ELSEVIER

Molecular & Biochemical Parasitology 117 (2001) 211–215

MOLECULAR & BIOCHEMICAL PARASITOLOGY

www.parasitology-online.com.

Short communication

Expression-site-associated-gene-8 (*ESAG8*) is not required for regulation of the *VSG* expression site in *Trypanosoma brucei*

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

Laboratory of Molecular Parasitology, The Rockefeller University, 1230 York Avenue, New York, NY 10021, USA

Received 30 May 2001; accepted in revised form 13 July 2001

Keywords: Trypanosoma brucei; ESAG8; Antigenic variation; Conditional expression; Expression site; Genetics

The expression sites (ES) of *Trypanosoma brucei* are multi-allelic loci from which the Variant Surface Glycoprotein genes (*VSG*) are expressed. Tight control of the ES transcriptional state ensures that only a single *VSG* is expressed at a time [1]. Although transcription initiates from multiple ES promoters, only a single ES is completely transcribed [2]. No reproducible differences in DNA sequence [3–5], in chromatin structure [6,7], or in DNA modification of repetitive flanking sequences [8,9] have been detected between the active and silent ESs. Since it is impossible to select for the complete simultaneous transcription of two ESs [10], and since deletion of the active ES promoter precipitates a switch to a previously inactive ES [7], some form of cross-talk is probably responsible for the exclusive transcriptional elongation of the active ES. Current models of ES control postulate the existence of a specific nuclear compartment from which only a single ES can be transcribed, or the presence of a limiting factor that is essential for the productive transcription of the active ES.

Co-transcribed from the single ES promoter are multiple expression-site-associated-genes (*ESAGs*), which provide the parasite with proteins important for growth in the bloodstream (such as the transferrin receptor encoded by *ESAGs 6* and *7*) and proteins that may function in ES regulation. We have investigated the role of the putative regulatory protein ESAG8 [11,12]. Since different variants of ESAG8 are very highly conserved and appear to be exclusively transcribed from ESs, and because ESAG8 is present exclusively in the bloodstream form at low levels (2000–4000 molecules per cell), we hypothesized that ESAG8 might be involved in ES regulation [13]. ESAG8 consists of an N-terminal RING Zn^{++}-finger domain and a C-terminal Leucine Rich Repeat (LRR) domain. The RING Zn^{++}-finger motif can mediate the polyubiquitylation and targeted degradation of proteins in several eukaryotic systems [14] and LRR domains are involved in a variety of protein–protein interactions [15]. ESAG8 elutes from a Sephacryl-300 gel filtration column with an apparent molecular weight of approximately 300 kDa, consistent with its presence in a multi-protein complex (Hoek and Cross, submitted for publication). Our recent finding that a small portion of ESAG8 was necessary and sufficient to target the protein to the nucleolus lent further support to the idea that ESAG8 might be involved in ES control, because the ES is transcribed by RNA polymerase I [13,16–18]. To test this hypothesis, we attempted to delete *ESAG8* from the actively transcribed ES.

In previous experiments, we were unable to target the active ES-linked copy using the highly conserved *ESAG8* coding sequence. Targeting was only successful in active ESs containing multiple copies of *ESAG8*, implying that the gene was essential [13]. We, therefore, designed a conditional disruption strategy to disrupt

Abbreviations: ES, expression site; *ESAG*, expression-site-associated gene; VSG, variant surface glycoprotein.

* Corresponding author. Tel.: +1-212-3277-571; fax: +1-212-3277-845.

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

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

PII: S0166-6851(01)00341-3

ESAG8 in the presence of an inducible ectopic copy of the gene. The ectopic copy of *ESAG8* was cloned from the 221 line by RT-PCR and conditionally expressed from a tetracycline controlled T7-promoter in the 'single marker' *T. brucei* cell line expressing T7-polymerase and the Tet-repressor [13,19,20]. In our first attempts, we again used the *ESAG8* coding sequence to target the deletions, but found that these cassettes invariably integrated into the ectopic copy instead of the ES-linked copy. It, therefore, became necessary to obtain high-quality sequence information for the 221 ES in order to obtain unique flanking regions we could use to disrupt the ES-linked copies of *ESAG8*. The 221 ES turns out to contain three copies of ESAG8 interspersed with *ESAG4* and *ESAG3* genes (S. Bowman, N. Hall and M. Berriman, personal communication), in a region that had been predicted to be triplicated when the 221 ES was originally mapped, before these genes were specifically identified [21] (Fig. 1A). 221-*ESAG8b* is almost identical to the *ESAG8* we cloned by RT-PCR and 221-*ESAG8c* contains an extra LRR, explaining the complex RNA and protein profile seen in the 221 clone. We identified flanking sequences unique to the upstream copy of *ESAG8*, as well as the downstream

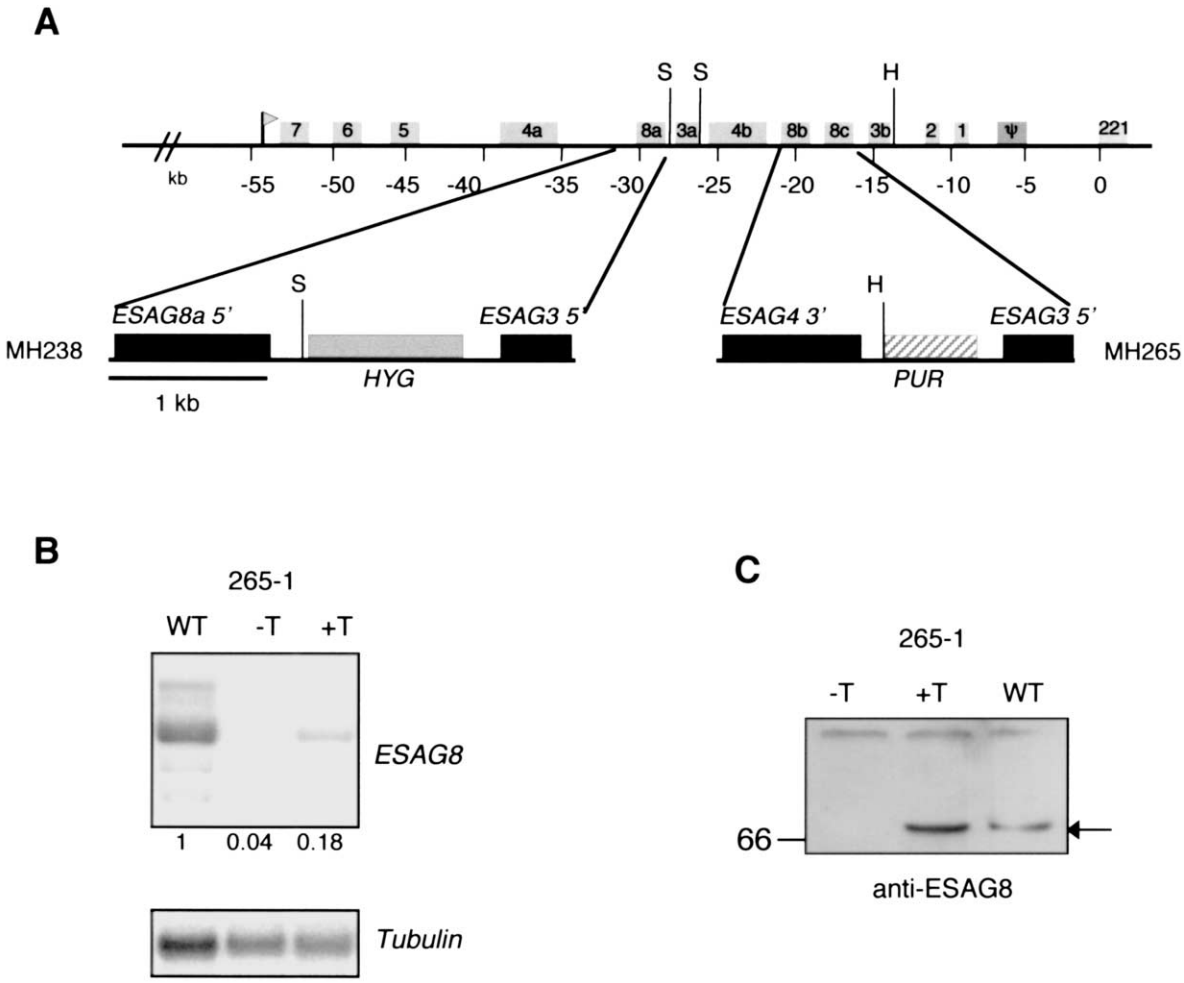

Fig. 1. Disruption of all copies of *ESAG8* in the 221 ES. (A) The disruption of *ESAG8* was done in the 'single -marker' cell line [20] expressing T7 RNA polymerase and the Tet-repressor plus an ectopic copy of *ESAG8* under the control of a regulatable T7 promoter (pLew111). The preliminary sequence of the 221 ES was obtained from BAC clone 25N7 from the RPCI-97 library (BACPAC Resource Center, Children's Hospital Oakland Research Institute), which covered almost the entire 221 ES. This BAC was isolated by G. Rudenko and sequenced by the Sanger Center (S. Bowman, N. Hall, and M. Berriman.) *ESAG8*-flanking regions were PCR-amplified from genomic or BAC DNA and sequenced. MH238 was used to target *ESAG8a*, selecting with 5 μg ml^{-1} hygromycin and MH265 was used to target the tandem duplication of *ESAG8b* and *c* in a second step using 100 ng ml^{-1} puromycin. All selection was done in the presence of 500 ng ml^{-1} tetracycline to maintain expression of the ectopic copy of *ESAG8*. (B) Northern analysis of RNA extracted from *ESAG8* knockout cell lines grown in the presence or absence of tetracycline for 3 days. RNA was isolated using RNA-STAT (Tel-test 'B') and 3 μg were loaded per lane on a 1% formaldehyde, 1.5% agarose gel. *ESAG8* levels were quantified by phosphorimaging and normalized to *TUBULIN*. The amount of *ESAG8* relative to wild-type is indicated below each lane. (C) Western analysis of ESAG8 protein levels of clone 1, grown in the presence or absence of tetracycline for 3 days. The arrow points to the ESAG8 protein in $-$Tet, $+$Tet and WT lanes. About 2×10^6 cells were loaded per lane, blotted to nitrocellulose and probed with anti-ESAG8 rat1 at 1:1000, followed by HRP-conjugated goat anti-rat (Roche) at 1:15 000.

tandem duplication of the gene, and sequentially targeted these two regions with the *HYG* and *PUR* selectable markers, respectively, in the presence of tetracycline to maintain transcription of the ectopic *ESAG8* (Fig. 1A).

Southern analysis of the regions 3′ of the integration site confirmed that the targeting occurred correctly in the hygromycin and puromycin resistant clone 265-1 (data not shown). When we removed tetracycline from the medium and examined ESAG8 levels, one clone

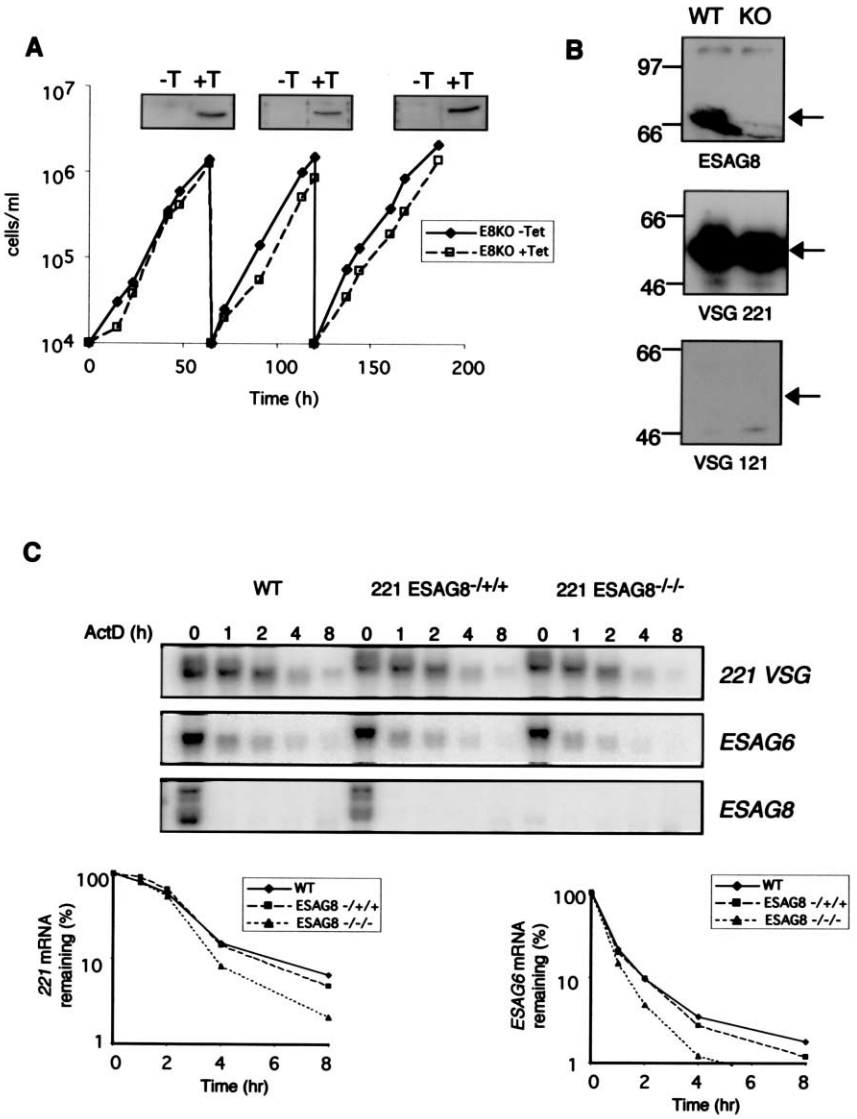

Fig. 2. Phenotypic analysis of *ESAG8* knockouts. (A) Cells grown with or without 500 μg ml^{-1} tetracycline grow equally well. Cell in the presence of tetracycline were washed twice in warm medium and inoculated at 1×10^4 cells ml^{-1}. Growth was monitored over the next week on a daily basis by counting in a hemocytometer. When cells reached $>1 \times 10^6$ ml^{-1}, protein samples were collected for western analysis as for Fig. 1 to show that the ESAG8 deletion was stable. ESAG8 blots from each peak are shown above the graph. (B) ESAG8 knockout cells express similar levels of VSG 221, and no detectable VSG 121. Arrows point to the protein band in each lane, and to the expected location of VSG 121. Western blots were processed as above for ESAG8. For VSG 221 blots, 2×10^5 cells were loaded per lane and the blot was probed with rabbit anti-221 at 1:10 000, followed by incubation with anti-rabbit (Amersham-Pharmacia) at 1:5000. VSG 121 blots were processed similarly to VSG 221 blots, except that 2×10^6 cells were loaded per lane. (C) *VSG* and *ESAG8* mRNAs are destabilized in the *ESAG8* knockout cells. Actinomycin-D was added to 150 ml of culture at 1.3×10^6 ml^{-1} at a final concentration of 10 μg ml^{-1}. Then, 30 ml of culture were isolated at the indicated times and processed for RNA as above. Cell lines used were either parental cells (WT), MH238 *221-ESAG8* single knockouts ($-/+/+$) or the MH238/MH265 *221-ESAG8* triple knockout ($-/-/-$). About 2 μg of RNA were loaded per lane and Northern blots were probed with *VSG 221*, *ESAG6* or *ESAG8* probes. Blots were quantified by phosphorimaging and log plots of the *VSG 221* and *ESAG6* mRNA decay were generated. *VSG 221* decayed with a half-life of 1.9, 1.7 and 1.3 h for the WT, $-/+/+$, and $-/-/-$ cell lines, respectively. *ESAG6* decayed with a half-life of 1.5, 1.4, and 1.2 h for the same three cell lines.

showed a significant reduction in both RNA and protein (Fig. 1B and C). Only 4% of wild-type levels of RNA and no protein were detectable in the absence of tetracycline, whereas normal levels of ESAG8 protein were seen when induced, indicating that the disruption was successful in this clone.

Clone 265-1 exhibited a slightly reduced in vitro growth rate in both the presence or absence of tetracycline, when compared with wild-type cells, but appeared otherwise unaffected by the lack of *ESAG8* (Fig. 2A). There was no reduction in VSG 221 protein levels and no increase in the levels of VSG 121, which is located in a silent ES in this background (Fig. 2B). Furthermore, *ESAG8* levels were clearly downregulated in the absence of tetracycline, implying that other ESs were still efficiently silenced. Cells grew normally for at least a week in the absence of ESAG8, and we observed no gross changes in switching frequency by immunofluorescence monitoring of the VSG profile in this population (data not shown). Thus, ESAG8 must be dispensable for tight ES regulation.

Our disruption data contradicts our previous conclusions that *ESAG8* is essential. Although *ESAG8* is almost universally ES-linked, sometimes in multiple copies, one functional ES has been observed that contains only *ESAGs 5*, *6* and *7* [22]. Nevertheless, we cannot rule out that *ESAG8* is essential and that the small amount of *ESAG8* RNA that we detect in the disrupted cell line is sufficient to allow the cells to survive. This RNA could emanate from the slight intrinsic leakiness of the ectopic expression system or might be generated at a low level from other ESs. The same might be true for the single example of an ESAG8-less ES [22]. The available antibody was not sensitive enough to detect the much smaller amount of protein that might be present in these cells. It is difficult to accept that a gene so uniquely linked to the ES would not play a role in natural infections.

We have recently identified an ESAG8-LRR interacting protein, TbPUF1, that falls into a well conserved class of RNA-binding proteins that regulate RNA stability and translation (Hoek and Cross, submitted for publication). *PUF1* is an essential gene and *PUF1* overexpressing cells are avirulent. PUF1 also appears to directly or indirectly affect the stability and steady state levels of several ES-derived mRNAs. The presence of a RING Zn^{++}-finger domain in ESAG8 suggested that the protein might negatively regulate interacting proteins. If this were true, then an *ESAG8* knockout might have similar phenotypes to a *PUF1* overexpressing strain, because a lack of ESAG8 might lead to an increase of PUF1 protein. However, we detected no significant stabilization of VSG mRNA in the *ESAG8* disrupted cells (Fig. 2C). Instead, *VSG 221* mRNA seemed to be destabilized in an ESAG8 dose-dependent manner, a result more consistent with the PUF1 knockout phenotype. We also noted only a slight and probably insignificant elevation of the rate at which these cells underwent a relapse in mice (data not shown). Moreover, we have not been able to detect any stabilization of PUF1 protein when cells were treated with the proteosome inhibitor lactacystin, nor have we been able to see any elevation in PUF1 levels in the ESAG8 knockout (data not shown). Since ESAG8 does not recapitulate the phenotype of a *PUF1* overexpressing cell line but displays characteristics of the *PUF1* knockout, we favor a model in which ESAG8 forms a tertiary complex with PUF1 and unidentified trypanosome mRNAs. The role of the RING Zn^{++}-finger domain of ESAG8 in this complex remains unclear, as does the predominantly nucleolar localization of ESAG8. Clearly, the identification of mRNAs that interact with this complex and a more sophisticated phenotypic analysis of the *ESAG8* and *PUF1* knockouts will be necessary to answer these questions.

Acknowledgements

This work was supported by the National Institutes of Health by grants AI21729 (George A.M. Cross) and training grant GM07982 (Maarten Hoek). We would like to thank all members of the Cross lab, as well as Miguel Navarro, for helpful discussions and Christine Clayton for suggesting the use of *ESAG8* flanking sequences in the knockout.

References

[1] Cross GAM. Antigenic variation in trypanosomes: secrets surface slowly. Bioessays 1996;18:283–91.
[2] Vanhamme L, Poelvoorde P, Pays A, Tebabi P, Van Xong H, Pays E. Differential RNA elongation controls the variant surface glycoprotein gene expression sites of *Trypanosoma brucei*. Mol Microbiol 2000;36:328–40.
[3] Zomerdijk JCBM, Ouellete M, ten Asbroek ALMA, Kieft R, Bommer AMM, Clayton CE, Borst P. The promoter for a variant surface glycoprotein gene expression site in *Trypanosoma brucei*. EMBO J 1990;9:2791–801.
[4] Navarro M, Cross GAM. DNA rearrangements associated with multiple consecutive directed antigenic switches in *Trypanosoma brucei*. Mol Cell Biol 1996;16:3615–25.
[5] Horn D, Cross GAM. Analysis of *Trypanosoma brucei vsg* expression site switching in vitro. Mol Biochem Parasitol 1997;84:189–201.
[6] Navarro M, Cross GAM. In situ analysis of a variant surface glycoprotein expression-site promoter region in *Trypanosoma brucei*. Mol Biochem Parasitol 1998;94:53–66.
[7] Navarro M, Cross GAM, Wirtz E. *Trypanosoma brucei* variant surface glycoprotein regulation involves coupled activation/inactivation and chromatin remodeling of expression sites. EMBO J 1999;18:2265–72.
[8] Gommers-Ampt JH, van Leeuwen F, de Beer ALJ, Vliegenthart JFG, Dizdaroglu M, Kowalak JA, Crain PF, Borst P. β-D-glucosyl-hydroxymethyluracil: a novel modified base present in the DNA of the parasitic protozoan *T. brucei*. Cell 1993;75:1129–36.

[9] van Leeuwen F, Wijsman ER, Kieft R, van der Marel GA, van Boom JH, Borst P. Localization of the modified base J in telomeric VSG gene expression sites of *Trypanosoma brucei*. Genes Dev 1997;11:3232–41.
[10] Chaves I, Rudenko G, Dirks-Mulder A, Cross M, Borst P. Control of variant surface glycoprotein gene-expression sites in *Trypanosoma brucei*. EMBO J 1999;18:4846–55.
[11] Revelard P, Lips S, Pays E. A gene from the VSG expression site of *Trypanosoma brucei* encodes a protein with both leucine-rich repeats and a putative zinc finger. Nucl Acids Res 1990;18:7299–303.
[12] Ross DT, Raibaud A, Florent IC, Sather S, Gross MK, Storm DR, Eisen H. The trypanosome VSG expression site encodes adenylate cyclase and a leucine-rich putative regulatory gene. EMBO J 1991;10:2047–53.
[13] Hoek M, Engstler M, Cross GA. Expression-site-associated gene 8 (ESAG8) of *Trypanosoma brucei* is apparently essential and accumulates in the nucleolus. J Cell Sci 2000;113:3959–68.
[14] Borden KL. RING domains: master builders of molecular scaffolds? J Mol Biol 2000;295:1103–12.
[15] Kobe B, Deisenhofer J. The leucine-rich repeat: a versatile binding motif. Trends Biochem Sci 1994;19:415–21.
[16] Laufer G, Schaaf G, Bollgonn S, Gunzl A. In vitro analysis of alpha-amanitin-resistant transcription from the rRNA, procyclic acidic repetitive protein, and variant surface glycoprotein gene promoters in *Trypanosoma brucei*. Mol Cell Biol 1999;19:5466–73.
[17] Laufer G, Gunzl A. In-vitro competition analysis of procyclin gene and variant surface glycoprotein gene expression site transcription in *Trypanosoma brucei*. Mol Biochem Parasitol 2001;113:55–65.
[18] Rudenko G, Lee MG-S, van der Ploeg LHT. The PARP and VSG genes of *Trypanosoma brucei* do not resemble RNA polymerase II transcription units in sensitivity to Sarkosyl in nuclear run-on assays. Nucl Acids Res 1992;20:303–6.
[19] Wirtz E, Hoek M, Cross GA. Regulated processive transcription of chromatin by T7 RNA polymerase in *Trypanosoma brucei*. Nucl Acids Res 1998;26:4626–34.
[20] Wirtz E, Leal S, Ochatt C, Cross GAM. A tightly regulated inducible expression system for dominant negative approaches in *Trypanosoma brucei*. Mol Biochem Parasitol 1999;99:89–101.
[21] Kooter JM, van der Spek HJ, Wagter R, d'Oliveira CE, van der Hoeven F, Johnson PJ, Borst P. The anatomy and transcription of a telomeric expression site for variant-specific surface antigens in *T. brucei*. Cell 1987;51:261–72.
[22] van Xong H, Vanhamme L, Chamekh M, Chimfwembe CE, van den Abbeele J, Pays A, van Meirvenne N, Hamers R, de Baetselier P, Pays E. A VSG expression-site-associated gene confers resistance to human serum in *Trypanosoma rhodesiense*. Cell 1998;95:839–46.