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Trypanosoma brucei expression-site-associated-gene-8 protein interacts with a *Pumilio* family protein☆

Maarten Hoek [1], Tyiesha Zanders, George A.M. Cross *

Laboratory of Molecular Parasitology, The Rockefeller University, Box 185, 1230 York Avenue, New York, NY 10021-6399, USA

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Abstract

The expression site (ES) loci of *Trypanosoma brucei* are a valuable model for allelic exclusion and post-transcriptional regulation in a highly divergent eukaryote. ES exist to facilitate the expression and switching of the variant surface glycoproteins (VSG) that are central to trypanosome virulence and persistence. A collection of other potential virulence determinants, known as expression-site-associated-genes (ESAGs), are co-transcribed from the single upstream promoter. ESAGs may be involved in regulating the transcriptional state of the ES, as well as contributing additional surface proteins and receptors. We have previously shown that a putative regulatory protein, ESAG8, accumulates within the nucleolus, although 20% of the protein is cytoplasmic. Here we identify TbPUF1, a cytoplasmic ESAG8-interacting protein that falls into the Puf family of regulators of mRNA stability. Our experiments show that, as in other Puf family proteins, the most C-terminal repeats of TbPUF1 mediate its interaction with ESAG8. TbPUF1 is essential for cell viability, and preliminary results suggest that its overexpression seriously affects parasite virulence. *T. brucei* is the most evolutionary divergent organism in which a Puf family protein has been identified, and our initial experiments suggest that this protein may also regulate RNA stability in trypanosomes.

Keywords: ESAG8; Expression site regulation; Pumilio; RNA regulation; *Trypanosoma brucei*; Virulence; Variant surface glycoprotein regulation

1. Introduction

Post-transcriptional regulation of mRNA is increasingly recognized as a central pathway controlling gene expression in eukaryotes, yet few regulatory factors have been identified that specifically control the turnover of transcripts in response to environmental signals and developmental programs. Post-transcriptional regulation may be particularly important in trypanosomes, where accumulated data provide little or no evidence for regulation of transcription initiation (reviewed in [1,2]).

The expression site (ES) loci of the parasitic protozoan *Trypanosoma brucei* may be a particularly interesting model in which to study post-transcriptional regulation, because transcripts from this well-characterized multigene locus are regulated post-transcriptionally by both global and transcript-specific mechanisms. Identification of pathways that specifically control transcript turnover in highly divergent eukaryotes may help identify and clarify the pathways used in other eukaryotes.

Bloodstream-form *T. brucei* is covered by the immunodominant variant surface glycoprotein (VSG), and the periodic switching of this coat to a new one allows the parasite to remain one step ahead of the immune system. This results in semi-clonal waves of parasitemia that characterize trypanosome infections. The *VSG* is expressed from an unusual and invariably telomeric locus called the ES [3]. ESs are polycistronic transcription units, with the VSG at the telomeric end and multiple ES-associated-genes (*ESAGs*) found between the *VSG* and the ES promoter, which is located ~50 kb upstream [3–5]. Polycistronic transcription is mediated

Abbreviations: ES, expression site; ESAG, ES-associated gene; LRR, leucine-rich repeat; UTR, untranslated region; VSG, variant surface glycoprotein.

☆ *Note:* Nucleotide sequence data reported in this paper are available in the GenBank database under the accession number AY034830.

* Corresponding author. Tel.: +1-212-327-7571; fax: +1-212-327-7845; http://tryps.rockefeller.edu.

E-mail address: george.cross@rockefeller.edu (G.A.M. Cross).

[1] Present address: Cold Spring Harbor Laboratory, James Building, 1 Bungtown Road, Cold Spring Harbor, NY 11724, USA.

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by RNA polymerase I (RNAP-I) and the nascent transcript is processed by trans-splicing a capped 39 nt sequence to every ES-derived mRNA [6–11]. Post-transcriptional processes that may or may not be coupled to the splicing reaction are very important for regulating steady state levels of *ESAG* encoded proteins, which are present at very low levels compared with the VSG. Productive transcription occurs in only one ES at a time, even though about 20 potentially active ESs are present in each cell. The mechanism by which this mutually exclusive transcription is maintained is unknown, but is unlikely to rely on mobile *cis* elements [12,13], changes in chromatin structure [14], or DNA modifications such as β-D-glucosyl-hydroxymethyluracil (also known as base J), which is found primarily in repetitive sequences flanking the ES [15,16] (P. Borst, personal communication).

We have been exploring the role of the putative regulatory gene *ESAG8* in ES regulation. *ESAG8* is distinct from most other *ESAGs* because it appears to be exclusively transcribed from the active ES, because it is very highly conserved, and because its expression is limited to the bloodstream forms. ESAG8 contains N-terminal RING Zn^{2+}-finger and C-terminal leucine-rich repeat (LRR) domains [17,18]. The LRR is a well known protein interaction module [19] and RING Zn^{2+}-fingers are present in proteins that mediate polyubiquitylation and targeted degradation of proteins in many systems, raising the possibility that ESAG8 is a negative regulator of other proteins in the bloodstream form [20].

We previously reported that the bulk of ESAG8 is nucleolar, which implied that the protein may be involved in regulating the transcription of the active ES by regulating RNA Polymerase I [21]. However, a significant fraction of ESAG8 was also found in the cytoplasm. We were, therefore, interested to explore whether ESAG8 might instead play a role in post-transcriptional or translational regulation of mRNA. Here, we demonstrate that ESAG8 interacts with a member of the Puf family of RNA regulatory proteins, which we named PUF1. An epitope-tagged version of PUF1 is cytoplasmic when over expressed, suggesting that ESAG8 has a function that may be distinct from any role in ES transcription. Modulations in PUF1 levels affect the stability of ES-derived mRNA, indicating that the function of Puf family proteins in mRNA regulation may be very ancient. PUF1 is the first Puf protein identified in an early branching eukaryote. In preliminary experiments we also show that PUF1 over-expression reduces or eliminates the virulence of *T. brucei*. A better understanding of the functions of PUF1 will enhance our knowledge of this interesting class of mRNA regulatory proteins and may also shed light on unique aspects of the pathogenesis of *T. brucei*.

2. Materials and methods

2.1. Trypanosome culture and transfection

Bloodstream forms of *T. brucei* strain 427 variant MITat1.2, expressing VSG 221 from a single-copy telomeric gene, in the '221 ES', were cultured in HMI-9 [22]. All transfections were performed as described previously [23]. For stable regulated expression of ectopic *ESAG*8 and *GFP* constructs, a T7 promoter-driven cassette was integrated into a *rDNA* spacer in trypanosome cell line 13–90, expressing T7 RNA polymerase (T7Pol) and the Tet repressor (TetR) [24]. For knockout experiments, the 'single-marker' regulatable cell line, which contains the *T7Pol* and *TetR* genes linked to a single *NEO* marker, was used as background [25]. Expression was induced by adding 2.5 ng ml^{-1} doxycycline or tetracycline to the medium as required. Transformants were selected with standard concentrations of drugs, as detailed elsewhere.

2.2. Plasmid construction

The cDNA library used for the two-hybrid analysis was custom-made by Clontech, from mixed bloodstream and procyclic total RNA provided by us. Clontech generated total mRNA by polyA+ selection, followed by cDNA synthesis using mixed poly dT and random primers to enhance the representation of RNAs containing long 3′ untranslated region (UTRs). The cDNA was cloned into the pGAD10 vector and amplified in bacteria. Bait and control plasmids used to screen the library were generated in the GBD fusion vector pAS2-1 or the GAD fusion vector pAct2-1. The *ESAG8* bait plasmid MH184 was prepared by cloning *Hind*III-digested and Klenow-treated MH126, containing full length *ESAG8*, into *Bam*HI-digested and Klenow-treated pAS2-1 to generate an in-frame fusion with the GBD. MH192, containing only the LRR region of *ESAG8*, was generated similarly using MH129 as a donor. MH193, which contains only the RING and linker domains of *ESAG8* fused to the GBD, was generated by amplifying this regions with appropriate primers, digesting with *Bam*HI and inserting this into similarly digested pAS2-1 To make sure the PUF1 two-hybrid interaction was reproducible, *PUF1* was re-cloned into MH215 by PCR-amplifying the gene from BAG 2H18 (library RPCI-102) using appropriate primers, cutting the product with *Bam*HI and inserting the fragment into similarly digested pAct2-1. Plasmids that mediate the expression of truncated derivatives of *PUF1* were made by digesting MH215 with either *Xho*I or *Sac*I and religating the plasmids to generate MH246 and MH247, which express PUF1 1–322 or PUF1 1–233, respectively.

T. brucei PUF1 expression vectors were derived from the two-hybrid cDNA 184:0412 clone, because expression of *PUF1* was only possible in the presence of a portion of its 3′ UTR (discussed later). MH217 containing HA-tagged *PUF1* was created by inserting the Klenow treated *Sal*I fragment from 184:0412 into MH181, a derivative of MH126 containing HA-tagged *ESAG8*, cut with *Sac*I–*Sma*I and treated with T4 polymerase to remove the 3′ overhang. This generated an in-frame *HA-PUF1* derivative. This cassette was then transferred into plasmids Lew82 or Lew100 to generate the *HA-PUF1* expression plasmids [24,25]. A similar approach was used to make the GFP–PUF1 derivative, MH228. To disrupt the *PUF1* gene, the 3 kb *Kpn*I fragment from BAC 2H18 (RPCI-102) (BACPAC Resource Center, Children's Hospital Oakland Research Institute) was subcloned into pBluescript II SK+ to generate MH232. The *PUF1* ORF was removed by *Nde*I digestion and replaced with either the *ALD5′ UTR–BSD–ALD* 3′ UTR cassette or the *ALD5′ UTR–PUR–ALD*3′ UTR from HD309-BSD or HD309-PUR to generate the blasticidin and puromycin knockout cassettes MH235 or MH236, respectively.

2.3. Two-hybrid interaction trap for ESAG8-interacting proteins

The Matchmaker-II (Clontech) interaction trap kit was used throughout these experiments and all plasmids and initial controls were used as derived from this kit. All yeast transformations were done using an optimized lithium acetate protocol [26], except that 50 μl of 2 mg ml^{-1} unsonicated salmon sperm DNA instead of 10 μl of 10 mg ml^{-1} sonicated salmon sperm DNA were used. Bait cell lines were established by transforming the tightly regulatable strain CG1945 ($MATa$, $ura3-52$, $his3-200$, $ade2-101$, $trp1-901$, $leu2-3$, 112, $gal4-542$, $gal80-538$, cyh^r2, $LYS2::GAL1_{UAS}-GAL1_{TATA}-HIS3$, $URA3::GAL4_{17\text{-}mers(x3)}-CYC1_{TATA}-lacZ$) with MH184; these cells expressed protein and did not autoactivate transcription of the β-Gal reporter gene. A total of 4×10^6 colonies were screened. Transformation reactions were plated directly onto -Leu/-Trp/-His plates containing 1 mM 3-amino triazole (3-AT), a minimal empirically determined amount to reduce background growth of non-activated cells. Colonies that grew out were patched onto -Leu/-Trp plates and were tested for β-Gal activity in a filter lift assay [27].

Positive clones were streaked onto -Leu plates containing 1 μg ml^{-1} cycloheximide, a dominant negative selectable marker present on the bait plasmid. Colonies that grew up on these plates contained only the bait plasmid. These colonies were tested again to ensure that the library plasmid by itself did not activate transcription. Plasmids were rescued from these cells by a 'smash and grab' procedure [28] and transformed into *Escherichia coli*, purified and sequenced using the Gal4AD primer supplied by Clontech. Library plasmids were transformed into fresh yeast with the original prey plasmids or a non-specific pLam5′ control plasmid and tested in a final round of filter lift assays for specific activation of the β-Gal reporter. As a final confirmation of interaction in the two-hybrid assay, the *PUF1* ORF that was isolated was recloned into the bait Gal4BD containing plasmid pAS2–1 and retested against *ESAG8* subcloned into the Gal4AD containing plasmid pAct2–1. Truncations of the *PUF1* gene and *ESAG8* were also tested against each other to identify the interacting domains.

2.4. RNA and DNA preparation and analysis

RNA was prepared from approximately 10^8 cells, using RNA STAT-60 (Tel-Test), according to the manufacturer's directions. Samples were denatured in formaldehyde/formamide premix at 65 °C for 10 min and run on a 1.5% agarose/1% formaldehyde gel, transferred to nylon and hybridized in Quickhyb hybridization buffer (Stratagene) with full-length *ESAG8* probe labeled with a random primer labeling kit. For Southern analysis, DNA was prepared from 10^7 cells using DNAzol according to the manufacturers directions (Molecular Research Center). Approximately 2 μg of DNA was digested using the appropriate enzyme and buffer (NEB) and run out on a 1% agarose gel for several hours. DNA was depurinated, denatured, neutralized and blotted according to standard techniques. Blots were cross-linked and hybridized with the appropriate probe as described above.

2.5. Gel filtration

Gel filtration chromatography was performed on a 10×360 mm, 28 ml Sephacryl S-300 column calibrated with several protein markers. The column was equilibrated with 50 ml GF Buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM dithiothreitol) at 0.2 ml min^{-1}. Total trypanosome extract from 2×10^8 cells was prepared by osmotic lysis in water (containing protease inhibitors and dithiothreitol), followed by dialysis against GF buffer for 2 h in a dialysis cassette (10 000 MWCO, Pierce). The extract was spun for 30 min at $100\,000 \times g$ and 500 μl (1×10^8 cells) was loaded onto the column. One milliliter fractions were collected from 8 ml until 25 ml, precipitated with 10% TCA for 1 h on ice, spun 10 min at $14\,000 \times g$, washed in 1 ml acetone, and resuspended in 50 μl SDS-PAGE sample buffer and boiled 5 min. In general, 5–10 μl were loaded on a gel for Western analysis.

2.6. Immunofluorescence and microscopy

For HA-PUF1 immunofluorescence analysis (IFA), cells were fixed as above and spun onto coverslips for 10 min at $400 \times g$, followed by permeabilization in 0.1% Triton X-100 and washing as above. Samples were blocked in PBG (PBS containing 0.02% cold-water-fish skin gelatin (Sigma), 0.5% BSA), incubated with anti-HA 12CA5 (Roche) overnight at 4 °C, followed by six washes in PBG at and incubation with FITC anti-mouse (Jackson Labs) at 1:50 or 1:200 dilution for 1 h and another six washes in PBG, all at room temperature. Cells were visualized with a 100 × objective using a Spot 2.3.1 camera attached to the same microscope as above, to capture the image. Images were imported into ADOBE PHOTOSHOP.

2.7. Antibody generation and Western blot analysis

The pET15b derived expression vector, MH227, containing the full length PUF1 gene from the cDNA clone, was transformed into BL21(λDE3) *E. coli.* Protein production was induced for 3 h in 1 mM IPTG and His_6-fusion protein purified from bacterial inclusion bodies was gel purified and used to inoculate two NZW rabbits. Initial inoculation was with 500 μg of antigen intradermally, followed by two boosts at 250 μg, two boosts with 125 μg protein and two boosts with 100 μg of antigen. All rabbit work was done by Covance, Inc. Specificity was confirmed by comparing pre-immune and immune sera against PUF1 knockout and HA-PUF1 overexpressing cell lines. A wild-type PUF1 band was seen just below the HA-PUF1 signal. This band disappeared in the knockout cell line, demonstrating reaction specificity. The antibody was cleared of cross-reacting activity by incubating with dry milk and *E. coli* BL21-derived acetone powders.

For Western blots, samples were run on either 8 or 10% SDS-PAGE gels and transferred to nitrocellulose (Amersham-Pharmacia). Crude lysates of 2×10^6 cells were loaded per lane. Western blots were probed with anti-PUF1 or anti-ESAG8 rat-1 antiserum at a 1:1000 dilution, and were secondarily probed with horseradish peroxidase-conjugated goat-anti-rabbit (Amersham-Pharmacia) or rat antibodies (Roche) at a 1:500 or a 1:15 000 dilution, respectively. For HA analysis, blots were probed with the anti-HA mouse monoclonal 12CA5 (Roche) followed by horseradish peroxidase-conjugated goat-anti-mouse antibody (Amersham-Pharmacia) both at 1:5000 dilutions. Blots were developed in SuperSignal West Pico substrate (Pierce), according to the manufacturer's directions.

2.8. Crosslinking and immunoprecipitation analysis

Cells stably transfected with the inducible HA-PUF1 expression vector, MH218, were induced overnight with 500 ng ml^{-1} tetracycline to obtain maximum expression levels. Approximately $3–4 \times 10^8$ cells were collected, washed once in trypanosome dilution buffer (5 mM KC1, 80 mM NaCl, 1 mM $MgSO_4$, 20 mM Na_2HPO_4, 2 mM NaH_2PO_4, 20 mM glucose, pH 7.7), resuspended to a concentration of 2×10^8 cells per ml in trypanosome dilution buffer and split into two microfuge tubes. Cells were crosslinked for 15 min at 4 °C with 0.5 mM dithiobis[succinimydyl propionate], followed by addition of Tris–Cl pH 7.5 to 50 mM for 30 min to quench the reaction. Cells were pelleted and lysed in the same volume of IP buffer (50 mM HEPES, pH 7.5, 100 mM NaCl, 0.4% NP-40) containing protease inhibitors (30 μg ml^{-1} TLCK, 10 μg ml^{-1} leupeptin, 10 μg ml^{-1} pepstatin-A, and 0.5 mM PMSF) for 5 min. Lysates were clarified by two successive 5 min spins at $14\,000 \times g$, followed by incubation with either 0.1 μl of normal rat serum as a control, or 100 ng of the high-affinity rat anti-HA monoclonal 3F10 (Roche) for 1 h with rotation. After a 5 min clearing spin, the cell lysate was transferred to a tube containing ~20 μl of protein A/G agarose beads (Pierce) and washed in IP buffer containing protease inhibitors. The beads were incubated with rotation for 1 h, washed four times for 2 min in 1 ml IP buffer and the final pellet was resuspended in 50 μl SDS-PAGE sample buffer, boiled 5 min and stored at −70 °C. Typically, 1/5th of the IP reaction (corresponding to $3–4 \times 10^7$ cell equivalents) was run for Western analysis and compared with 2×10^6 cell equivalents of the input and output.

2.9. In vivo virulence assays

Inbred trypanosome-resistant C57BL/10J mice (Jackson Labs) were used for all virulence analyses. Mice were inoculated intraperitoneally with 10–15 trypanosomes in culture medium and parasitemias were monitored by tail bleeds. When tetracycline induction was desired, mice were watered with 200 μg ml^{-1} doxycycline in 5% sucrose, a procedure previously shown to give high levels of induction in vivo [24].

2.10. RNA stability assays

For *PUF1* knockout cell lines, cells were washed twice with HMI-9 and resuspended in fresh medium ±500 ng ml^{-1} tetracycline and grown for 72 h to be sure PUF1 had disappeared. *PUF1* overexpressing cells were incubated with 500 ng ml^{-1} tetracycline overnight. Cells were always grown to between 1 and 1.3×10^6 cells per ml because variation in cell density increased the variability of the assay. Actinomycin-D (Sigma) was

pre-diluted 1:10 from a 10 mg ml^{-1} stock into HMI-9 at 37 °C, followed by a final 1:100 dilution into the cell culture for a final concentration of 10 μg ml^{-1}. The culture was maintained at 37 °C and 30 ml samples were removed at the indicated time-points for RNA purification as above.

3. Results

3.1. ESAG8 is present in a high molecular weight complex

Since ESAG8 is predicted to contain two protein interaction domains, we estimated the native size of ESAG8 by gel filtration chromatography. We used an osmotic lysis procedure, which solubilizes the bulk of the cellular protein and generates a crude cell extract, followed by dialysis against a HEPES buffered saline. The cell extract was centrifuged at 100 000 × *g* for 30 min, to remove any non-specific aggregate, and loaded onto a Sephacryl S-300 column, which separates proteins from ~25 kDa to 1 MDa. Under these conditions, ESAG8 was predominantly found in a 300–600 kDa fraction (Fig. 1, upper panel). This fractionation profile was reproducible and ESAG8 was clearly separable from the void volume in these experiments. In some experiments (data not shown), we detected a small amount of protein eluting at the monomer position, consistent with previous data that the protein is present in two different pools in the cell. We do not know the identity of the cross-reacting band in fractions 16–18. There is also a very weak background at the same position as ESAG8 in all the later fractions. It remains a formal possibility that this represents low levels of ESAG8 throughout the fractions, but preimmune serum also gave a very faint band at this position.

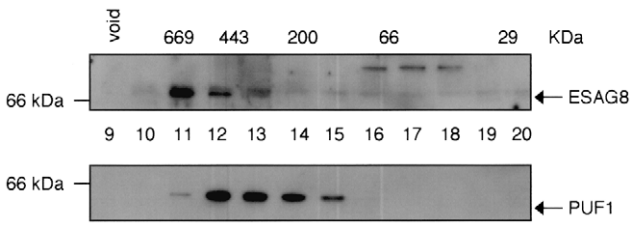

Fig. 1. ESAG8 exists in a high-molecular-weight complex. Gel filtration of an osmotic lysate of *T. brucei* overexpressing the HA-PUF1 protein shows that the bulk of the extracted ESAG8 is present as a complex in the 300–600 kDa range. The HA-PUF1 protein is seen in an overlapping region. Protein was precipitated from 1 ml column fractions, electrophoresed on reducing SDS-PAGE, blotted onto nitrocellulose, and probed with anti-ESAG8 or anti-HA (for PUF1) antibodies.

3.2. 2-Hybrid screening for ESAG8 interacting proteins

The gel filtration results showed that most of the ESAG8 exists as a high molecular weight complex. We initially sought to take a biochemical approach to identify ESAG8-interacting proteins. However, we were unsuccessful in generating soluble recombinant protein for an affinity matrix, because the RING Zn^{2+}-finger domain had a strong tendency to aggregate, a known problem with these proteins [20]. To overcome problems with the biochemical approaches, we turned to a yeast two-hybrid interaction trap [29], using the commercially available Matchmaker II system (Clontech). The Gal4-activation domain (GAD)–DNA fusion library used in these experiments was constructed by reverse-transcribing mixed polyA+ *T. brucei* RNA from cultured bloodstream and insect midgut stages. Both oligo dT and random primers were used for cDNA synthesis, because some 3′ UTRs in *T. brucei* are very long and might not generate full length cDNA when extended from the polyA tail. The library was not normalized and testing showed that about 5–10% of the library was made up of VSG cDNA and 2% of the library consisted of *TUBULIN* cDNA, similar to levels of these transcripts in the cell (J.L. Muñoz-Jordán; P.K. Patnaik, personal communication).

We screened the GAD–cDNA fusion library in a strain containing the Gal4 DNA-binding domain (GBD)–ESAG8 bait plasmid (MH184). 4.9×10^6 colonies were screened and 69 His+ colonies were tested for β-Gal activity by a filter lift assay. Five colonies reproducibly activated the β-Gal reporter and one of these, 184.0412, did not autoactivate β-Gal transcription or activate the reporters in the presence of the negative control plasmid, pLam5′, but activated the β-Gal reporter when co-transformed with MH184 or with MH192, which contained just the LRR domain of ESAG8 (Fig. 2A). This clone consisted of a 2.2 kb cDNA containing an ORF of 1.5 kb, in frame with the GAD, encoding a protein showing strong homology to the Pumilio/FBF (Puf) family of RNA binding proteins. No polyA tail was identified, so the cDNA was probably randomly primed in the 3′ UTR.

3.3. Cloning and initial characterization of the ESAG8 interacting protein, TbPUF1

The 184:0412 cDNA was used as a probe to identify the genomic copy of *TbPUF1* on Southern blots using various enzymes. *PUF1* is a single copy-gene present on a 3 kb *Kpn*I fragment. This fragment was cloned from a size-selected genomic library and sequenced. One ORF corresponding to the cDNA was identified. Sequencing confirmed that the 184:0412 cDNA contained the full ORF as well as a significant portion of the 3′ UTR, hinting that the full-length protein is required for a

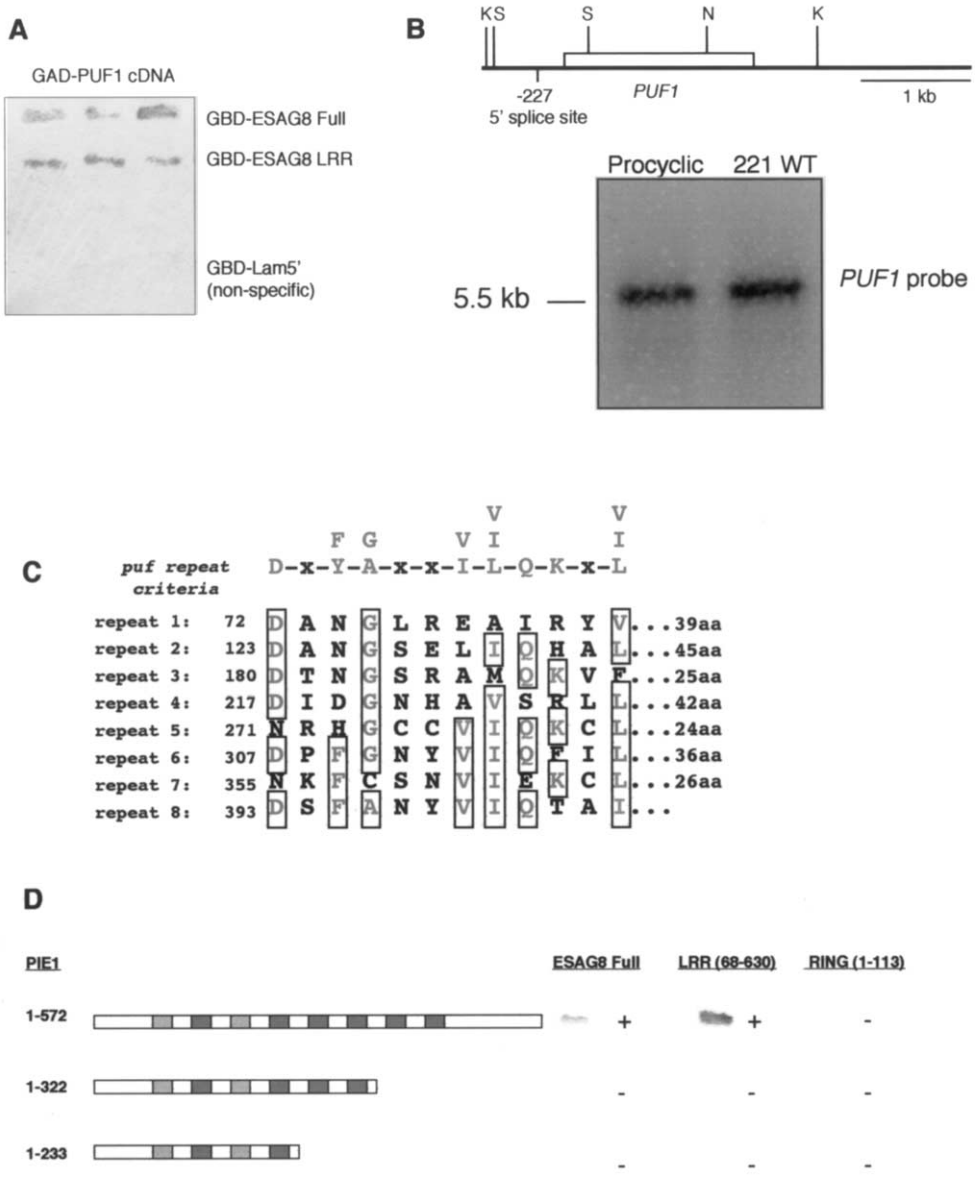

Fig. 2. Isolation of PUF1 as an ESAG8-interacting protein. (A) Filter lift β-galactosidase assay shows that the GAD–PUF1 fusion interacts with full-length ESAG8 or just the LRR of ESAG8 (amino acids 68–630), but does not interact with the control Lam5′ fusion protein. Three independent clones were analyzed for each test plasmid against the 184:04121 cDNA clone. (B) Northern analysis of the PUF1 mRNA profile in insect and bloodstream cells. The PUF1 3′ UTR is abnormally long because the 5′ splice site is found at −227 and the mRNA is > 5 kb. (C) PUF1 contains eight Puf repeats that conform to the consensus sequence as defined by Zhang et al. There are eight amino-acid identity criteria and three spacing criteria for the Puf domain. In previous publications, a repeat sequence had to conform to eight of 11 criteria to be represented [30]. Repeats one and three conform to only five or six criteria, but still resemble Puf repeats. (D) C-terminal truncations that remove two or more of the Puf repeats abrogate binding to ESAG8 and interaction of the reporter gene. The interaction maps exclusively to the LRR domain since RING Zn^{2+}-finger and nucleolar localization region (amino acids 1–113) did not support the interaction.

productive interaction with ESAG8. This is further supported by the fact that no truncated *PUF1* cDNAs were isolated in the screen, although they should have been present and more abundant in the library, due to incomplete reverse transcription. *PUF1* is expressed at equal levels in both insect and bloodstream-form parasites. The RNA is approximately 5.5 kb, much longer than expected for an ORF of 1.5 kb (Fig. 2B). RACE analysis showed that the 5′ splice site was 227 nt upstream from the initial ATG, so the extra sequence most likely comes from the 3′ UTR. PUF1 is clearly a member of the Puf family of RNA binding proteins. Proteins in this family are sequence-specific RNA-binding proteins that classically bind the 3′ UTR of their target genes and regulate stability and translation of their target RNAs [30,31]. These proteins generally contain eight Puf repeat elements separated by 20–40 amino acids. TbPUF1 has eight repeats that conform to

this consensus, although repeats one and three are somewhat divergent (Fig. 2C). The flanking regions around the Puf repeats are also shorter compared with other family members and the protein consists almost exclusively of this repeat structure.

3.4. Confirmation of the ESAG8–PUF1 interaction

To further investigate the interaction between PUF1 and ESAG8, we generated a set of C-terminal deletions of GAD–PUF1 and re-tested these for interaction in the two-hybrid assay. Whereas full-length PUF1 was able to activate transcription of the β-Gal reporter when co-transformed with either full-length or ΔRING GBD–ESAG8, truncated versions of PUF1 were unable to interact in this assay (Fig. 2D). None of the PUF1 fusion proteins interacted with the RING+nucleolar localization domain (amino acids 1–113) of ESAG8, indicating that the interaction was mediated exclusively with the LRR repeats. Together with the fact that no N-terminal truncations of PUF1 were isolated in the 2-hybrid screen, these data indicate that full-length protein is likely required for productive interaction with ESAG8 and that the interaction is likely to be specific. Recent evidence from mutational and structural analysis indicates that the C-terminal region of the *Drosophila* Puf protein, Pum, is essential for mediating interactions with two other proteins, Nos and BRAT [32]. Our data indicate that the function of this region of the Puf repeats may be evolutionarily conserved.

To show that the interaction identified in the two-hybrid screen occurs in vivo, we attempted to co-immunoprecipitate ESAG8 with an HA-tagged version of PUF1. We generated a strain that expressed HA-tagged PUF1 in a tetracycline-inducible system [24]. Upon induction this strain overexpressed a 3 kb *PUF1* mRNA approximately eight to ten fold when compared with the native mRNA. HA-PUF1 protein was also present at a considerably higher level than the endogenous PUF1 (see Fig. 7B). Attempts to establish cell lines without part of the *PUF1* 3′ UTR failed, implying that the 3′ UTR contains elements that are important for regulating PUF1 expression. These may be negative regulatory elements. Since overexpression of PUF1 is deleterious (see later), including some of the 3′ UTR may be necessary to prevent overexpression that might otherwise occur from the 3′ UTR provided by the pLew expression vector. It is possible that PUF1 regulates itself by binding to its 3′ UTR, but it would require much work to test this speculation.

Initial immunoprecipitation experiments, in which HA-PUF1 was immunoprecipitated from *T. brucei* lysates using an anti-HA monoclonal antibody, showed an enrichment for ESAG8 (data not shown), but some ESAG8 signal was also present in the negative control immunoprecipitation. Attempts to diminish the background, by increasing the stringency of the wash steps or by adding higher amounts of detergent to the lysate, reduced overall signal without increasing the specific ESAG8 signal (data not shown). However, some PUF1 did overlap with the high-molecular weight ESAG8 complex in gel filtration experiments (Fig. 1, lower panel). Although, most of the ESAG8 and PUF1 were not found in the same gel-filtration fraction, it should be noted (see below) that HA-tagged PUF1 is overexpressed by a factor of about eight-fold. This overexpression was associated with a very small increase in ESAG8. We took the material from fraction 12 and ran it through a second cycle of gel filtration. Although, the signals were weaker, the results (not shown) suggested that the two proteins stayed together in the second cycle, implying that a proportion of ESAG8 and PUF1 are present as a complex.

These preliminary experiments suggested that ESAG8 does interact in vivo with HA-PUF1, but that the tendency of ESAG8 to precipitate or aggregate was interfering with the assay. The membrane permeable, dithiothreitol-cleavable, crosslinking agent dithiobis[succinimidyl propionate] has been used to identify an in vivo interaction between c-Myc and TATA-box binding protein, as well as between $p21^{ras}$ and p60 [33,34]. This crosslinking agent is reactive to amino groups and has a spacer arm of 12 Å. We reasoned that it might be possible to use cross-linking to enrich specifically for the ESAG8–PUF1 complex, allowing the stringency of the lysis and washing steps to be increased without dissociating the complex.

HA-PUF1 expressing cells were induced overnight with 2.5 ng ml^{-1} doxycycline. Cross-linking conditions were determined empirically (see Section 2), to allow for the highest level of extraction of ESAG8 and TbPUF1 from the cell. In the presence of anti-HA monoclonal antibody, but not of normal rat serum, an ESAG8-reactive band was specifically co-immunoprecipitated with HA-PUF1 (Fig. 3A). No ESAG8 co-precipitated when the parental cell line (not expressing HA-PUF1) was used in these immunoprecipitations. As a further control to be sure that proteins were not aggregating non-specifically, we probed the immunoprecipitates with antibody against 221 VSG, one of the most abundant proteins in and on the cell, and the best control we have in the absence of antibodies against abundant cytoplasmic proteins. We did not see a significant enrichment for 221 VSG when compared with ESAG8, confirming that the ESAG8–PUF1 interaction is specific (Fig. 3B). Immunoprecipitation of HA-PUF1 was not quantitative, but we have not been able to increase the yield by increasing the amount of antibody or protein A/G beads, perhaps because much of the protein is inaccessible to the antibody, due to the cross-linking step.

There are two isoforms of ESAG8 expressed from the three copies of *ESAG8* in the 221 ES, leading to an

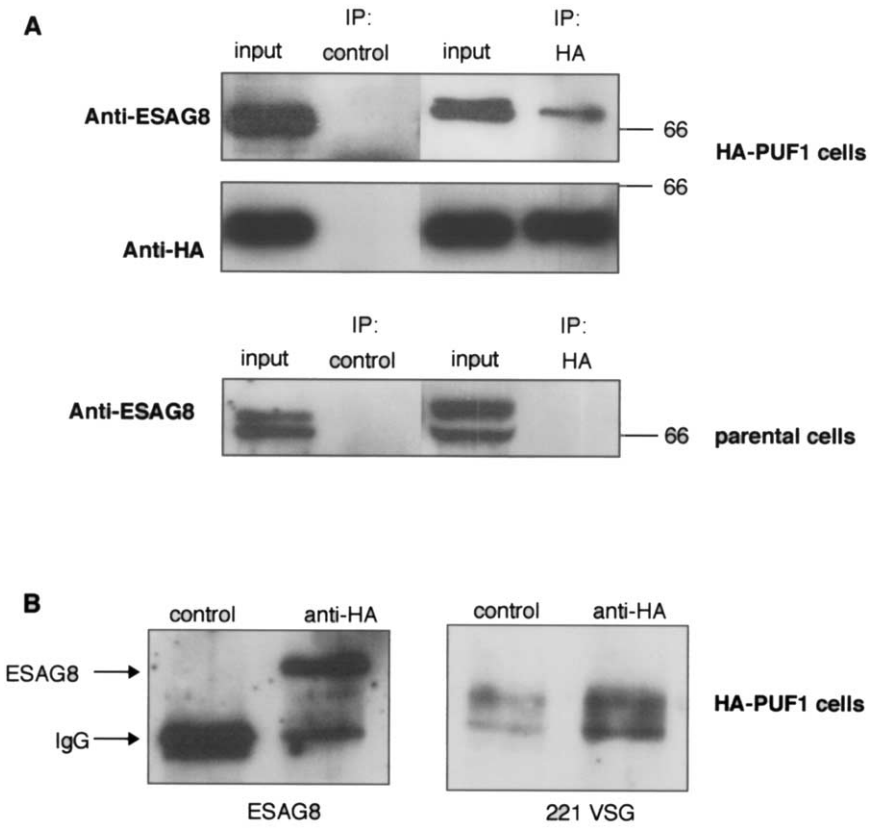

Fig. 3. ESAG8 co-precipitates with HA-PUF1. All experiments were done in HA-PUF1 overexpressing cells using a high-affinity anti-HA rat monoclonal 3F10 or normal rat serum as a control. Western blots were probed with anti-HA mouse monoclonal 12CA5 or with anti-ESAG8 (rat-1) or anti-VSG221. Typically, 20% of the IP, corresponding to $3–4 \times 10^7$, cells was loaded in the IP lane, whereas 2×10^6 cell equivalents were always loaded in the control lanes. (A) ESAG8 specifically co-precipitates with HA-PUF1 when using dithiobis[succinimydyl propionate]-assisted immunoprecipitation. ESAG8 is not non-specifically precipitated in wild-type cells. (B) VSG is not highly enriched in the anti-HA immunoprecipitate, demonstrating that non-specific interactions do not contribute to PUF1-ESAG8 co-precipitation.

ESAG8 doublet in western blots of cells in which this ES is active [35]. It is interesting to note that only one of the two ESAG8 bands co-precipitated in this experiment. Due to small differences in mobility in different extracts, we cannot be sure which copy of ESAG8 is the predominant interacting one. However, because the two-hybrid screening was done with the smaller version of ESAG8, we tentatively suggest that this is the variant that is co-precipitated. Since the larger copy of ESAG8 in the 221 ES contains an extra LRR (data not shown), this would imply that alterations within the LRR region can affect protein interaction, and supports data from the two-hybrid analysis that the ESAG8–LRR is the interacting domain.

3.5. Localization and quantification of PUF1

PUF1 might interact with ESAG8 in either the cytoplasm or the nucleolus. We examined cells over-expressing HA-PUF1 by immunofluorescence analysis and found that the protein is restricted to the cytoplasm (Fig. 4). HA-PUF1 is functional because it can replace substitute for PUF1, which is essential (see later), after

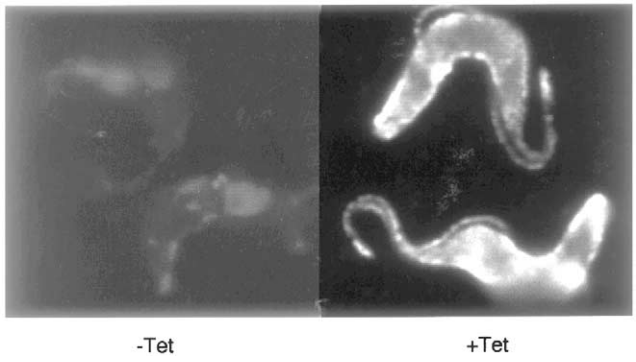

Fig. 4. HA-PUF1 is not a nucleolar protein. HA-PUF1 overexpressing cells were incubated in the presence or absence of doxycycline and processed with the 12CA5 antibody. A strong HA signal is present throughout the cytoplasm, but not in the nucleus.

both endogenous *PUF1* alleles are deleted. No nuclear signal was evident, implying that the cytoplasmic pool of ESAG8 is the PUF1-interacting pool. The same distribution was seen when using GFP-tagged PUF1, although the functionality of this fusion protein has not been established. This may explain why immunopreci-

pitation of ESAG8 was difficult: if only 20% of ESAG8 is present in the cytoplasm and if only a portion of PUF1 is precipitated, a very small amount of specific signal would be expected.

ESAG8 is present at low levels, 2000–4000 copies per cell [21]. Quantitative distribution analysis of GFP–ESAG8 implies that only 400–800 copies of ESAG8 are present in the cytoplasm [21]. To begin to understand the functional relevance of this interaction, it would be interesting to know whether PUF1 is present in excess in the cytoplasm. We produced a rabbit antiserum specific for PUF1 and determined the amount of PUF1 protein in the cell. Our initial experiments showed that PUF1 is present at very low levels. It was difficult to detect with an antiserum that had a detection threshold of ~ 0.5 ng on Western blots (data not shown). This corresponds to an upper limit of roughly 2000 molecules of PUF1 per cell, similar to total levels of ESAG8, meaning that PUF1 may be present in five-fold excess in the cytoplasm. A more accurate analysis of PUF1 distribution will depend on the generation of higher affinity anti-PUF1 antisera.

3.6. Genetic manipulation of PUF1 levels

The most straightforward path to the functional analysis *PUF1* is to examine the consequences of manipulating PUF1 levels in the cell. Disruption cassettes were able to target both *PUF1* alleles (five transfections with four to five clones for each construct), but cell lines in which both cassettes had integrated (five transfections with no clones) could not be obtained, implying that *PUF1* is essential in the bloodstream form. Similar experiments suggest that *PUF1* is also essential in tsetse midgut culture forms (S. Leal, personal communication).

To confirm that *PUF1* is essential, a conditional disruption strategy was used [25]. In this system, a very tight Tet_R-regulated PARP promoter drives expression of *HA-PUF1*. Disruption of both wild-type alleles of *PUF1* was possible when the ectopic HA-PUF1 was induced with 500 ng ml^{-1} tetracycline. The double disruption was also possible in a cell line that expressed *PUF1* from the more poorly regulated expression system used for the overexpression studies. These data conclusively establish that *PUF1* is an essential gene and demonstrate that HA-tagged PUF1 is functional.

Cells did not die when tetracycline was removed from the medium, however, contradicting the conclusion that the *PUF1* is essential. *PUF1* mRNA disappeared from these cells by 24 h and the growth rate slowed by approximately 50% by 40 h (Fig. 5A). When the cells were subcultured, however, they always needed to be fed 1 day later than wild-type, indicative of slower growth. *PUF1* message was undetectable in these cells, demonstrating that the expression system is tight. We suspect that the more gradual decrease of *PUF1* RNA and protein using this conditional strategy may have allowed cells time to adjust by upregulating compensatory pathways. This might not be possible in a direct disruption. Interestingly, cells that overexpress *HA-PUF1* also have a significant growth defect (Fig. 5B). Thus, either over- or under-expression of *PUF1* reduces growth in culture.

3.7. Infectivity of PUF1-perturbed cells

Since changing PUF1 levels affected growth in vitro, we were interested to see whether there was a more pronounced effect in animals. We infected a trypanosome-resistant inbred mouse line (C57BL/10J) with the parental single-marker cell line, the knockout cell line grown in the absence of tetracycline for 3 days, or the overexpressing cell line induced with tetracycline overnight. The latter group of mice received 200 μg ml^{-1} doxycycline in their drinking water for 1 week prior to inoculation [24,36]. Trypanosomes overexpressing PUF1 were unable to establish infection in mice, whereas parental or 'knockout' cell lines killed mice in 7–10 days (data not shown).

To confirm this effect, we inoculated three groups of five mice with the overexpressing cell line. Group 1 received no doxycycline, group 2 received doxycycline immediately after inoculation and group 3 received doxycycline after the parasitemia reached about 1×10^6 trypanosomes per ml (Fig. 6). Induction after inoculation drastically reduced infectivity of these cells (three mice survived indefinitely). When overexpression of HA-PUF1 was induced after a substantial infection had been established, almost all mice went into remission and one was cured. Control mice that had been fed doxycycline for 1 week and were inoculated with the parental trypanosome strain died with normal kinetics, demonstrating that the effect was not simply due to doxycycline toxicity. In the absence of induction, the HA-PUF1 overexpressing trypanosomes killed mice with somewhat slower kinetics, perhaps due to the leakiness of the expression system. These experiments show that overexpression of PUF1 significantly reduces the infectivity of a virulent trypanosome strain.

3.8. Perturbation of PUF1 affects the stability and levels of specific mRNAs

The effect of PUF1 on growth in vivo might be mediated by an effect on the stability or steady state levels of target mRNAs. Since PUF1 interacts with ESAG8, certain ES derived mRNAs might be specific targets of PUF1. To test this hypothesis, we examined the effect of PUF1 perturbation on the levels and stability of *VSG*, *ESAG6* and *ESAG8* mRNA. There was little change in the steady state levels of either *VSG*

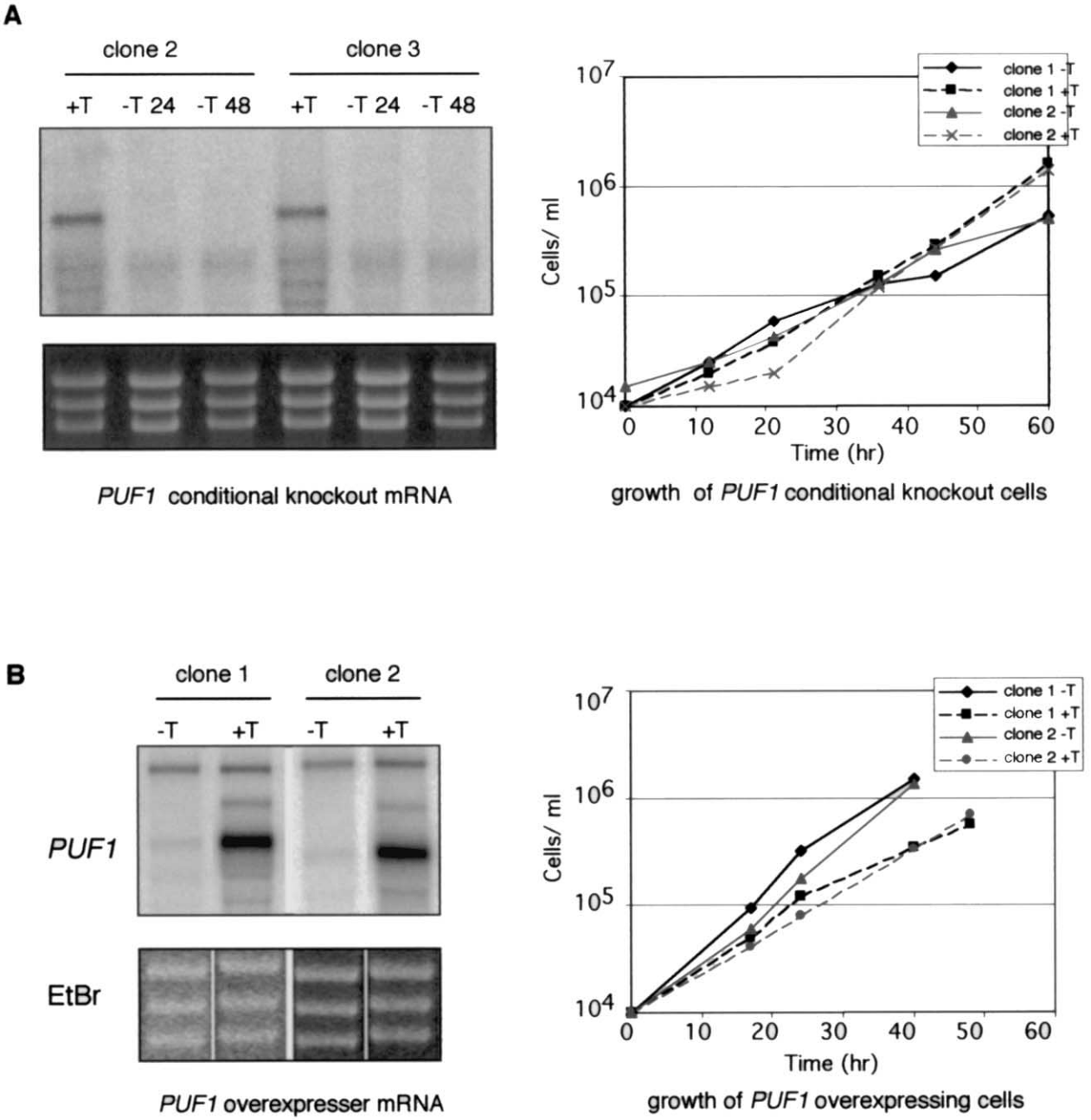

Fig. 5. Perturbation of PUF1 levels leads to an in vitro growth defect. (A) Northern blot analysis and growth curves of PUF1 conditional knockout cell lines showing that PUF1 mRNA levels decrease and growth slows after removal of tetracyline (—). Doubling time increased from 7.5 h ± 0.1 with tetracycline to 11.5 h ± 0.4 in the absence of tetracycline. (B) Northern blot analysis and growth curves of HA-PUF1 overexpressing cell lines indicating that PUF1 mRNA is overexpressed eight to ten fold and that growth slows in the presence of tetracycline (---). Doubling time of the overexpressing cell lines increased from 6.4 h ± 0.6 without tetracycline to 9.3 h ± 1.3 with tetracycline.

or *ESAG6* mRNA. However, a reproducible increase of *ESAG8* mRNA and protein was seen in cells that overexpressed HA-PUF1 (Fig. 7A and B). This effect strengthens the conclusion that ESAG8 and PUF1 interact, and indicates that there may be a positive feedback loop where high levels of PUF1 increase the levels of *ESAG8* mRNA and protein.

We examined the effect of PUF1 over- or under-expression on the stability of this same pool of ES-derived mRNAs by following RNA levels after shutting down transcription with actinomycin-D. There was a small effect of PUF1 perturbation on the half-life of *VSG* mRNA, which increased when PUF1 was over-expressed and decreased in the 'knockout' cell lines (Fig. 7C and D). There was considerable variability in VSG half-life in PUF1 overexpressing cells in different experiments, but the overall effect is reproducible. The decay curve of *VSG* mRNA is normally biphasic, but in

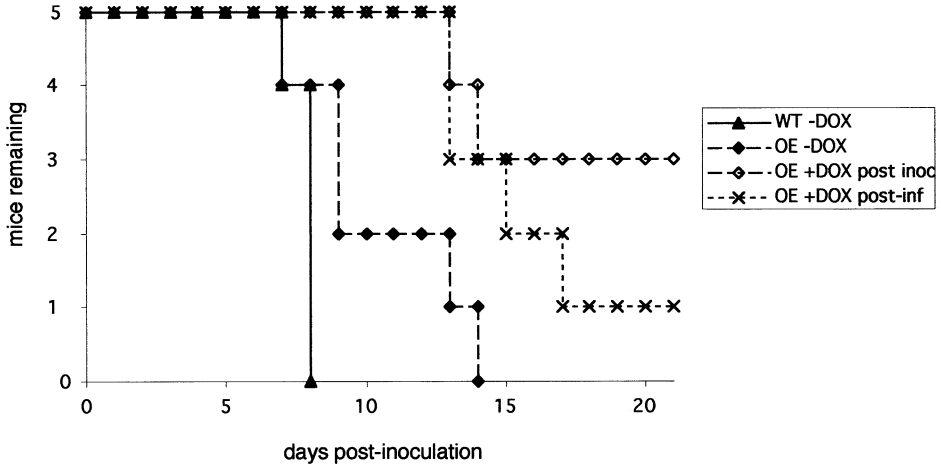

Fig. 6. Survival of mice infected with wild-type or overexpressing (OE) cells in the presence or absence of doxycycline. Wild-type cells killed C57BL/10J mice with a mean survival time (MST) of 8.6 days. Overexpressing cells killed mice more slowly, even without doxycycline (MST = 10.6 days), whereas doxycycline given to mice after infection or after inoculation decreased virulence drastically. Four of ten mice survived indefinitely and death was delayed in the others.

PUF1 knockout cells this character was less pronounced, providing further evidence that PUF1 stabilizes *VSG* mRNA. This effect may be direct or indirect and it is currently unclear whether this might explain the in vivo phenotype of PUF1 overexpression. *ESAG6* mRNA was de-stabilized in either low or high PUF1 levels; this distinct behavior demonstrates that the effect on *VSG* mRNA half-life is a specific effect (Fig. 7C and D). Since the half-life of *VSG* mRNA is so long in comparison to other transcripts, it is difficult to accurately compare to other housekeeping genes. Nevertheless, we saw absolutely no effect on half-life or steady state levels of *TbSIR2* and *TUBULIN* and the 5S rRNA (data not shown), further indicating that the effects on *VSG* are specific.

4. Discussion

The ES locus of *T. brucei* is a model for post-transcriptional regulation because, although the active ES is transcribed at high levels in a polycistronic manner, the individual mRNAs are present in very different amounts. For example, the *VSG* represents approximately 5–10% of total cellular mRNA, whereas *ESAG8*, is sometimes barely detectable by Northern analysis. For this reason, the identification of a putative translational regulator as an ESAG8-interacting protein may be very significant.

We isolated the ESAG8-interacting protein PUF1 by a two-hybrid approach. We think the interaction is genuine because the interaction in the two-hybrid assay was robust and because our immunoprecipitation data demonstrate that the interaction occurs in vivo.

Interestingly, PUF1 contains only 1 weak consensus nuclear localization signal in its C-terminus, and tagged versions of the protein are not nuclear, implying that the interaction with ESAG8 must occur within the cytoplasm. This argues that the cytoplasmic pool of ESAG8 is functional, but does not address the function of the nucleolar pool of the protein. It is possible, for example, that ESAG8 is sequestered within the nucleolus and is released to regulate cytoplasmic targets such as PUF1. The strategy of nucleolar sequestration is used in other eukaryotic systems such as the yeast RENT complex and $p19^{ARF}$ mediated sequestration of MDM2 in human cells [37–39]. To further test the possibility that ESAG8 functions in the cytoplasm, we attempted to generate a non-nucleolar version of GFP–ESAG8, but this was expressed at levels much lower than wild-type ESAG8, thwarting this attempt to confirm the functionality of the cytoplasmic pool by dominant negative means.

TbPUF1 represents the earliest diverged example of a Puf protein yet identified. This class of sequence-specific RNA-binding proteins regulates developmental transitions and germ-cell development in metazoa, but is clearly involved in more global regulation of RNA stability in yeast. The presence of Puf family proteins in very early branching eukaryotes such as *T. brucei* suggests that this class of proteins represents an ancient and important approach to the control of RNA metabolism in eukaryotes. Since *T. brucei* depends largely on post-transcriptional controls to regulate steady state levels of mRNA, further study of TbPUF1 in this organism may illuminate functions of this class of proteins that have been previously unrecognized.

The founding member of the Puf family is the *pumilio* gene originally identified as a maternal-effect gene important in establishing polarity in the *Drosophila* embryo. It acts by binding the 3′ UTR of *hunchback* (*hb*) mRNA, to negatively regulate this transcript [40].

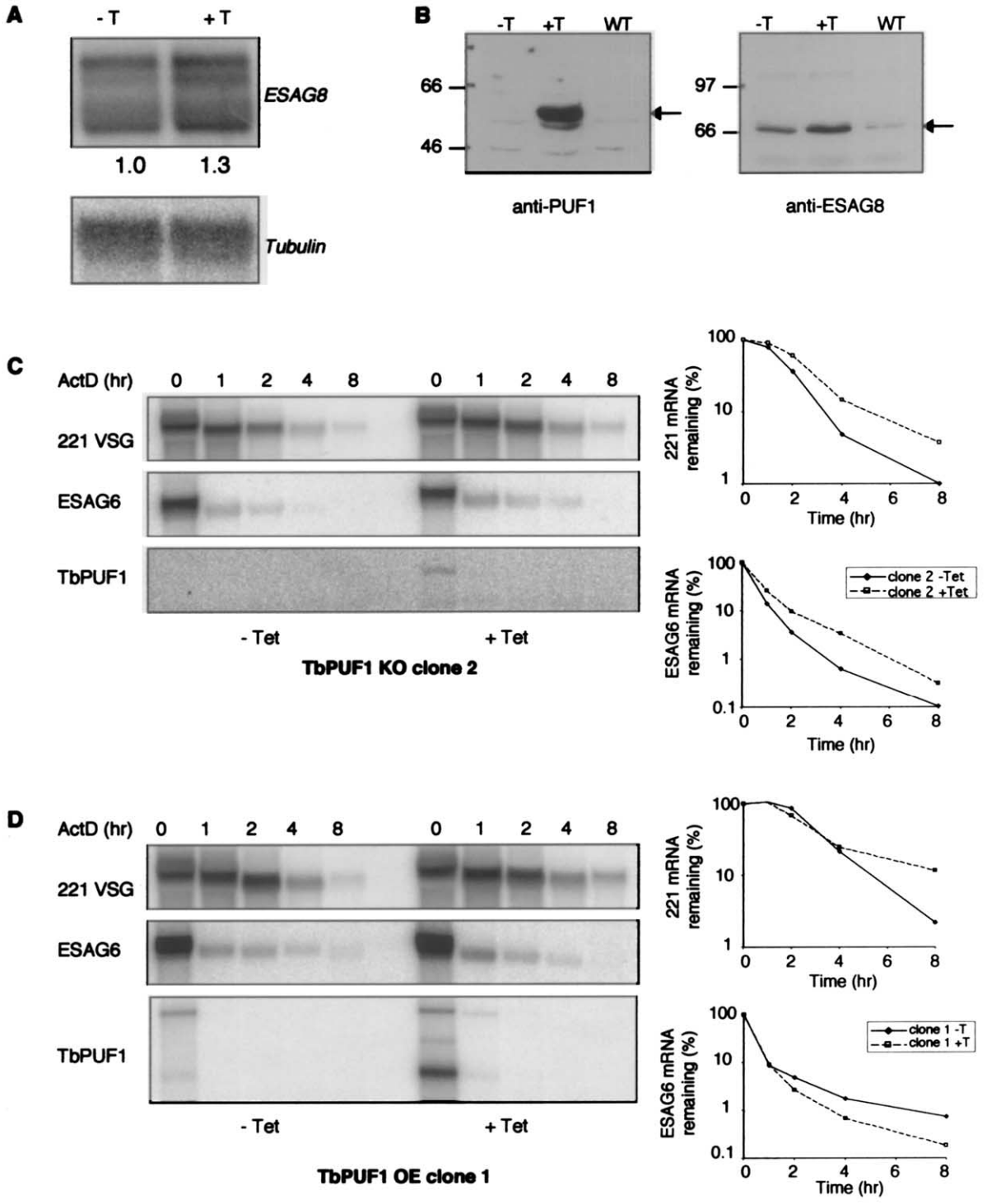

Fig. 7. Perturbation of PUF1 levels affects specific ES-derived mRNAs. (A) Overexpression of PUF1 reproducibly increases steady state levels of *ESAG8* mRNA by at least 1.3-fold. (B) ESAG8 protein levels are elevated in PUF1 overexpressing strains. Western analysis for PUF1 or ESAG8 in PUF1 OE−Tet, +Tet, or wild-type cell lines. Cells (2×10^6) were loaded per lane. (C) Disruption of PUF1 decreases the stability of 221 *VSG* and *ESAG6* mRNA by 20%. (221 *VSG*: +Tet $t_{1/2} = 1.5$ h ± 0.03; −Tet $t_{1/2} = 1.2$ h ± 0.04). RNA was prepared from cells treated with 10 μg ml^{-1} actinomycin-D for the indicated times, northern blotted, and probed with the ESAGs indicated. (D) Overexpression of PUF1 increases the stability of 221 *VSG* but not *ESAG6* mRNA by 35% (221 *VSG*: −Tet $t_{1/2} = 1.4$ h ± 0.05; +Tet $t_{1/2} = 1.9$ h ± 0.4). Half-life calculations are based on an exponential model and are, therefore, approximations because decay of VSG, but not ESAG6, is biphasic.

The Pumilio (Pum) protein binds sequence specifically to a small repeated element in the *hb* mRNA, but is only able to negatively regulate expression in combination with the Nanos protein (Nos) and another protein,

BRAT; hence these elements are called nanos response elements (NRE). While Pum is able to bind the NRE in the absence of Nos, the quaternary complex of RNA, Nos, Brat, and Pum is more stable and mutations that decrease the stability of this complex decrease the efficiency of repression [32,41]. Interestingly, although Pum is a large protein of approximately 150 kDa, the Puf repeat domain is necessary and sufficient to carry out all of the translational regulation functions of the protein and the role of the remaining sequence is still unclear [42]. This argues that the smaller tbPUF1 protein, which consists almost exclusively of Puf repeats, might have similar functions.

The most recently characterized members of the Puf family are from yeast. A comprehensive study by Olivas and Parker [31] used microarray analysis to identify genes that were misregulated in a strain deficient in all five yeast Puf proteins. They identified 168 mRNAs that were misregulated in these strains, clearly demonstrating that Puf proteins play a role in global post-transcriptional regulation. Although, a more detailed analysis of the collection of putative targets for each Puf protein has not been reported, it seems likely that Puf proteins in yeast regulate classes of mRNAs as opposed to one or two specific transcripts. More than 90% of the affected mRNAs were downregulated rather than upregulated, which implies that Puf proteins in yeast generally act negatively, as is true for Pum and FBF. Further analysis of the Puf3 regulated mRNA for *COX17* provided support for the model that Puf proteins act by promoting deadenylation of their target transcripts, thus downregulating expression and translation [31]. At least one function of *Drosophila* Pum is to promote deadenylation of the *Hb* mRNA, suggesting that this may a common mechanism of action of the Puf family [43]. In our hands, PUF1 overexpression tended to stabilize rather than destabilize the mRNAs we examined. This result was unexpected and it will be interesting to examine more carefully how directly PUF1 is involved in regulating the stability of ES-derived mRNAs.

A related study on yeast *PUF5*/*MPT5* implicates this gene in the post-transcriptional regulation of the HO endonuclease mRNA. MPT5 binds the 3′ UTR of the *HO* gene, to a sequence that contains a UUGU box (at position +24 from stop) that is also essential for the binding of Pum (+107 and +117) and FBF (+126) to their respective targets [44]. Thus, UUGU may represent a conserved RNA motif to which Puf proteins bind. While few 3′ UTRs have been accurately mapped in *T. brucei*, it is interesting to note that one copy of this sequence exists in 3′ coding sequence of VSG 221 (at position −43) and several copies exist in the 221 *ESAG8b* 3′ region (+161, +174, +238, and +246) (although the polyadenylation site of *ESAG8* is not known), both of which are affected by perturbation of PUF1 levels. Many but not all *VSGs* contain a 3′ UUGU sequence, but all contain the sequence UUGCU and the invariant 14-mer UGAUAUAUUUUAAC, whose function is unknown.

Gel filtration analysis of wild-type ESAG8 indicates that the bulk of the protein is present in high molecular weight fractions, implying the existence of one or more ESAG8-containing complexes. One fraction co-elutes with HA-tagged PUF1, in HA-PUF1-overexpressing strains, suggesting that only a fraction of ESAG8 and PUF1 may interact. ESAG8 and PUF1 together account for only 120 kDa, so perhaps other components are present in this complex, which eluted at 300 kDa. We could not detect any homodimerization of ESAG8 in a two-hybrid assay, arguing against multiple copies of ESAG8 being present in this complex. It is interesting to consider either that this complex contains constituents of the ubiquitylation machinery or that it contains RNA, but we have not directly tested either possibility. We have not seen any effect of the proteosome inhibitor lactacystin on HA-PUF1 levels, as would be expected if the PUF1 were destabilized by ESAG8.

Perturbing the levels of PUF1 has a reproducible impact on the levels and stability of several ES-associated mRNAs. These types of effects are consistent with a role of PUF1 in the regulation of certain bloodstream-form RNAs, although we stress that the ES-associated sequences that we tested need not be the primary targets of this protein. Alternatively, the effect on *VSG* and *ESAG* mRNAs that we have noted may be a secondary effect, and PUF1 may regulate other RNAs important in establishing persistent infection in the bloodstream. Puf repeats also play a role in mediating protein–protein interactions, and it is important to remember that the RNA-binding functionality of Puf repeats may not have evolved until after trypanosomes diverged from the more studied eukaryotic lineage. Indeed, recent structural evidence indicates that the Puf fold is similar to peptide binding folds in the helical repeat family such as the ARM and HEAT repeats, which themselves are similar to other peptide binding folds such as the LRR repeats important in the ESAG8–PUF1 interaction [45,46]. It will thus be interesting to determine whether PUF1 binds to RNA and whether PUF1 has other interacting partners besides ESAG8. The conserved basic resides towards the C-terminus of the Puf repeat are important in mediating RNA binding in Pum. These residues are also conserved in TbPUF1, suggesting that the RNA-binding activity will be conserved.

PUF1 overexpression results in a drastic reduction in infectivity. This effect is specific and not due merely to a growth defect in these strains, since growth rates of the overexpressing strains appeared largely unchanged in vivo when compared with wild-type cells. Moreover, the PUF1 knockout cells, which have a similar in vitro growth defect to the overexpressers, kill mice with

normal kinetics. It is unclear whether this in vivo phenotype is directly related to PUF1 function and to its interaction with ESAG8. Since PUF1 is overexpressed, it is difficult to demonstrate that this phenotype is specific and not pleiotropic. It will be important to overexpress mutants of PUF1 that do not interact with ESAG8 to determine whether the virulence phenotype correlates with ESAG8 interaction, but such experiments are beyond the scope of the current work. Since cells lacking ESAG8 remain virulent ([21] and data not shown), this interaction may not be directly linked to parasite virulence, and the virulence defect of the PUF1 overexpressers may be through an ESAG8-independent pathway. Nevertheless, PUF1 is a putative regulatory gene whose misregulation results in an in vivo and an in vitro phenotype and determining the reason for this effect will enhance our understanding of growth of the parasite in the mammalian bloodstream and may help define new therapeutic targets. As a first step, we would like to identify misregulated mRNAs by microarray analysis in the overexpressing strain, an approach that has been successful in yeast [31], once *T. brucei* microarrays can be generated. This type of analysis will also be essential in determining more convincingly whether PUF1 has a novel RNA-stabilizing activity.

Acknowledgements

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