The role of the 5'–3' exoribonuclease XRNA in transcriptome-wide mRNA degradation

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ABSTRACT

The steady-state level of each mRNA in a cell is a balance between synthesis and degradation. Here, we use high-throughput RNA sequencing (RNASeq) to determine the relationship between mRNA degradation and mRNA abundance on a transcriptome-wide scale. The model organism used was the bloodstream form of *Trypanosoma brucei*, a protist that lacks regulation of RNA polymerase II initiation. The mRNA half-lives varied over two orders of magnitude, with a median half-life of 13 min for total (rRNA-depleted) mRNA. Data for poly(A)⁺ RNA yielded shorter half-lives than for total RNA, indicating that removal of the poly(A) tail was usually the first step in degradation. Depletion of the major 5'–3' exoribonuclease, XRNA, resulted in the stabilization of most mRNAs with half-lives under 30 min. Thus, on a transcriptome-wide scale, degradation of most mRNAs is initiated by deadenylation. Trypanosome mRNA levels are strongly influenced by gene copy number and mRNA half-life: Very abundant mRNAs that are required throughout the life-cycle may be encoded by multicopy genes and have intermediate-to-long half-lives; those encoding ribosomal proteins, with one to two gene copies, are exceptionally stable. Developmentally regulated transcripts with a lower abundance in the bloodstream forms than the procyclic forms had half-lives around the median, whereas those with a higher abundance in the bloodstream forms than the procyclic forms, such as those encoding glycolytic enzymes, had longer half-lives.

Keywords: RNASeq; *Trypanosoma*; Xrn1; mRNA decay; polycistronic

INTRODUCTION

The steady-state level of mRNAs in a cell is a balance between synthesis and degradation. In order to understand mRNA homeostasis, it is therefore essential to measure not only promoter activity and RNA processing but also the kinetics of mRNA decay. Genome-wide measurements of mRNA expression and decay, made using microarrays, have documented mRNA stability differences over at least two orders of magnitude in individual organisms. The median half-lives of mRNAs in bacteria (Bernstein et al. 2002) and yeast (Wang et al. 2002; Grigull et al. 2004) were 5 and 21 min, respectively. Mammalian cells have yielded median half-lives of 10 h (Yang et al. 2003), 7.1 h (Sharova et al. 2009), and 4 h (t Hoen et al. 2011) and *Arabidopsis*, 3.8 h (Narsai et al. 2007), with up to 50-fold differences between the most stable and unstable mRNAs. All publications noted that mRNAs encoding proteins with related or linked functions often have similar half-lives.

In eukaryotes, decay of most mRNAs is initiated by 3'–5' exonucleolytic removal of the poly(A) tail. Once the poly(A) has been shortened to a threshold length, there is either degradation of the remaining mRNA in the 3'–5' direction, by the exosome, or decapping followed by 5'–3' exonucleolytic degradation (Parker and Song 2004). In yeast, plants, and mammalian cells, the cytoplasmic 5'–3' exonuclease XRN1 catalyzes mRNA degradation (He et al. 2003; Souret et al. 2004; Stoecklin et al. 2006). The steady-state transcriptome of yeast lacking Xrn1 has been described (He et al. 2003), but the role of Xrn1 in transcriptome-wide mRNA degradation has yet to be studied.

Trypanosoma brucei is a unicellular flagellated protist parasite that grows extracellularly in the gut of tsetse flies (procyclic form) and in the blood and tissue fluids of mammals (bloodstream form). The parasite undergoes extensive control of gene expression to adapt to the different environments in the two hosts. Almost all protein-coding genes are organized in long polycistronic units that are transcribed by RNA polymerase II (Palenchar and Bellofatto 2006). Precursor mRNAs are processed through *trans*-splicing coupled to polyadenylation to generate mature mRNAs (Liu et al. 2004). Multiple lines of evidence

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Article published online ahead of print. Article and publication date are at <http://www.rnajournal.org/cgi/doi/10.1261/rna.2837311>.

indicate that for protein-coding genes in both trypanosomes and Leishmanias (which are closely related Kinetoplastids), there is no control of the transcription initiation by RNA polymerase II at the individual promoters (for review, see Palenchar and Bellofatto 2006; Clayton 2002). Regulatory transcription factors are missing from the genomes (Ivens 2005); mRNAs with different patterns of developmental regulation are cotranscribed (Gibson et al. 1988; Vijayasathay et al. 1990); and nuclear run-on assays detect no differences in the transcription rates within or between transcription units, or for individual genes in different life-cycle stages (Vijayasathay et al. 1990; Martinez-Calvillo et al. 2003, 2004). Finally, RNASeq and microarray surveys of the transcriptomes of bloodstream-form and procyclic-form trypanosomes revealed no relationship between the genomic locations of open reading frames (ORFs) and their expression levels (Jensen et al. 2009; Kabani et al. 2009; Queiroz et al. 2009; Siegel et al. 2010). The last two lines of evidence also yielded no evidence for any regulation of transcriptional elongation. African trypanosome genes encoding the dominant surface proteins are exceptions: Their transcription is by RNA polymerase I (Gunzl et al. 2003) and is subject to epigenetic control (e.g., Hughes et al. 2007; Figueiredo et al. 2008).

As a consequence of their “strange” gene organization, post-transcriptional processes, especially mRNA degradation, are exceptionally important in the control of Kinetoplastid gene expression (for review, see Clayton and Shapira 2007; Ouellette and Papadopoulou 2009; Fernández-Moya and Estévez 2010; Kramer and Carrington 2011). Trypanosomes are therefore an ideal model system for the analysis of mRNA turnover. They have homologs of deadenylases (Milone et al. 2004; Schwede et al. 2008, 2009) and the exosome (Clayton and Estevez 2010) and three potential homologs of Xrn1, XRNA, XRNB, and XRNC (Li et al. 2006). Of these, XRNA was shown to be essential for the growth of both life cycle stage forms of *T. brucei* and for the degradation of two highly unstable developmentally regulated mRNAs (Li et al. 2006). More detailed analysis revealed that the unstable *EP* mRNA was degraded from both the 5′ and 3′ ends (Schwede et al. 2009).

High-throughput RNA sequencing (RNASeq) is far more sensitive than microarrays for transcriptome analysis and can measure mRNA levels over several orders of magnitude (Mortazavi et al. 2008; Agarwal et al. 2010; Metzker 2010). With respect to *T. brucei*, it has already been used to map RNA processing sites (Kolev et al. 2010; Nilsson et al. 2010) and to examine developmental regulation (Siegel et al. 2010; Zhang et al. 2010) and control of mRNA levels through the cell cycle (Archer et al. 2011). So far, however, RNASeq has not been used to measure mRNA decay rates. In this article, we have used RNASeq to determine the roles of deadenylation and XRNA in trypanosome mRNA degradation.

RESULTS

Data reproducibility

To measure mRNA half-lives, RNA was prepared from exponentially growing cells without an RNAi construct (wild type [WT]), or cells with a tetracycline-inducible XRNA RNAi construct. Cells were grown with or without tetracycline and with or without a 30-min RNA synthesis inhibition. We selected either poly(A)+ RNA or rRNA-depleted total RNA (total RNA). The RNA was fragmented prior to the preparation of cDNA libraries and high-throughput sequencing. Between 16 and 30 million single reads were obtained per library, and 45%–96% of them could be mapped to the genome (Supplemental Tables S2, S3). The correlations between replicates were 0.95–0.99 (Supplemental Fig. S1A,B,G,J). The addition of 100 ng/mL tetracycline to WT bloodstream trypanosomes had no effect on the transcriptome (Supplemental Fig. S1C–F), so we treated “WT+tetracycline” results as WT replicates in the subsequent analyses. Results from the XRNA RNAi line grown without tetracycline were also mostly similar to those from the WT control (Supplemental Fig. S1K,L) but were not used because such lines usually show a low level of RNAi “leakage.”

Many ORFs in the trypanosome genome are present as two or more virtually identical copies. For subsequent analysis, we considered only one representative of each ORF, using a list of 6787 unique ORFs compiled by Siegel et al. (2010); these ORFs are found in under 200 transcription units (Siegel et al. 2009). Our results showed that over 1000 mRNAs were present at less than one copy per cell (Fig. 1; Supplemental Table S3). At the other extreme, the mRNAs encoding α - and β -tubulin, elongation factor 1 α , and histones H2A, H3 and H4 together comprised $\sim$ 10% of the total mRNA. Sequences encoding known Lister 427 variant surface glycoproteins (VSGs) constituted 1%–2% of the total.

Our total mRNA and poly(A)+ mRNA transcriptomes had an overall correlation coefficient of 0.98 (Supplemental Fig. S2A–C). The correlation between our poly(A)+ data set and that previously obtained by Siegel et al. (2010) was not quite so good (Supplemental Fig. S2D), probably due to technical differences. Our methodology, which involved fragmentation of the RNA prior to cDNA synthesis, prevented bias caused by differences in ORF lengths (Supplemental Fig. S2E,F).

Transcriptome-wide mRNA half-lives

To estimate the mRNA half-lives, we compared the read densities across each ORF with and without a 30-min transcription inhibition. The single time point, with two to three replicates, was chosen as a compromise between accuracy and affordability. Known values for tubulin and histone H4, measured relative to a stable standard (the 7SL

FIGURE 1. Most ORFs are represented as less than three mRNAs per cell. Each bar represents the number of distinct ORFs with an mRNA abundance within the indicated range.

RNA), were used for normalization. To calculate the half-lives for every ORF, we assumed simple exponential decay kinetics. Very low abundance RNAs rpk (0 reads per kilobase of gene [ORF] length per million reads rpk at any time point) were excluded.

Of the 6787 unique mRNAs included, half-lives for total mRNA varied from 7 min upward (Fig. 2A; Supplemental Table S3). To test the accuracy of our estimates, we compared our RNASeq results with the published values for six genes, and measured the decay rates for 12 further ORFs using quantitative RT-PCR (RT-qPCR) (Table 1; Supplemental Fig. S3). The estimated WT half-lives by RT-qPCR correlated ($R = 0.95$) with those from RNASeq. For seven out of 10 mRNAs with half-lives over 8 min, the discrepancies between RNASeq and RT-qPCR were $<20\%$ (Table 1). Discrepancies were, as expected, worst (average 40%) for the eight mRNAs with half-lives of ≤ 8 min, two of which (*EP* and *PGKB*) are known to show biphasic kinetics (Schwede et al. 2009). At the other extreme, 98 stable mRNAs returned negative half-lives from RNASeq. This probably reflected the inherent inaccuracy of our single-time point estimate of half-life. However, a mysterious increase in some mRNAs after actinomycin D treatment has also been documented previously, using more classical methods, for several different Kinetoplastid mRNAs (for review, see Clayton and Shapira 2007). For some calculations, we assumed a maximum half-life of 240 min for the 150 most stable mRNAs; a list of all mRNAs with half-lives above 120 min (“total long half-lives”) is included in Supplemental Table S3. For statistical comparisons among WT, poly(A)+, and XRNA-depleted-cell mRNA, we excluded all ORFs with WT half-lives showing SDs of more than half the arithmetic mean and also those with WT rpk of <10 at time = 0 (Supplemental Table S4, sheet “filtered half-lives and rpk,” 5950 ORFs). Details, including a flowchart, are provided in the Supplemental Methods.

Trypanosome mRNAs are, overall, surprisingly unstable in comparison with those of yeast (Fig. 2A). The median half-life was 13 min, and the arithmetic mean half-life was 16 min; only 5% had half-lives of over 1 h. The 243 mRNAs that are at least 2.5-fold developmentally up-regulated in the bloodstream forms were more stable than average

(median half-life 27 min, 29% over 1 h) (Supplemental Table S4). The 159 mRNAs that are at least 2.5-fold up-regulated in the procyclic forms, in contrast, had half-lives (median 16 min) similar to those of the whole population.

Many trypanosome mRNAs have multiple spliced leader addition sites and polyadenylation sites (Kolev et al. 2010; Nilsson et al. 2010; Siegel et al. 2010). To examine the relationship between mRNA half-life and length, we generated a complete data set of processing sites using all accessible data. We identified the most frequently used splice acceptor sites for 8210 genes and poly(A) sites for 5961 genes (Supplemental Table S3). The average 5' UTR and 3' UTR lengths

FIGURE 2. Transcriptome-wide mRNA half-lives. (A) Half-lives for total mRNA from WT (dark gray bars) and XRNA RNAi (black bars) cells and for poly(A)+ mRNA (light gray bars) from WT cells. For each ORF, we calculated the arithmetic mean half-life (WT total, three measurements; others, two measurements). Negative half-lives were classified as >60 min, ORFs with <10 rpk at time = 0 are removed. The number of mRNAs in each half-life category was counted. (B) Scatter plot showing half-lives for individual ORFs, total mRNA, and poly(A)+ mRNA. Data were filtered as in A; half-lives were removed if the mean was less than twice the SD (Supplemental Table S4). Only half-lives between 5 min and 120 min are shown. The correlation coefficient was calculated in KaleidaGraph; the dashed line indicates the line that would be obtained from a perfect 1:1 correlation. (C) As in B but using a $\log_2$ scale.

TABLE 1. Comparison between RNASeq half-lives and measurements by Northern blot or real-time RT-PCR

	Locus	Product	Half-lives			
			Total expt	Total Seq	XRNA expt	XRNA Seq
a		VSG222	30 [?]	30	NA	159
b	Tb09.211.0620	Actin A	12–25	28	15	22
c	Tb927.2.6000	Glycosyl phosphatidylinositol phospholipase C	25	433	NA	28
d	Tb927.10.10250	EP1/2 procyclins	2–14	13	31	17.5
e	Tb927.1.700	PGKC	90	93	NA	39.5
f	Tb927.1.710	PGKB	5–7	24	NA	45.3
g	Tb11.01.7990	Myosin heavy chain	14	16.0	16	51.6
g	Tb11.02.2320	Hypothetical conserved	8.2	9.3	22.5	22.9
g	Tb11.03.0340	Hypothetical conserved	6.5	11.3	25.0	13.5
g	Tb11.12.0005	Hypothetical conserved	5.8	10.4	20.8	20.7
g	Tb927.6.2170	Co-chaperone GrpE	45.0	38.3	24.5	19.5
g	Tb927.6.2810	ABC transporter	4.8	10.6	16.3	33.4
g	Tb927.6.4340	LSm5	9.0	10.1	18.5	20.2
g	Tb927.7.3780	Poly(A) polymerase-like	7.7	8.8	18.8	16.2
g	Tb927.8.2810	XRNC	6.2	11.0	25.5	41.1
g	Tb927.4.410	CAF40	30.3	25.8	32.3	17.5
g	Tb11.01.8320	EAP2	8.0	8.8	29.0	39.5
g	Tb927.7.6980	Hypothetical conserved	23.3	14.7	58.5	45.3

Results designated “expt” were taken from the literature or measured by real-time PCR.

^aSingle measurement, Northern blot, and transcription inhibition (Vanhamme et al. 1999), and no degradation after 30 min but RNA reduced to 25% after 60 min.

^bNorthern results at 12 min (Li et al. 2006) and 25 min (Schwede et al. 2008, 2009).

^cNorthern data (Webb et al. 2005).

^dRNASeq—mean of Tb927.10.10250 and Tb927.10.10260; Northern blotting, biphasic degradation—50% 2–4 min, 50% 14–30 min (Schwede et al. 2009).

^eRNASeq value from unique parts of 3′ UTR only; Northern data from Schwede et al. (2008), but Colasante et al. (2007) suggested a half-life of >90 min.

^fAs in footnote e; Northern (Quijada et al. 2002; Li et al. 2006; Schwede et al. 2008).

^gQuantitative RT-PCR (this article) for 12 mRNAs, using four time points and three replicates. NA indicates not available.

were 347 nt and 594 nt, in agreement with published values. We found no relationship between mRNA half-lives and the lengths of ORFs, 5′ UTRs, or 3′ UTRs (Supplemental Fig. S4).

The results for poly(A)⁺ mRNA in effect give us the half-lives of the poly(A) tails attached to each mRNA. As seen in yeast (Wang et al. 2002), the poly(A)⁺ and total WT data sets correlated well (Fig. 2B,C), but the poly(A)⁺ mRNA half-lives were shorter than those for total mRNA (Fig. 2A–C). Thus, as expected, for most mRNAs poly(A)⁺ shortening precedes the decay of the body of the transcript. About 90% of the total mRNAs had half-lives under 30 min, whereas ~90% of the poly(A)⁺ mRNAs had half-lives under 20 min (Fig. 2A). For mRNAs with total mRNA half-lives between 10 and 30 min, the difference between the poly(A)⁺ and total half-lives was 8.6 ± 4.5 min. This value, which is similar to that previously seen in yeast (Wang et al. 2002), must be the average time interval between poly(A) tail removal and destruction of the mRNA by exoribonucleases. The average does conceal some differences: For the 1000

least stable mRNAs, the average difference between the poly(A)⁺ half-life and the total RNA half-life was 3.8 ± 1.0 min, whereas for the 1000 most stable mRNAs, it was 10 ± 15 min.

We next investigated whether there was any correlation between the stability of a transcript and the function of its protein product. Messenger RNAs encoding complexes involved in gene expression (SF3b, exosome, and Lsm proteins) showed decay rates that were similar to the modal value (15 min) (Supplemental Fig. S5). In contrast, of the 63 mRNAs encoding ribosomal proteins, only 12 had half-lives of <60 min (different from the population with a Mann-Whitney *P*-value < 0.0001) (Supplemental Table S3). Similarly, mRNAs encoding all but one enzyme of the bloodstream-form glycolysis were exceptionally stable (50 min to >240 min) (Supplemental Table S3). The core histone genes have copy numbers between six and 25, and mRNA half-lives are between 50 and 130 min (*P*-value < 0.004). Gene ontology (GO) term enrichment revealed short half-lives (<20 min) for mRNAs encoding proteins capable of nucleotide binding or hydrolysis, enzymes involved in post-translational protein modification, and RNA methylases. Amino acyl tRNA synthetases, translation initiation factors, enzymes involved in amino acid metabolism, dy-

neins, and components of the proteasome were enriched in the 20- to 40-min range (Supplemental Table S5).

Correlation between half-lives of mRNAs and their abundances

If trypanosomes truly rely primarily on mRNA degradation to regulate their transcriptome, the abundance of an mRNA should be directly proportional to both the half-life and the gene copy number. We estimated gene copy numbers using data from random shotgun sequencing (G Cross, unpubl.) and corrected the read densities according to the gene copy numbers in order to get an rpkm/ORF (Supplemental Table S4). We found a significant correlation between mRNA abundance (rpkm/ORF) and mRNA half-life (Fig. 3). If the mRNA half-life was the only important factor, however, a doubling of the half-life should double the RNA abundance (log plot slope of 1): In fact, it had considerably less effect (Fig. 3B,D). Although some deviations from the expected relationship could be

due to errors in our half-life and copy number estimates, other factors probably contribute. The 200 ORFs producing mRNAs with the lowest abundance/half-life ratios were not clustered within particular transcription units (data not shown), and previous analyses have ruled out control of initiation (see Introduction). However, trypanosome transcription start sites are not discrete, but spread over several kb (Kolev et al. 2010). If starts and stops actually spread through into coding regions, the affected ORFs should be less transcribed than the rest of the transcription unit. We therefore looked to see whether the 200 mRNAs with particularly low abundances, relative to half-life, were preferentially located near start or stop sites; but no such enrichment was found (data not shown).

Our data suggest that although mRNA abundances are strongly influenced by gene copy number and mRNA turnover and although transcriptional effects cannot be ruled out completely, the efficiencies of mRNA processing and export probably also influence final mature mRNA levels. Detailed measurements with more time points will be required for the definitive identification of mRNAs that are subject to these additional layers of control.

XRNA depletion increases the half-lives of unstable mRNAs

Three enzymes, XRNA, XRNA, and XRNC, are candidates for roles in 5′–3′ degradation of mRNAs. Deletion of XRNA in the bloodstream trypanosomes did not affect trypanosome multiplication and showed no effects by microarray (data not shown). XRNC RNAi had no effect on growth (Li et al. 2006); our attempts to delete the gene have failed (data not shown). We therefore focused on XRNA.

XRNA depletion caused slight increases in the relative abundances of low-copy number mRNAs and corresponding decreases in some more abundant ones (Fig. 4A). A comparison of the total mRNA half-lives revealed stronger differences (Fig. 4B). First, the median half-life was increased (Fig. 2A). No fewer than 741 mRNAs (16%) showed a two-fold or more increase in mRNA half-life in the XRNA-

depleted cells (Fig. 4C,D). The most notable observation was that stabilization after XRNA depletion was almost completely restricted to mRNAs with half-lives of <30 min (Fig. 4C). For the 1224 mRNAs with total half-lives under 10 min, XRNA depletion increased the half-life by 7.4 ± 5.2 min. The half-life changes correlated with changes in relative read density for many mRNAs (data not shown). The effects of XRNA depletion could be confirmed qualitatively by RT-qPCR (Table 1; Supplemental Fig. S3), although even with this method, measurements of half-lives <45 min were rather variable. There was no correlation between the effect of XRNA depletion and the time taken to remove the poly(A) tail.

We also looked to see if developmentally unstable mRNAs were preferentially affected by XRNA depletion. For example, the 590 mRNAs that were most stabilized by XRNA depletion (10% of the data set in Supplemental Table S4) have an average WT half-life of 12 min and an average half-life increase after XRNA depletion of 3.1-fold (range, 2.3- to 22-fold). For this group, the ratio of mRNA abundances in the bloodstream forms relative to the procyclic forms, as measured by Siegel et al. (2010), was 0.90 ± 1.43 (arithmetic mean $\pm$ SD). The group included just eight mRNAs that are twofold more abundant in the bloodstream forms relative to the procyclic forms and only 28 that are twofold more abundant in the procyclic forms relative to the bloodstream forms. Therefore, XRNA depletion does not preferentially affect developmentally regulated mRNAs.

In contrast to the many stabilized mRNAs, a few (2%, 140 in total) showed more than twofold decreases in mRNA half-life after XRNA depletion (Fig. 4D; Supplemental Table S4). These 140 mRNAs were weighted toward mRNAs with longer half-lives (mean, 74 ± 44 min). The products of the 140 destabilized mRNAs included 12 ribosomal proteins, seven cytoskeletal components, eight proteins involved in vesicular transport, and three glycolytic enzymes, including PGKC (Table 1). Since many of these proteins are required for cell growth and division, we speculate that the mRNA destabilization could be a secondary effect of growth inhibition.

We finally investigated whether the direction of mRNA degradation could be seen as a gradient of read density. Such

FIGURE 3. Relationship between mRNA abundance and half-life. Data were filtered as for Figure 2B and Supplemental Table S4. For each individual ORF, the copy number–normalized rpkm was plotted against the half-life. Results are shown for total RNA (A,B) and poly(A)+ RNA (C,D). Plots B and D use log scales; regression lines were plotted in KaleidaGraph. With control by degradation alone, using the log scale, the slope should be 1 (dotted line).

FIGURE 4. XRNA depletion preferentially affects less stable mRNAs. (A) Abundance data for total RNA of WT and XRNA-depleted cells, filtered as in Figure 2B, and for half-lives under 120 min only; regression line plotted using KaleidaGraph, and dotted line shows 1:1 correlation. (B) As in A, but showing half-life results. (C) The fold effect of XRNA depletion was calculated (half-life in XRNA-depleted cells divided by WT half-life) and then plotted (y-axis) against the WT half-life (x-axis). (D) mRNAs were divided into classes according to the fold effect of XRNA depletion; bars indicate the mean $\pm$ SD. The number of ORFs in each class is indicated above the bars.

a gradient would only be seen if the degradation were nonprocessive. Read densities for 200-nt windows at various positions in each mRNA were extracted and the results compared. There was an excellent correlation between read densities in neighboring segments, with slightly lower densities at the extremities caused by the intrinsic end-bias in the method, but no evidence for any systematic gradient in read densities under any condition (data not shown). This indicates that degradation by XRNA and the exosome is highly processive.

DISCUSSION

We have used high-throughput transcriptome sequencing to analyze the role of XRNA in the decay of mRNAs in *T. brucei*. We relied on a single (30 min) time point after inhibition of mRNA processing and transcription: This was inadequate for very long and short half-lives, could not reveal complex kinetics, and would be insufficiently accurate for quantitative modeling. Nevertheless, when tested against other methods, our half-life estimates were within a range of $\pm 50\%$, and predictions for roles of XRNA in degradation could be confirmed. The use of the single time point is therefore a useful approach; a time point near to the median should give optimal information for the majority of mRNAs. The use of rRNA depletion, as opposed to poly(A) selection, allows one to examine the roles of enzymes that operate at various points in the degradation pathway. For detailed quantitative analysis and modeling, inclusion of several time points is clearly essential: In order to plan such projects, results from single-point measurements will be invaluable since they will guide the choice of sampling times.

When we analyzed total RNA, we found that transcripts from most ORFs were degraded very fast, with a median half-life of 13 min; the mean half-life for total cellular mRNA (after correction for the abundance of each mRNA) was 30 min, identical to an estimate made by extrapolation from two mRNAs (Haanstra et al. 2008). It has been postulated that mRNA decay rates correlate with cell division times (Yang et al. 2003), based on microarray

data from *Escherichia coli* (Bernstein et al. 2002), yeast (Wang et al. 2002), and human cells (Yang et al. 2003). It is already clear, however, that this is not a universal rule, since relatively short mRNA half-lives were observed in a slowly dividing cyanobacterium (Steglich et al. 2010) and half-lives varied from 9.5–65 min during *Plasmodium* development (Shock et al. 2007). Our results from trypanosomes, which divide once every 8 h, also fail to support this relationship.

The abundances and half-lives of trypanosome mRNAs were approximately correlated, as expected given the lack of transcriptional control, but the correlation was far from perfect. Although some of the deviation may stem from the imprecision of our analysis, additional regulatory processes are likely to be present—most obviously, variations in the efficiency of mRNA processing (e.g., Stern et al. 2009). Although it has been postulated that long 3' UTRs predispose toward faster mRNA degradation (e.g., Kebaara and Atkin 2009; Ramani et al. 2009), no overall relationship was found between 3' UTR length and half-life in human cells ('t Hoen et al. 2011). In trypanosomes also, mRNA half-lives showed no correlation with the lengths of 3' UTRs, 5' UTRs, or ORFs.

Previous studies of mRNA decay in yeast and a mammal have found that mRNAs encoding the proteins required for cellular maintenance and structure were particularly stable, whereas those responsible for regulation of gene expression were rapidly degraded (Yang et al. 2003; 't Hoen et al. 2011). In trypanosomes, in contrast, the most stable mRNAs, with half-lives that often exceeded 120 min, were those encoding the translation apparatus (ribosomal proteins, translation elongation factors), histones, and the glycolytic enzymes. The differences for mRNAs encoding histones and ribosomal proteins were dramatic: In yeast, these mRNAs have average half-lives of 7 min and 22 min, respectively (Wang et al. 2002). But yeast can turn transcription of these genes on and off, whereas trypanosomes cannot. The trypanosome transcription rate has to be fast enough to allow the production of abundant mRNAs, while allowing the degradation machinery, which has finite capacity, to keep pace and destroy unwanted transcripts. The high degradation rate of

the majority of mRNAs in trypanosomes is a consequence of this fine balance. The trypanosome has two strategies to ensure high mRNA copy numbers: high stability, and gene amplification. Since the latter cannot be regulated, it is reserved for proteins that are required constitutively, such as tubulin and histones (half-lives ~ 1 h); for the 28 ORFs with copy numbers exceeding 3.5, the median half-life was 49 min. Meanwhile, genes encoding the translation apparatus are present in one or two copies, with mRNA half-lives exceeding 2 h. For glycolysis, extreme mRNA stability allows high expression in the bloodstream form, while low (one to three) gene copy numbers allow for strong down-regulation through rapid mRNA degradation in the procyclic form.

A main aim of our study was to examine pathways of mRNA decay. By analyzing both poly(A)⁺ RNA and total RNA, we could compare deadenylation kinetics with the decay of the RNA body. The overall correlation between half-lives confirmed that deadenylation is the first step in degradation of the majority of trypanosome mRNAs. A previous survey of yeast mutants showed that many mRNAs that were increased in an Xrn1 deletion mutant were also increased in mutants of the nonsense-mediated decay pathway; these tended to be low-abundance transcripts, but no correlation with mRNA half-life was observed (He et al. 2003). Also, the mRNAs that were increased in an *xrn4* mutant of *Arabidopsis* were not particularly unstable (Rymarquis et al. 2011). In contrast, by using the more sensitive RNASeq methodology and measuring half-lives as well as abundances, we found that highly unstable mRNAs were disproportionately dependent on 5'–3' exonucleolytic degradation. Interestingly, though, even within this group, some mRNAs were affected far more than others. This may reflect differences in recruitment of different components of the degradation machinery by regulatory mRNA binding proteins. The mRNAs that are less affected by XRNA depletion may be degraded by the exosome—or alternatively, their decapping may be much slower than XRNA recruitment. Our previous suggestion that XRNA recruitment might be specifically linked to developmental regulation, however, proved incorrect. For longer-lived mRNAs, the initiation of deadenylation is presumably so slow that even after RNAi, the rate of 5'–3' exonuclease digestion does not become limiting.

Transcript-internal cDNA tag profiles indicated that once the poly(A) tail has been removed, exonucleolytic degradation of mRNAs is usually an all-or-nothing event, implying that in vivo, degradation of both XRN1 and the exosome is processive, rather than distributive. This is consistent with earlier observations. In both yeast (Muhlrad et al. 1994; Jacobs Anderson and Parker 1998) and trypanosomes (Irmer and Clayton 2001), degradation intermediates can be seen only if exonuclease progression is inhibited by strong secondary structures, and yeast Xrn1 shows processive activity in vitro (Stevens 1980). The contribution of decapping to the overall kinetics remains a mystery, since

the complex that removes the cap from full-length, but deadenylated mRNAs has not yet been identified.

In conclusion, our transcriptome-wide data indicate that in trypanosomes, most mRNAs are deadenylated prior to degradation. Deadenylation of the least stable mRNAs is complete within 4 min. The average interval between deadenylation and mRNA destruction is 8 min; in this time, the slowest steps must be recruitment of the decapping complex and exoribonucleases. Processive XRNA activity was important for destruction of the more unstable mRNAs.

MATERIALS AND METHODS

T. brucei brucei cell lines

All experiments were done using the bloodstream form of *T. brucei brucei* of the Lister 427 strain, expressing the tet repressor. XRNA experiments were done using a published RNAi cell line (Li et al. 2006); RNA interference was induced with tetracycline (100 ng/mL) for 24 h. All were grown in HMI-9 medium at 37°C (van Duersen et al. 2001).

RNA degradation assay

To investigate the mRNA half-lives, mRNA formation was inhibited by addition of sinefungin (2 μ g/mL) and then, 5 min later, actinomycin D (10 μ g/mL) for a further 30 min. RNA was prepared using peqGOLD-Trifast (peqLab). “Total” mRNA was prepared using the Ribominus Eukaryotic kit for RNA-seq (Invitrogen, no. 10837-02 or -08), and poly(A)⁺ mRNA was obtained using Oligo(dT) beads (Illumina). After RNA fragmentation, libraries were constructed and sequenced at the Genecore (EMBL, Heidelberg) using Illumina kits. All tags were 72mers except for one of the replicates of the induced XRNA RNAi strain at steady state, tags were 36mers.

For real-time PCR, mRNA synthesis was inhibited for 10, 20, 30, and 60 min and total RNA prepared without further selection. Real-time PCR was done from three biological replicates using Fermentas kits K1642 and K0252; primers are in Supplemental Table S1. Each cDNA sample was analyzed in triplicate.

Alignment of sequence tags to the genome

DNA tags (76 or 36 bp in length) were aligned to the *T. brucei* TREU 927 genome using Bowtie (Langmead et al. 2009; Siegel et al. 2010). Manipulation and processing of the aligned tags were done using SAMtools (Li et al. 2009) and in-house Perl scripts. Aligned sequence tags were visualized using Artemis (Rutherford et al. 2000; Carver et al. 2008). “Relative abundance” or “gene expression” was expressed as rpkm. For some analyses, we used the non-redundant gene list provided by Siegel et al. (2010). To obtain steady-state mRNA copy numbers (number of mRNAs per cell), we used the rpkm data for the non-redundant gene list to estimate the proportion of mRNA that was represented by each mRNA, combined with the previous estimate of 19,000 RNA polymerase II-encoded mRNAs per cell (Haanstra et al. 2008).

To obtain ORF copy numbers, the output from shotgun sequence libraries of Lister 427 trypanosomes (kindly provided by George Cross) was aligned in the same way as for RNA tags, and the gene copy number was deduced from the rpkm values.

5' and 3' UTRs were inferred based on a collection of splice sites identified from all accessible sources, including published data (Kolev et al. 2010; Siegel et al. 2010; Archer et al. 2011) and the 10 WT sets generated for this publication. The predominant splice acceptor and poly(A) site for each gene were identified. To determine the positions of ORFs relative to transcription start and stop sites, the first and last five ORFs in each transcription unit were identified manually, using epigenetic markers (Siegel et al. 2009) annotated in TritrypDB.

mRNA half-life calculation

The averages of “rpkm left at 30 min” for two or three biological replicates were normalized, using known values for histone H4 (0.6, eight experiments) and tubulin (0.8, five experiments). The same values were used for all data sets since these two mRNAs are not detectably deadenylated after 30 min and their degradation is not affected by *XRNA* RNAi (Li et al. 2006). The mRNA half-life was calculated assuming exponential decay using the following equation:

$$t^{1/2} = 30 / -\ln(\text{normalized “rpkm left at 30 min”}) / \ln 2.$$

For the WT total, half-lives were calculated for the three replicates, and the genes showing SDs of more than half the arithmetic mean were excluded. For RT-qPCR, the expression (*E*) at different times was computed, an exponential model ($E = ae^{-kt}$) was fitted using the least squares method, and the average across replicate experiments was taken. Model fitting was done with *R*.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (Cl112/9-3, T.M.) and the Bundesministerium für Bildung und Forschung (Sysmo “The silicon trypanosome,” A.F.). We thank George Cross (Rockefeller University, New York) for communicating the unpublished shotgun sequencing data for *T. brucei* strain Lister 427. We thank Matt Berriman and Matthew Rogers (Sanger Centre) for teaching T.M. how to do the sequence alignments, and Martha-Lena Müller for assistance with RT-qPCR. We thank Omar Harb and Brian Brunk (TritrypDB) and Jan Korbel and Vladimir Benes (EMBL) for useful discussions and advice.

Received May 26, 2011; accepted August 16, 2011.

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RNA 2011 17: 2039-2047 originally published online September 26, 2011

Access the most recent version at doi:[10.1261/rna.2837311](https://doi.org/10.1261/rna.2837311)

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