |

| Property                                      | U2AF35 down-regulated genes | U2AF35 up-regulated genes |
|-----------------------------------------------|-----------------------------|---------------------------|
|                                               | Regulated genes (mean)      | Other genes (mean)        | P-value (MWU) | Regulated genes (mean) | Other genes (mean) | P-value (MWU) |
| SL dispersion                                 | 7.67377                     | 15.0843                   | 0.38903       | 6.85968                  | 15.0174                  | 0.007493      |
| SL diversity                                  | 0.555143                    | 0.590394                  | 0.334671      | 0.55205                  | 0.590046                 | 0.394579      |
| PPT length                                    | 18.1364                     | 18.1611                   | 0.800356      | 18.6667                  | 18.1411                  | 0.067292      |
| PPT-AG distance                               | 39.7543                     | 37.5139                   | 0.27337       | 38.0922                  | 37.6580                  | 0.993399      |
| Pyrimidine fraction upstream of splice site   | **0.631655**                | **0.636548**              | **0.179025**  | **0.644547**             | **0.636**                | **0.08821**   |
| Purine fraction downstream of splice site     | 0.556911                    | 0.544382                  | 0.018609      | 0.53318                  | 0.545438                 | 0.011103      |
| PolyA dispersion                              | 59.9467                     | 75.2329                   | 0.969529      | 99.4762                  | 73.3347                  | 0.660629      |
| PolyA diversity                               | 2.04453                     | 1.88617                   | 0.036495      | 1.82942                  | 1.90017                  | 0.432948      |
| Distance from polyA site to downstream splice site | 184.471                    | 224.173                   | 0.039195      | 349.615                  | 217.687                  | 0.463979      |
mass spectrometry analysis. Because in *L. tarentolae*, the protein is expressed from an episomal vector, the association of each factor with RNP complexes was compared with that of the *T. brucei* factor tagged by PTP at the authentic site of the gene (35). The results in Fig. 5B demonstrate that the distribution of the tagged proteins is similar in *T. brucei* and in *L. tarentolae*. SF1 and U2AF65 were found in complexes at a size range of 10–40 S, which are devoid of ribosomal complexes, whereas U2AF35 is also associated with ribosomal complexes, which were identified by the presence of the 60 S large subunit small srRNA-4 (Fig. 5B, panels i and ii). The 10–40 S complexes contain spliceosomal RNAs identified by the presence of U2 and SL RNAs.

Next, the proteins associated with SF1 and U2AF35 were purified in *L. tarentolae*. Unfortunately, the expression of U2AF65 was toxic. However, because U2AF65 and SF1 interact tightly with each other, we anticipate that their purification outcome should be almost identical. The proteins from the final step of purification of U2AF35 and SF1 were fractionated on 12% SDS-polyacrylamide gel and stained with silver (Fig. 5C). The proteins were subjected to mass spectrometry, and the results are presented in supplemental S-4. Among the proteins identified in a complex with SF1 are splicing factors such as U2AF65 and TSR1IP, and two proteins that are like many proteins carrying RRM domains are implicated to function in mRNA stability (TRRM2 and RBP29). Of special interest is

FIGURE 4. Changes in stability of mRNAs following silencing of U2AF65, SF1, and U2AF35. Uninduced and U2AF65- or SF1- or U2AF35-silenced cells (3 days after induction) were treated with sinefungin (2 μg/ml) and, after 10 min, with actinomycin D (30 μg/ml). RNA was prepared at the time points indicated above the lanes, separated on a 1.2% agarose-formaldehyde gel, and subjected to Northern analysis with the indicated gene-specific probes. 7SL RNA was used to control for equal loading. A, half-life of up-regulated transcripts: panel i, U2AF65; panel ii, SF1; and panel iii, U2AF35. B, half-life of down-regulated genes. Panel i, U2AF65; panel ii, SF1; and panel iii, U2AF35. The hybridization signals were measured by densitometry. The decay curves are shown with the blots, and the half-life (as obtained by linear interpolation) is illustrated by black line, and following induction (+ Tet) by gray dashed line. The experiments were repeated three times; each data point corresponds to the average, with the standard deviation also indicated. The half-life was then also calculated by fitting the normalized RNA levels to an exponential decay. The half-lives (averaged over the three experiments) are shown as bars with standard deviations, along with p values (t test) for the difference between the half-lives in uninduced compared with silenced cells.
Tb11.03.0530, which is most probably an RAP55 homologue; in mammalian cells RAP55 is found in stress granules (49). The mRNA-binding proteins associated with SF1 complexes support the role of this factor not only in splicing but also in mRNA stability.

A much larger group of U2AF35-associated proteins was revealed. The U2AF35-associated proteins involved in mRNA processing and ribosome biogenesis are listed in supplemental S-4, U2AF35-A, and the ribosomal proteins in supplemental S-4, U2AF35-B. Interestingly, although the SF1 purification detected TSR1IP but not TSR1, the U2AF35 purification detected TSR1 but not TSR1IP. Another splicing factor detected in the purification is the SR protein, TRRM1 (16). Of special interest are the proteins RBP29, DRBD2, DRBD10, which are implicated in mRNA stability but no studies exist to date describing their function. These factors seem to be unrelated to known splicing factors, and most probably do not function in splicing (7, 50, 51). PUF proteins detected in this purification are also implicated in mRNA stability, translation regulation, and rRNA processing (52, 53). The most well studied protein detected in U2AF35 purification is UBP1, which was shown in T. brucei to regulate the stability of mRNAs encoding transmembrane proteins and proteins associated with cell cycle control (54, 55). Surprisingly, a very distinct group of proteins associated with rRNA processing was detected among the purified proteins, including the small nucleolar RNP proteins NOP58, SNU13, fibrillarin, or NOP1, and CBF5 (56, 57), as well as NOG1, a GTP-binding protein that is required for 60 S ribosome biogenesis (58). NRBD2/p37, which co-purified with U2AF35, is a trypanosome-specific factor that functions in the transport of the large ribosome subunit from the nucleus to the cytoplasm (59). A number of hypothetical proteins that were found in the purification of other factors were also detected in the purification of U2AF35 (supplemental S-4, U2AF35-A). Interestingly, many of the metabolic proteins detected here were previously shown to be associated with translating ribosomes in Trypanosoma cruzi (60). In conclusion, the biochemical data strongly support the function of SF1 and U2AF65 in mRNA stability and the association of U2AF35 with ribosomes and with factors involved in rRNA processing.

How Can Splicing Factors Affect mRNA Stability? An Example from a U2AF65 Target Gene—The binding of U2AF65 to mature mRNA was unexpected, because there was no prior evidence in any system for the binding of such factors to sequences besides the splicing signals. The key to understanding the mechanism of such binding in T. brucei may be the alternative polyadenylation that was observed in T. brucei transcripts (4). Indeed, the data presented in supplemental S-3 suggest that a significant number of the transcripts regulated by U2AF65 are differentially polyadenylated. Because trans-splic-
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A mini-gene model demonstrating that the splicing signal present in the 3′ UTR of a differentially polyadenylated transcript regulates the stability of the mRNA. A, panel i, schematic representation of the asparagine synthetase gene model (Tb927.7.1110). The size of the alternative 3′ UTRs, the locations of SL and poly(A) sites, and the distances between the sites are given. Panel ii, schematic representation of the asparagine synthetase construct carrying the 3′ UTR of the proximal splice site. The positions of the probes used in B are indicated. The sequence of the domain carrying the PPT is shown. Panel iii, sequence of the 3′ UTR around the PPT of the proximal splice site. The positions of the probes used in B are indicated. The sequence of the domain carrying the PPT is shown. Panel iv, sequence of the 3′ UTR around the PPT of the proximal splice site. The positions of the probes used in B are indicated.

Note that this PPT is the one present upstream of the SL1 addition sites. The 3′ UTRs were cloned into the pNS21b vector (34). Cells carrying the U2AF65 silencing construct were transfected to each of the two transgenes. To verify that the cells lines were efficiently silenced, the level of SL RNA and Y structure intermediates were examined, and the results (Fig. 6B, panel i) indicate that the effect on both the SL RNA and Y structure was exactly as observed in Fig. 1, showing 80% reduction in the level of SL RNA and of the Y structure intermediate as a result of silencing, suggesting that these cells were properly silenced. Next, the effect on trans-splicing of luciferase reporter gene expressed from the mini-gene was examined. As expected, the trans-splicing of luciferase mRNA (both WT and mutated forms) was affected under U2AF65 silencing, as revealed by an almost 5-fold increase in the level of luciferase pre-mRNA as result of silencing (Fig. 6B, panel ii). Next, the effect of U2AF65 silencing on the level of the luciferase mature mRNA was examined. RNA was subjected to Northern analysis with the luciferase probe (probe I) and a gene-specific probe that can detect only the transcripts processed at poly(A2) (probe II). The results, presented in Fig. 6B, panels iii and iv, suggest that two transcripts can be detected using the luciferase probe I. The longer transcript also hybridizes with the gene-specific probe II indicating that this transcript is polyadenylated in the distal site poly(A2). Under U2AF65 depletion, the level of the longer transcript detected using probes I and II increased (lanes 1 and 2) by 1.5-fold (Fig. 6B, panels iii and iv). However, when the PPT site was mutated, this up-regulation was abolished (Fig. 6B, panels iii and iv, lanes 3 and 4), and the level of the transcript above the lanes, separated on a 1.2% agarose-formaldehyde gel, and subjected to Northern analysis with luciferase probe I. 7SL RNA was used to control for equal loading. The hybridization signals were measured by densitometry. The decay in the uninduced cells (−Tet) is indicated by black line, and the decay upon (+ Tet) induction is designated by dashed gray line. Panel v, cells expressing wild type mini-gene under U2AF65 silencing; panel vi, cells expressing mutated mini-gene under U2AF65 silencing. The experiments were repeated three times, and the average of each time point is shown with its corresponding standard deviation. Half-lives were calculated both using linear interpolation (shown on top of the decay curves) and by fitting an exponential decay (shown as bars). The bars correspond to the average of the three experiments. The standard deviation is also plotted, along with p values (t test) for the difference between the half-life in uninduced compared with silenced cells.

FIGURE 6. A mini-gene model demonstrating that the splicing signal present in the 3′ UTR of a differentially polyadenylated transcript regulates the stability of the mRNA. A, panel i, schematic representation of the asparagine synthetase model gene (Tb927.7.1110), whose alternative polyadenylation (two 3′ UTRs of sizes 733 and 1186 nt) was described previously (Fig. 6A, panel i) (4). To investigate whether the PPT site present downstream to poly(A1) is used for U2AF65 binding and its effect on the stability of the mRNA, two cell lines were generated, one expressing a luciferase gene with the wild type Tb927.7.1110 3′ UTR (Fig. 6A, panel ii) and one in which the PPT was mutated (Ts were converted to A and G) (Fig. 6A, panel iii). Note that this PPT is the one present upstream of the SL1 addition sites. The 3′ UTRs were cloned into the pNS21b vector (34). Cells carrying the U2AF65 silencing construct were transfected to each of the two transgenes. To verify that the cells lines were efficiently silenced, the level of SL RNA and Y structure intermediates were examined, and the results (Fig. 6B, panel i) indicate that the effect on both the SL RNA and Y structure was exactly as observed in Fig. 1, showing 80% reduction in the level of SL RNA and of the Y structure intermediate as a result of silencing, suggesting that these cells were properly silenced. Next, the effect on trans-splicing of luciferase reporter gene expressed from the mini-gene was examined. As expected, the trans-splicing of luciferase mRNA (both WT and mutated forms) was affected under U2AF65 silencing, as revealed by an almost 5-fold increase in the level of luciferase pre-mRNA as result of silencing (Fig. 6B, panel ii). Next, the effect of U2AF65 silencing on the level of the luciferase mature mRNA was examined. RNA was subjected to Northern analysis with the luciferase probe (probe I) and a gene-specific probe that can detect only the transcripts processed at poly(A2) (probe II). The results, presented in Fig. 6B, panels iii and iv, suggest that two transcripts can be detected using the luciferase probe I. The longer transcript also hybridizes with the gene-specific probe II indicating that this transcript is polyadenylated in the distal site poly(A2). Under U2AF65 depletion, the level of the longer transcript detected using probes I and II increased (lanes 1 and 2) by 1.5-fold (Fig. 6B, panels iii and iv). However, when the PPT site was mutated, this up-regulation was abolished (Fig. 6B, panels iii and iv, lanes 3 and 4), and the level of the transcript above the lanes, separated on a 1.2% agarose-formaldehyde gel, and subjected to Northern analysis with luciferase probe I. 7SL RNA was used to control for equal loading. The hybridization signals were measured by densitometry. The decay in the uninduced cells (−Tet) is indicated by black line, and the decay upon (+ Tet) induction is designated by dashed gray line. Panel v, cells expressing wild type mini-gene under U2AF65 silencing; panel vi, cells expressing mutated mini-gene under U2AF65 silencing. The experiments were repeated three times, and the average of each time point is shown with its corresponding standard deviation. Half-lives were calculated both using linear interpolation (shown on top of the decay curves) and by fitting an exponential decay (shown as bars). The bars correspond to the average of the three experiments. The standard deviation is also plotted, along with p values (t test) for the difference between the half-life in uninduced compared with silenced cells.
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was even reduced by 60%. The reduction in the level of the transcript likely results from the fact that U2AF65 lost its effect on the stability of the transcript. Under these conditions, the effect of splicing inhibition is unmasked, leading to a decrease in transcript level. This result therefore suggests that the regulation of the asparagine synthetase gene by U2AF65 depends on the PPT sequences found in its 3' UTR; therefore, when the PPT was mutated, this regulation was eliminated. As expected, because trans-splicing and polyadenylation are coupled, the mutation on PPT abolished the formation of the shorter transcript, which can be detected only using probe I (Fig. 6B, panels iii and iv, lanes 3 and 4). Because our results show that mutating the PPT site in this 3' UTR affected the stability of mRNA utilizing poly(A2) under U2AF65 depletion, this suggests that U2AF65 binding to the 3' UTR destabilizes the mRNA under wild-type conditions. Moreover, this experiment demonstrates the strength of the effect of stability on the level of mRNAs; despite the splicing defect under U2AF65 silencing, the luciferase mRNA that was nevertheless produced under U2AF65 depletion was stabilized to such an extent that it resulted in a net up-regulation of transcript levels.

To unequivocally prove that the mutation in the PPT affected luciferase stability, the half-life of wild-type and mutated transcripts was examined under silencing. Only when the PPT site was present in the 3' UTR was there a significant increase in the half-life observed as a result of the silencing (Fig. 6B, panel v). However, no change in half-life of the mRNA was observed when the PPT was mutated (Fig. 6B, panel vi).

These results led us to ask whether genes regulated by splicing factors should be enriched with transcripts that undergo differential polyadenylation. Of the genes reported in supplemental S-3, 297 were found in Ref. 4 to be the subject of alternative polyadenylation. From the 1412 genes that are regulated by at least one splicing factor (supplemental S-3), 93 undergo alternative polyadenylation versus only 69 expected (p value 0.001, Fisher's exact test). This significant enrichment suggests that transcripts regulated by splicing factors are enriched with those that undergo alternative polyadenylation. Although this result and the data presented in Fig. 6 strongly support the notion that the effect of splicing factor on mRNA stability results from direct binding of the factor to the mRNA, we cannot, however, exclude the possibility that other scenarios may co-exist. For instance, the effect on stability may be due to a secondary effect, resulting from the reduction in the level of a master regulator(s) of mRNA stability, which may then affect the stability of numerous mRNAs.

DISCUSSION

The study identifies novel and unconventional roles for basal splicing factors in trypanosomes. All the splicing factors studied, U2AF65, SF1, and U2AF35, were shown to affect both trans-splicing and mRNA stability. The involvement of these factors in mRNA stability is based on the following: 1) up-regulation of transcripts in the silenced cells even before the detection of splicing defects; 2) localization of splicing factors also in the cytoplasm; 3) mRNA decay assay demonstrating changes in half-life during silencing; 4) co-purification of splicing factors with proteins implicated in mRNA stability; 5) mature mRNA were shown to bind to the splicing factors; and 6) studies using a mini-gene demonstrating that mutating a PPT at the 3' UTR affects the stability of the mRNA. Despite the direct interaction of splicing factors with mature mRNAs, we cannot at this point exclude the possibility that the transcriptome under these depletions may also be influenced by a change in key master regulators that exert an effect on their target genes.

We propose that splicing factors bind to splicing signals present in the 3' UTR of target genes, many of which undergo differential polyadenylation. Indeed, transcripts regulated by U2AF65 and SF1 were shown to have higher polyadenylation heterogeneity (Table 1). In addition, transcripts regulated by splicing factors are enriched with transcripts recently shown to undergo differential polyadenylation (4). Our data suggest that different splicing factors control different subsets of biological functions, including metabolic enzymes, chaperones, transporters, RNA processing, signaling factors, and proteins involved in movement and trafficking. Many of these regulated genes were shown to be differentially regulated during cycling between the two hosts. Our data support the existence of RNA operons whose co-expression dictates the transcriptome during parasite differentiation (43, 61).

Are All Splicing Factors Important for Trans-splicing of Each mRNA?—The most surprising result presented in this study is that the down-regulation of splicing factors by RNAi resulted in up-regulation of selected mRNAs, suggesting a very dominant role of these factors in regulating the stability of mRNAs in these parasites. In a similar study in mammalian cells, where the transcriptome following U2AF35 and U2AF65 depletion by RNAi was examined, it was found that the majority of the mRNAs were down-regulated (62, 63), suggesting that the dominant role of splicing factors on mRNA stability is unique to trypanosomes. This might be due to the very special mechanisms that regulate trypanosome gene expression and obligate linkage between trans-splicing and polyadenylation, giving rise to 3' UTRs that contain splicing signals that can bind splicing factors (3).

This unique feature explains how mRNAs can be elevated in trypanosomes during silencing of major splicing factors. Such regulated transcripts most probably bind splicing factors that, under normal conditions, destabilize the mature mRNAs. Splicing defects detected by accumulation of precursors were observed only after 4–5 days of silencing (Fig. 2), whereas the effect on stability was already observed at the 2nd day of silencing.

Although the effect of splicing factor depletion on splicing is masked by its effect on mRNA stability, these factors are clearly required for trans-splicing (Fig. 1). Inhibition of trans-splicing did contribute significantly to the results observed in this study, because all depletions affected the Y structure intermediate. However, differential effects on transcripts were observed that are significantly correlated with the splicing signals of the genes (Table 1). Indeed, among the transcripts down-regulated in U2AF65-depleted cells are transcripts with short PPT that are likely to be affected more severely when this factor is limited. In addition, transcripts down-regulated by U2AF35 have low splice site diversity, suggesting that these transcripts are more reliant on the presence of U2AF35. This may suggest that not all
transcripts rely on all factors for their splicing. This was also shown in other systems such as yeast, Drosophila, and mammals. In mammals, the depletion of U2AF65 mainly affects transcripts with a weak 3‘ splice site (64). Moreover, U2AF35 silencing in mammals leads to reduction in the level of only 400 transcripts and not of the entire transcriptome (63). In yeast, depletion studies on 18 different mRNA processing factors revealed groups of genes with different degrees of dependence on these factors (65). Combined data from this study and from the studies in mammals and yeast suggest that transcripts may differ in their need for basal splicing factors.

It should be noted that this study only addresses cases in which the depletion severely affected splicing, mRNA stability, or both. Thus, the full extent of changes in the transcriptome is far more complex. For instance, there could be cases, not detected in our experiments, in which the level of the mature mRNA did not change because the splicing led to a decrease in the transcript, but mRNA stability increased its level, thus leading to no net change in the level of mRNA. Indeed, our results revealed changes in only a few hundred genes in each depletion and not in all ~9000 trans-spliced transcripts present in the T. brucei genome. RNA-seq analysis of the transcriptome of the splicing-depleted factors studied here should yield a more quantitative picture, demonstrating effects on both mature and intergenic precursors.

Involvement of Splicing Factors in mRNA Stability and Translation—The involvement of basal splicing factors in functions other than splicing was reported in only a few studies. The splicing factor ASF/SF2, an SR protein, was shown in mammals to regulate the stability of PKC-1 by binding to a regulatory sequence in the 3‘ UTR of its transcript (66). In addition, mammalian U2AF65 and PTB were shown to bind to mature mRNAs (62). More recent studies used cross-linking and immunoprecipitation to demonstrate that mammalian SF1 binds not only to pre-mRNA at the branch site but also mRNAs spanning exon junctions (67). CLIP data also demonstrated the binding of mammalian SF2/ASF to mature mRNA in the cytoplasm (68). Moreover, the mammalian splicing factor, SFRS1, was shown to bind targets within mRNAs, miRNA, snoRNA, and noncoding RNA (69). Most recently, the RNA-binding protein HuR was shown to bind not only to 3‘ UTR but also to intronic sequences, and its knockdown suggests its function not only in mRNA stability but also in splicing (70).

Although the studies cited above suggest binding of splicing factors to mature mRNAs, only one study demonstrated the role of these factors in mRNA stability (66). In contrast, the effect observed in this study regarding the role of basal splicing factors on mRNA stability is very profound and significant. Recently, it was suggested that certain factors function as master coordinators of gene expression. One such complex is yeast Rbp4/6, which is part of the polymerase II complex but also represents a class of mRNA coordinators that integrate the various stages of gene expression (71). The trypanosome factor U2AF35 controls splicing, mRNA decay, and possibly translation and thus may serve as such coordinators of gene expression in these parasites.

The factors known to regulate mRNA stability in trypanosomes are proteins carrying RRM domains, proteins containing a CCH type zinc finger, and PUF proteins (7). Each of the RRM-containing proteins seems to affect the stability (stabilization or destabilization) of a different subset of proteins. These cumulative data suggest the existence of RNA operons, i.e. genes that participate in a single pathway or biological function that are co-regulated by a common factor. The presence of RNA operons in T. brucei gene expression was also noted in a study that determined the transcriptome during differentiation and demonstrated that ribosomal proteins, flagellar biogenesis proteins, and factors from the same metabolic pathways are often co-regulated (43). Our study also supports the operon type of regulation in the trypanosome transcriptome. Over 20% of the transcripts regulated by U2AF65/SF1 are metabolic enzymes. Interestingly, many of the genes regulated by these splicing factors are transcripts whose expression is changed during the differentiation of the parasites (supplemental S-3). As stated above, we cannot exclude the possibility that some of the effects observed on the transcriptome emerge from a perturbation in expression of a master regulator(s), also controlling the stability of many mRNAs.

How Could a Single Factor Both Stabilize and Destabilize mRNAs?—The mRNAs that are destabilized during factor silencing may carry hypersensitive sites that are protected by the factor. Conversely, those that are stabilized during silencing may be recruited by the factor to the degradation machinery. But how can the same protein be recruited for such different machineries? This depends solely on the other factors that bound the mRNA. The combinatorial binding of other binding proteins may generate a multitude of complexes each formed under different physiological and developmental conditions. Indeed, a variety of RNA-binding proteins co-purified with these splicing factors; these proteins can either directly interact with the factor or, more likely, interact with the transcripts bound by the factor. Interestingly, U2AF65- and SF1-regulated transcripts are also rich in purine-rich exons, suggesting that regulated transcripts are bound by several different factors.

Purification of Complexes Containing the Splicing Factors Supports Their Role in mRNA Decay and Translation—Our mass spectrometry data identified several RNA-binding proteins that may also function in regulating mRNA stability and even translational control. The factors examined in this study are known for their splicing activity. However, the majority of proteins associated with these splicing factors are implicated in mRNA stability, ribosome function, and biogenesis (especially those associated with U2AF35). Spliceosomal complexes were comprehensively identified by purifying Sm proteins from both T. brucei (38, 72) and Leishmania (27). In yeast, spliceosomal complexes were also identified by tagging Sm or snRNP-specific complexes (73). This study failed to detect components of the spliceosomal complex, including core proteins such as Sm proteins, and U snRNPs-specific proteins. These results may indicate either that the spliceosomal complexes carrying the studied factors are fragile and dissociate during purification or that the level of spliceosomal complexes containing these factors is low compared with subspliceosomal complexes that include these factors. Indeed, studies in mammals identified a large pool of extra-spliceosomal complexes containing U2AF65 and SF1 (74).
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Mechanism by Which Splicing Factors Affect mRNA Stability in Trypanosomes—By what mechanism could splicing factors bind so extensively to sequences other than the splicing signals present on mRNAs? In trypanosomes, such interactions might be prevalent due to two major specific characteristics of gene expression. First, extensive alternative polyadenylation was observed, with over 400 cases already described in the procyclic stage (4). Indeed, U2AF65- and SF1-regulated transcripts were shown in this study to have higher polyadenylation heterogeneity and to be enriched with transcripts previously shown to undergo differential polyadenylation (4). Second, trans-splicing and polyadenylation are linked (19–22). In trypanosomes, trans-splicing determines which poly(A) site will be selected and hence the size of the 3′ UTR; this choice in turn dictates the stability of the mRNA due to the presence of binding sites for a variety of RNA-binding proteins. However, orphan splicing signals that are not coupled to polyadenylation also may exist at the 3′ UTR of genes and serve as binding sites that exert their effect on mature mRNAs.

Regulation of mRNA stability by splicing factors in trypanosomes might be general and not trypanosome-specific, and splicing factors may also affect the stability of mRNA by binding to the 3′ UTR in other systems. Indeed, support for this notion was recently provided for mammalian SF1, which was shown to bind the 3′ UTR of mammalian genes (67). Thus, the role of basal splicing factors in mRNA stability, suggested for the first time in this study, might operate in other systems as well. Although the data presented in this study support the direct binding of the splicing factor to mature mRNA, most probably to the 3′ UTR, we must also consider the possibility that the mRNA stability changes observed may stem not only from the direct binding of the splicing factors to the 3′ UTR but may result from secondary perturbations. For instance, the effect on stability may result from perturbation induced by splicing defect of a factor(s) involved in mRNA stability. Extensive genome-wide CLIP-Seq assays are expected to differentiate between direct binding and secondary effects.

In sum, this study highlights the contribution of splicing factors not only to the efficiency of trans-splicing but also to mRNA stability, which seems to be the most robust process determining the parasite transcriptome. The decision regarding the choice of splicing and polyadenylation signals determines the composition of the 3′ UTR, and thus the half-life of the target mRNA. This regulation is most probably pivotal for the adaptation of gene expression during the developmental cycle of the parasite and for responding to external or internal stimuli.

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