Journal of Cell Science 113, 3959-3968 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
JCS1796

Expression-site-associated gene 8 (*ESAG8*) of *Trypanosoma brucei* is apparently essential and accumulates in the nucleolus

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Accepted 19 September; published on WWW 31 October 2000

SUMMARY

***Trypanosoma brucei* variant surface glycoprotein expression sites are interesting examples of genomic loci under complex epigenetic control. In the infectious bloodstream stage, only one of about 20 expression sites is actively transcribed. In the Tsetse midgut (procyclic) stage, chromatin remodeling silences all expression sites. We have begun to explore the function of one of the expression-site-associated genes, *ESAG8*. Gene knockout experiments implied that ESAG8 is essential. ESAG8 is present at a very low level and apparently accumulates in the nucleolus. A 32-amino-acid domain, which contains a putative bipartite nuclear localization signal (NLS), is both necessary and sufficient to target fusions of ESAG8, with *Aequorea victoria* green fluorescent protein, to the trypanosome nucleolus. This same sequence functioned only as an NLS in mammalian cells, supporting the idea that nucleolar accumulation requires specific interactions. These results have implications for models of ESAG8 function.**

Key words: ESAG8, Nucleolus, GFP localization, *Trypanosoma brucei*

INTRODUCTION

The variant surface glycoprotein gene (*VSG)* expression sites (ESs) of *Trypanosoma brucei* represent a potentially instructive example of genomic loci under the control of two distinct epigenetic mechanisms. In the infectious bloodstream form of the parasite, an unknown system of allelic exclusion ensures that only one among the approximately 20 potentially active loci is productively transcribed (reviewed in Borst et al., 1998; Cross et al., 1998; Rudenko et al., 1998; Vanhamme and Pays, 1998; Borst and Chaves, 1999). The periodic inactivation of one ES and simultaneous activation of another is one of the two main mechanisms by which a trypanosome can switch its VSG (reviewed in Cross, 1996). When the parasite differentiates to the insect midgut (procyclic) form, no VSG is expressed (Horn and Cross, 1995). The regulatory mechanisms in bloodstream and procyclic forms are thought to be functionally distinct. No significant chromatin differences have been detected among the bloodstream ESs, but bacteriophage T7 RNA polymerase promoters inserted into ESs, and only in ESs, are rendered inoperable in procyclic forms (Navarro and Cross, 1998; Navarro et al., 1999), indicating a major change in ES chromatin structure after differentiation from bloodstream to tsetse forms.

Each ES is a structurally and functionally haploid polycistronic telomeric locus, probably transcribed by RNA polymerase I from a promoter approximately 50 kbp upstream of the sub-telomeric *VSG*. To date, no specific factors regulating either epigenetic mechanism have been identified. A nucleotide modification, resulting in the substitution of about 15% of T by J, β-D-glucosyl-hydroxymethyluracil, is found primarily in the repetitive telomeric and upstream 50-bp repeat sequences that flank all ESs in the bloodstream stage (Gommers-Ampt et al., 1993; van Leeuwen et al., 1997). J is present in ES-internal repetitive sequences in silent but not in active ESs (van Leeuwen et al., 1997) and is absent from the tsetse stages (van Leeuwen et al., 1998). Despite these studies, and the cloning of a gene encoding a J-binding protein (Cross et al., 1999), there is no direct evidence that J is involved in ES regulation. The ES promoter has been well characterized (Vanhamme et al., 1995; Pham et al., 1996; Qi et al., 1996) and promoter binding factors have been demonstrated but not characterized, so their role remains unclear (Pham et al., 1997; Berberof et al., 2000). Finally, *cis* elements that play a specific role in ES transcription stability or switching have not been identified, although deletion of the promoter results in a rapid switch in ES transcription (Navarro et al., 1999) and deletion of the region immediately upstream of the *VSG* can destabilize the currently active ES (Davies et al., 1997). In sum, there are few clues to the mechanisms that regulate ES transcription.

To enhance our understanding of ES regulation, we have begun to focus on some of the genes that are cotranscribed from the ES promoter. There are about ten Expression-Site-Associated Genes (*ESAG*), which are mainly predicted to encode surface proteins (Paindavoine et al., 1992). We were prompted to investigate *ESAG8* for several reasons. The

coordinated inactivation and activation of ESs (Chaves et al., 1999; Horn and Cross, 1997; Navarro and Cross, 1998) suggests that a product of ES transcription might play an autoregulatory role. ESAG8 appears to be transcribed exclusively from the active ES and there appears to be minimal sequence variation among *ESAG8* genes, in contrast to other *ESAG* families. The predicted structure of ESAG8 consists of an amino-terminal RING Zn^{2+} -finger domain and a large Leucine-Rich-Repeat (LRR) domain, separated by a short region that harbors a putative nuclear localization signal (NLS) (Fig. 1A) (Revelard et al., 1990). In other proteins, LRR and RING Zn^{2+}-finger domains can mediate protein-protein interactions (Kobe and Deisenhofer, 1994; Borden, 2000), suggesting that ESAG8 could potentially encode an adapter protein. To clarify the function of *ESAG8*, we initiated a series of genetic experiments. Initial results imply that *ESAG8* is essential. We have also examined the cellular distribution of ESAG8 and found that a small region of the linker domain was both necessary and sufficient to target GFP to the nucleolus of *T. brucei*.

MATERIALS AND METHODS

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 (Hirumi and Hirumi, 1989). All transfections were performed as described previously (Navarro and Cross, 1998). For stable regulated expression of ectopic *ESAG8* and *GFP* constructs, a T7 promoter-driven cassette was integrated into a rDNA spacer in trypanosome cell line 13-90, expressing T7 RNA polymerase and the Tet repressor (TetR) (Wirtz et al., 1998). Expression was driven by the addition of 2.5 ng/ml doxycycline or tetracycline to the medium as required.

Plasmid construction

ESAG8 was cloned from *T. brucei* MITat1.2 clone 221 by RT-PCR, using a primer specific for the 5′ and 3′ ends of the coding sequence from another strain (Revelard et al., 1990). The PCR product was cloned into pBluescript II SK+ to generate plasmid MH103, which was sequenced using a nested deletion strategy (New England Biolabs, Inc). Portions of MH103 were re-amplified, using specific primers, to generate MH126, which contained *ESAG8* on a *Hin*dIII fragment, with a 3′ stop codon, to facilitate subsequent cloning steps. MH156 is a plasmid containing PCR-amplified eGFP (Haas et al., 1996) inserted into pBluescript. pLew111 is a derivative of the TetR-regulated T7 promoter-driven expression plasmid, pLew82, that has a *Hin*dIII-*Bam*HI linker replacing the *Luciferase* gene (Wirtz et al., 1998). To generate the *GFP-ESAG8* full-length expression plasmid, *ESAG8* was liberated from MH126 with *Hin*dIII, filled in with Klenow and inserted into *Sma*I-digested MH156, to generate an in-frame fusion where *GFP* and *ESAG8* were separated by 9 bp encoding the tripeptide PSL. The *GFP-ESAG8* fusion was then transferred into *Hin*dIII-digested pLew111, to generate MH161. All other expression plasmids were constructed by similar means or by PCR amplification from the full-length fusion protein. Two of the truncations were transferred to pCDNA-3 as *Hin*dIII-*Bam*HI fragments, to test localization in mammalian cells. All expression constructs were sequenced to confirm integrity.

Immunofluorescence and microscopy

For immunofluorescence analysis, $1\text{-}2\times10^7$ washed cells induced with 2.5 ng/ml of doxycycline were fixed in suspension in 4% paraformaldehyde/0.05% glutaraldehyde for 1 hour, washed twice in 5 ml of phosphate-buffered saline (PBS) and resuspended in 400 µl of 1% bovine serum albumin (BSA). Cells were spun onto glass slides in a tabletop centrifuge for 1-2 minutes at 400 ***g***. For visualization of nucleolar antigens, cells were permeabilized with 0.1% Triton and washed for 5 minutes in PBS. The samples were blocked with 1% BSA for 5 minutes, followed by incubation with an antinucleolar antibody (Ersfeld and Gull, 1997) (a kind gift of K. Ersfeld and K. Gull) for 1 hour at a 1:1000 dilution. After washing three times for 5 minutes, cells were blocked again in 1% BSA and incubated with rhodamine-conjugated goat anti-mouse (Roche) at a 1:300 dilution. Cells were mounted in Fluoromount PVA-antifade mounting solution (Fisher). Confocal microscopy was performed on a Zeiss LSM510 system at the Rockefeller University Imaging Center. GFP was visualized using the 488 nm line and rhodamine was visualized in a separate scan using the 568 nm line of the Krypton-Argon laser. Images were directly imported into the Zeiss LSM510 software and exported as TIFF files for assembly in Adobe Photoshop.

Image acquisition for quantitative deconvolution microscopy was done with a motorized Zeiss Axiophot2 widefield microscope equipped with a Zeiss 63×/1.40 Oil DIC objective and a Princeton Instruments Micromax cooled (–15°C) slow scan CCD camera (Kodak KAF-1400 CCD chip). For acquisition of 3D-image stacks, a PIFOC objective Z-stepper was driven by the Piezo-Amplifier E662 LVPZT (Physik Instrumente, Germany). Living trypansomes were transiently immobilized on an object cooling stage. The microscopic set-up was driven by the IPLab vs.3.2 software (Scanalytics, Fairfax). For measuring the quantitative distribution of fluorescence, 126 living cells were chosen randomly. Following 3D image acquisition (10×200 nm *z*-distance), the raw data were exported to the Huygens vs.2.8 software (SVI, Hilversum) and digital deconvolution was performed using the Tikhonow-Miller algorithm. Segmentation analysis of the deconvolved data set was done with IPlab vs.3.2. Three segments were used in the calculation. Intensity segment 1 was the image area with more than 10 counts per pixel defining the region of interest used for calculation of the total fluorescence value. Segment 2 was the result of intensity/texture segmentation within the region of interest, yielding a subnuclear signal. Segment 3 was defined by texture segmentation describing a nuclear fluorescence signal. The summed intensity values from the three segments were used for calculation of fluorescence distribution in individual cells. The percentage signal in the nucleus was calculated as (Seg2+Seg3/Seg1)×100. The subnuclear signal was described by (Seg3/Seg1)×100.

Mammalian cell culture and transfection

Human HEK-293T cells were cultured in DMEM, high glucose, with 10% FCS. Transfections were performed using FuGene6 transfection reagent (Roche), exactly according to the manufacturer's protocol, using a reagent to DNA ratio of 3:1. For fluorescence analysis cells were grown on coverslips for 24 hours prior to transfection.

Antibody generation and western blot analysis

Antibodies were generated to the N terminus of ESAG8 (amino acids 1-114) by inoculating rats with a His_6-fusion protein purified from bacterial inclusion bodies. Rats were immunized with macerated gel slices containing the protein and boosted on a 21-day schedule. The primary inoculation was intradermal with Freund's Complete Adjuvant and 250 µg of protein, followed by four subcutaneous inoculations with Freund's incomplete adjuvant and 125 µg of protein. Specificity was confirmed by comparing pre-immune and immune sera on western blots against wild-type cells and cell lines expressing truncated forms of ESAG8, as well as against procyclic cell extracts that express no ESAG8. Background was too high for use in immunofluorescence and attempts at purification of the specific activity were only partially successful, resulting in significant loss of avidity and/or specificity.

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. For quantification, dilutions of full-length ESAG-8 His_6-fusion protein, purified from bacterial inclusion bodies, were prepared in 8 M urea/1× DTT sample buffer. The concentration of the initial solution of ESAG-8 His_6 was estimated at 100 ng/μl by comparison to known amounts of BSA in a Coomassie-stained SDS-PAGE gel. Western blots were probed with the anti-ESAG8 rat-1 antiserum at a 1:1000 dilution, and were secondarily probed with horseradish peroxidase-conjugated goat anti-rat antibody (Amersham-Pharmacia) at a 1:5000 dilution. Blots were developed in SuperSignal substrate (Pierce), according to the manufacturer's directions.

Northern blot 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 minutes and run on a 1.5% agarose/1% formaldehyde gel, transferred to nylon and hybridized with full-length *ESAG8* probe labeled with a random primer labeling kit (Stratagene).

RESULTS

Cloning and analysis of ESAG8 from the 221 ES

Because of small sequence variations between different copies of *ESAG8*, we began our analysis by cloning *ESAG8* expressed from the 221 ES, in the cell line in which our studies were to be performed. We obtained a full-length cDNA for *ESAG8*a by RT-PCR. This construct formed the basis for all subsequent experiments on ESAG8. Sequence analysis confirmed that this product contained an intact *ESAG8*. We compared the 221-ESAG8a protein sequence to other confirmed ESAG8 sequences (Fig. 1A). As we expected, the amino acid sequences are highly conserved, with an overall identity of 96%. The variability was not uniformly scattered throughout the protein, but was clustered towards the carboxy terminus. Clusters of variability have been seen in other ESAG sequences, but the overall conservation of ESAG8 is much higher.

As expected, *ESAG8* was strongly developmentally regulated (Fig. 1B). In contrast to previous reports (Revelard et al., 1990), however, we were easily able to detect mRNA on northern blots, suggesting that steady-state levels of *ESAG8* mRNA vary between strains and may be higher in more virulent lines (Lips et al., 1996) and/or in cell lines that have lower rates of ES switching (Lamont et al., 1986; Horn and Cross, 1997; Robinson et al., 1999). The *ESAG8* mRNA profile depended upon which ES was active. When the 221 ES was active, the mRNA profile showed several bands, whereas cells in which the 118 ES was active revealed a single band. Only one ES has been largely sequenced so far (Lips et al., 1993), although others are in progress (S. Bowman, G. Rudenko, M. H. and G. A. M. C., unpublished data). Our analysis of bacterial artificial chromosome 40D16 (BACPAC Resource Center, Children's Hospital Oakland Research Institute), containing most of the 221 ES, identified two copies of *ESAG8*, organized as a tandem repeat of *ESAG8* and *ESAG3*, interspersed by *ESAG4* (data not shown). We speculate that the complex RNA profile reflects multiple copies of *ESAG8* in the 221ES, perhaps complicated by the utilization of alternative splicing and polyadenylation sites. It is still unclear, however, whether there is much variability among ESs in terms of the organization of *ESAG8* so we have continued to work with the clone 221 because it grows well, is easily transfected, and is the background for our regulated cell lines.

Disruption of *ESAG8*

Since only one ES is active in a given cell and *ESAG8* has not been found outside an ES, we reasoned that the gene might be functionally haploid if only a single copy was present in the active ES, and thus amenable to a direct disruption strategy. We therefore designed a disruption cassette that would allow us to target both active and silent ESs. The disruption cassette consisted of the 5′ and 3′ *ESAG8* coding sequences flanking a double drug-resistance cassette in which the upstream *BLE* gene lacked a promoter whereas the downstream *NEO* gene was driven by the rDNA promoter (Horn and Cross, 1995). Growth in high levels of phleomycin allowed us to select for integration into the active ES, whereas G418 selects for integration into any ES, because of the inclusion of the rDNA promoter.

By selecting with G418, we were easily able to obtain transformants in which various silent ESs had been targeted (data not shown). We were also able to integrate the cassette at two locations in the actively transcribed 221 ES, which contains multiple copies of *ESAG8*, although these transformants were obtained at a somewhat lower frequency. However, when we attempted to integrate the cassette into the active site in cells in which the 118 ES was active, an expression site that we believe contains only a single copy of *ESAG8*, we were unable to obtain any clones after extensive efforts, although we were able to obtain clones with integrations into multiple silent ESs when we selected for the promoter-driven *NEO* marker. We also attempted to select for switches to activate silent ESs into which we had introduced the cassette. In no case were we able to activate an ES that lacked *ESAG8*, and all switches still expressed *ESAG8*. These results, and extensive experience in targeting other trypanosome genes, strongly imply that expression of *ESAG8* is essential for cell viability. Moreover, there does not appear to be any compensatory *ESAG8* expression from other loci, as has been experienced in both *ESAG1* and *ESAG6* knockout experiments (Carruthers et al., 1996; Ansorge et al., 1999).

Localization of GFP-tagged ESAG8

Because of the potential role of ESAG8 in regulating VSG expression, we wanted to determine the subcellular distribution of the protein. Previous attempts to localize ESAG8 suggested that protein was enriched in the nucleus (Lips et al., 1996). We had difficulty raising antisera that could specifically recognize ESAG8, even in western blot analysis. Although we were eventually able to raise a rat antiserum that was capable of recognizing ESAG8 on a western blot, this antiserum was unsuitable for immunofluorescence analysis, because of high background. Attempts at immunolocalization of HA epitope-tagged versions of ESAG8 were similarly unsuccessful, due to high background (data not shown).

It may have been difficult to specifically visualize ESAG8 because of its low abundance. We quantified the amount of ESAG8 in a single cell by comparing known amounts of purified recombinant ESAG8 to levels in trypanosome lysates, in a western blot (Fig. 2A). We estimate that there are only 2000-4000 copies of ESAG8 in cells transcribing the 221 ES,

which was probably below the detection threshold for our antisera in an immunofluorescence assay, and two- to fourfold less when the 118 or 117 ESs were active.

To overcome these limitations, we designed a fusion construct in which 'humanized' eGFP (Haas et al., 1996) was fused to the N terminus of ESAG8 and expressed in trypanosomes using an inducible expression system (Wirtz et al., 1998). We reasoned that the signal-to-noise threshold should be lower with GFP-tagged proteins, because of the high quantum efficiency of GFP and the absence of any secondary detection reagents that could potentially add to background. Moreover, background fluorescence in bloodstream-form trypanosomes is very low, and the detection limit of GFP was estimated to be approximately 500 molecules per cell when concentrated in a discrete location (data not shown). All constructs were designed with a PSL tripeptide linker between GFP and the protein, to minimize potential effects on ESAG8 function.

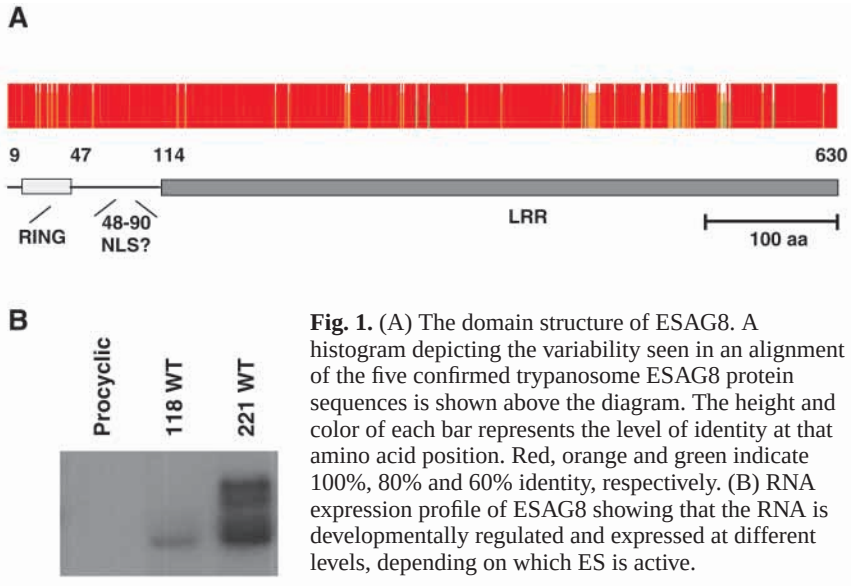

Fig. 1. (A) The domain structure of ESAG8. A histogram depicting the variability seen in an alignment of the five confirmed trypanosome ESAG8 protein sequences is shown above the diagram. The height and color of each bar represents the level of identity at that amino acid position. Red, orange and green indicate 100%, 80% and 60% identity, respectively. (B) RNA expression profile of ESAG8 showing that the RNA is developmentally regulated and expressed at different levels, depending on which ES is active.

The GFP-ESAG8 fusion was expressed well in the tetracycline-regulated cell line 13-90 (Wirtz et al., 1998). In the uninduced cells, no tagged protein was seen in western analysis. In the presence of 2.5 ng/ml of tetracycline, approximately wild-type levels of a protein running at the expected molecular mass of 97 kDa were seen when probed with anti-ESAG8 antibody (Fig. 2B). The native ESAG8 protein runs at approximately 70 kDa.

High-resolution deconvolution experiments demonstrated that GFP-ESAG8 was localized to a subdomain of the trypanosome nucleus that resembled the nucleolus (Fig. 3A-D). The trypanosome nucleolus is easily visible as a dark region in the DAPI stained nucleus (Das et al., 1998). To confirm this localization, we performed confocal microscopy on the GFP-ESAG8-expressing cell lines that were counterstained with a monoclonal antibody specific for an uncharacterized *T. brucei* nucleolar protein (K. Gull, personal communication) (Ersfeld and Gull, 1997). There was good correlation between the GFP signal and the nucleolar marker, indicating that GFP-ESAG8 did accumulate within the nucleolus (Fig. 3E-G).

In experiments designed to look at the distribution of proteins throughout karyogamy, Marchetti and colleagues (Marchetti et al., 2000) noted the presence of a thin connection between the two separating nucleoli in a subset of mitotic cells. We only rarely detected such a structure, most likely because the amount of GFP-ESAG8 is extremely low, but its occasional detection reinforces the idea that ESAG8 is an intrinsic component of the nucleolus (Fig. 3H-J).

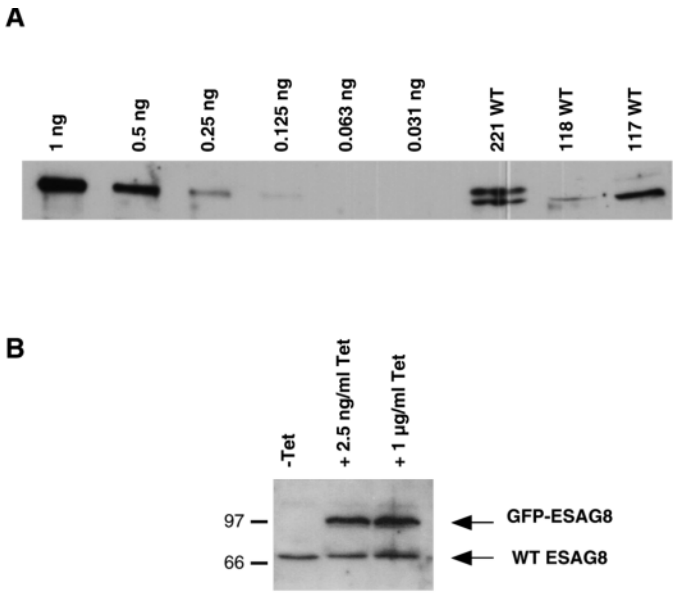

Fig. 2. (A) Quantification of ESAG8. A twofold dilution series of His_6-tagged ESAG8 was compared to 2×10^6 trypanosomes from cell lines in which different ESs are active. Note the second ESAG8 band in the 221 extract. (B) Expression of GFP-ESAG8 in the 13-90 cell line containing the T7 polymerase and the Tet-Repressor. The double band of 221 ESAG8 was not resolved in this gel.

Quantification of nuclear GFP-ESAG8

ESAG8 might have a specific function in the nucleolus or be sequestered there, as is true for several components of mammalian and yeast nucleoli (Shou et al., 1999; Weber et al., 1999). From our confocal analyses, it appeared that a fraction of ESAG8 is cytoplasmic, raising the possibility that this represents the active pool of the protein. Because trypanosomes are so small (approximately 1-2 µm diameter), it is difficult to accurately estimate protein distribution using confocal microscopy. To quantify the subcellular distribution of GFP-ESAG8, we examined living unfixed trypanosomes using digital deconvolution.

126 randomly selected cells were examined for the quantitative analysis. Raw images were collected through a *z*-stack of 10×200 nm and subjected to deconvolution. Stringent criteria for sampling and deconvolution were applied to assure that all data sets were correctly aligned and within the linear range of photon counting. The nuclear signal

was segmented from the overall cellular fluorescence by texture segmentation, while a subnuclear region of interest was defined by intensity segmentation. 82% of the total GFP signal was found within the nucleus. The signal within the subnuclear region was 2.5-fold above the mean nuclear intensity values. These results suggest that the bulk nuclear GFP-ESAG8 localizes to the nucleolus and that there may be a significant fraction (18%) of GFP-ESAG8 that is found outside of the nucleus (Fig. 4). We have some evidence from cell extraction experiments that a pool of ESAG8 is resistant to extraction, corroborating our observation that the protein exists in at least two distinct pools in the cell (data not shown).

Identification of a minimal nucleolar localization signal

To gain further insight into the relevance of the nucleolar accumulation of ESAG8, we sought to identify the features responsible for this localization. Nucleolar accumulation can occur through various mechanisms in mammalian cells, and no consensus signal has been identified. Initially we hypothesized that either the RING domain or the LRR domain might be required, in addition to the NLS region, to mediate nucleolar accumulation, since a two-step import pathway has been shown to be used by some mammalian proteins (Schmidt-Zachmann and Nigg, 1993). Thus we designed constructs that contained portions of the putative NLS region and the two domains separately (Fig. 5). The ΔRING construct (amino acids 68-630 fused to GFP) showed a diffuse cytoplasmic staining whereas the ΔLRR construct (amino acids 1-79) showed robust nucleolar accumulation (Fig. 6A,C). Constructs containing the RING domain alone (1-47) were not able to target GFP to the nucleolus (Fig. 6B). If a two-step pathway were required, however, this would not be unexpected. Yet full-length ESAG8 harboring a double mutation in the metal binding residues of the RING domain was able to efficiently target GFP to the nucleolus, suggesting that the RING domain was not necessary for nucleolar targeting (Fig. 6D). In contrast, a small region downstream of the RING domain (amino acids 48-79) was sufficient to target GFP to the nucleolus, although no appreciable cytoplasmic signal was observed in these cells (Fig. 6E). Neither smaller segments of this region nor an overlapping construct expressing GFP-ESAG8 68-114 were functional for nucleolar targeting (Fig. 6F-H). Northern analysis suggested that most of these fusions were not significantly overexpressed (data not shown).

To test whether the apparent nucleolar targeting region was necessary for nucleolar accumulation in the context of the full-length protein, we generated a version of ESAG8 in which amino acids 50-67 were deleted. Deletion of this region resulted in a predominantly cytoplasmic localization of GFP (Fig. 6I). Thus the linker region from amino acids 48-79 contains elements that are both necessary and sufficient for nucleolar accumulation of GFP-ESAG8 in trypanosomes.

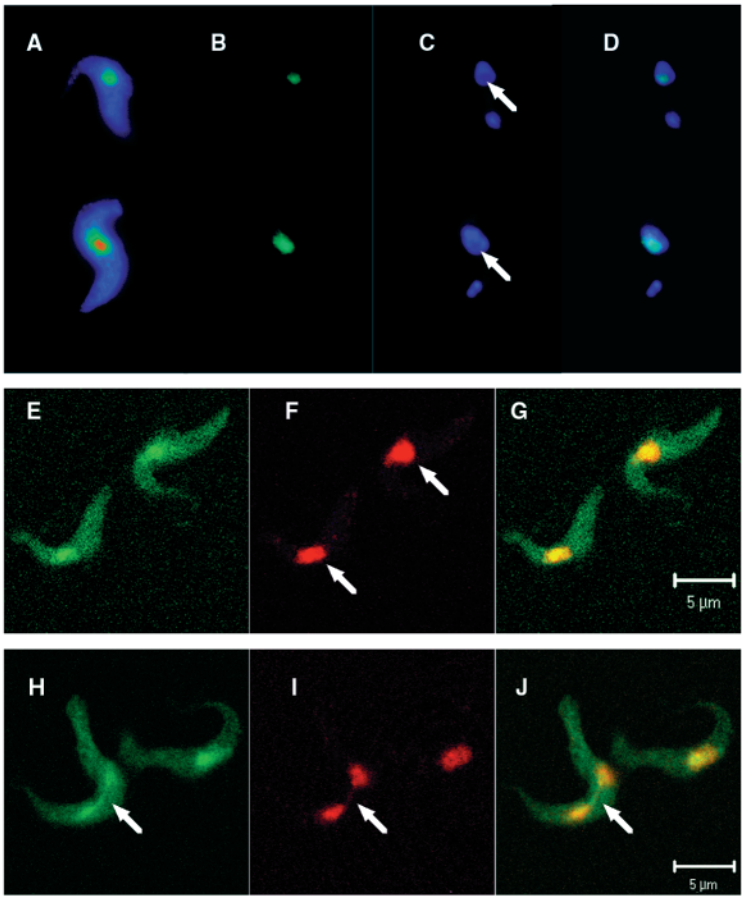

Fig. 3. (A-D) Visualization of GFP-ESAG8 in fixed and DAPI-stained trypanosomes. The image represents the summed view through a 3D-dataset (25×200 nm *z*-distance) after digital deconvolution using an advanced Maximum Likelihood Estimation-based restoration. The image was pseudocolored by intensity (A) and subjected to threshold segmentation to visualize the peak GFP signal (B). The dark area in the DAPI counterstain defines the nucleolar localization (arrows; C). The merged color coordinates of GFP and DAPI fluorescence reveal a localization within the nucleolar region (D). (E-G) Confocal analysis of GFP-ESAG8 expressing cells counterstained with a nucleolar marker in red (arrows at nucleolus). (H-J) Confocal micrograph showing the thread of GFP-ESAG8 (arrow) between two nucleoli in a mitotic cell. Scale bars, 5 µm.

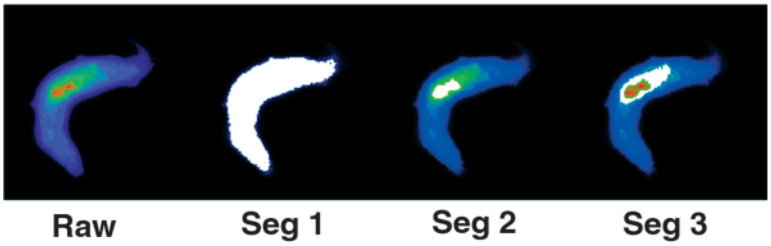

Fig. 4. Distribution of ESAG8 in living cells. We used three segments to calculate the ESAG8 distribution in a living cell. Intensity segment 1 was the image area, with more than 10 counts per pixel defining the region of interest used for calculation of the total fluorescence value. Segment 2 was the result of intensity/texture segmentation within the region of interest, yielding a subnuclear signal. Segment 3 was defined by texture segmentation describing a nuclear fluorescence signal. The summed intensity values from the three segments were used for calculation of fluorescence distribution in individual cells.

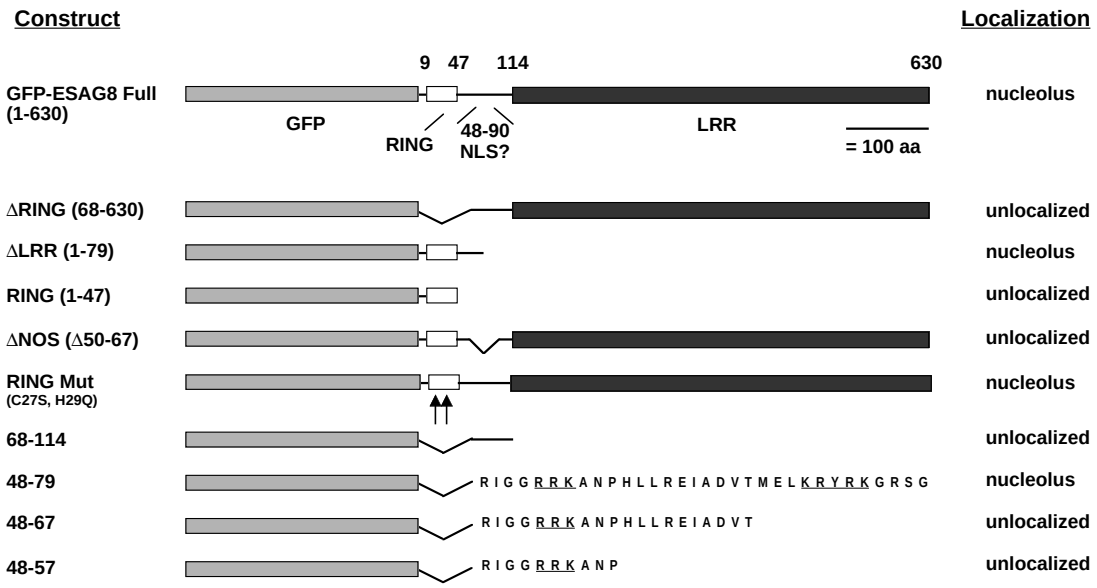

Fig. 5. GFP-ESAG8 constructs tested in this study. The minimal targeting sequence and truncations of it are shown. Basic regions that might contribute to nuclear localization are underlined. Arrows indicate the location of two point mutations in the RING domain.

Localization of GFP-ESAG8 fusions in mammalian cells

To examine whether the ESAG8 nucleolar signal (NOS) works in mammalian cells and might shed light on conserved nucleolar import pathways, we transferred two *GFP-ESAG8* fusions into the mammalian expression vector pCDNA, in which the viral CMV promoter drives expression. We transfected HEK-293T cells and observed the localization of the fusion proteins (Fig. 7). The minimal element we identified, from amino acids 48-79, conferred a predominantly nuclear localization on the GFP reporter whereas the shorter sequence, 48-57, which contains only a single basic element of the putative bipartite NLS, showed a uniform cytoplasmic and nuclear distribution. The ESAG8 NOS thus appears to function predominantly as an NLS in mammalian cells, supporting the idea that nucleolar localization requires species-specific interactions.

DISCUSSION

ESAG8 is a potential expression-site regulator. It contains two motifs that are found in several interesting regulatory proteins of mammalian cells and yeast. Unlike other ESAGs, its sequence differs little and probably insignificantly between the various ESs. It appears to be found only in the context of an ES and our preliminary genetic analysis suggests that ESAG8 is essential to the parasite. Our experiments show that ESAG8 is present at a very low abundance, 2000-4000 molecules per cell, and it may be lower in other strains or clones (Lips et al., 1996). There is one report of a functional ES that lacks *ESAG8*, suggesting that the gene is not essential in all situations, but the structure of this ES was highly atypical and lacked several *ESAGs* (van Xong et al., 1998). Although no mRNAs corresponding to the 'missing' *ESAGs* could be detected, these negative results contrast with other situations in which *ESAG* deletions could be compensated by low-level expression of multiple copies from other ESs or from non-ES locations (Carruthers et al., 1996; Ansorge et al., 1999).

We have shown that GFP-tagged ESAG8, expressed at wild-type levels, is concentrated within the trypanosome nucleolus.

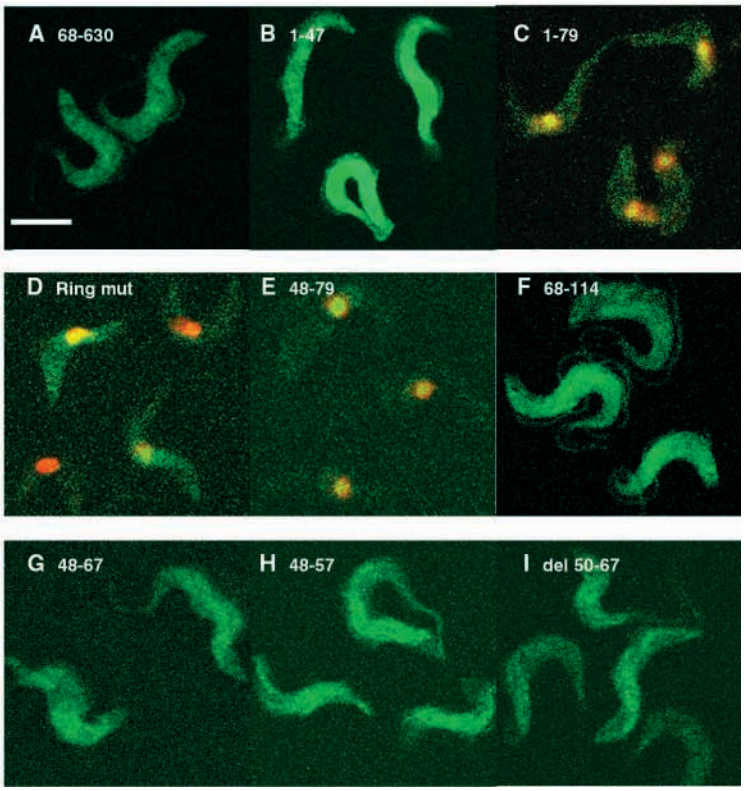

Fig. 6. (A-I) Confocal analysis of representative cells showing the distribution of selected GFP-ESAG8 truncations (see Fig. 5). Nucleoli are counterstained, where necessary, to show colocalization. (A) ΔRING (68-630), (B) RING (1-47), (C) ΔLRR (1-79), (D) RING Mut (C27S, H29Q), (E) 48-79, (F) 68-114, (G) 48-67, (H) 48-57, (I) ΔNOS (Δ50-67). Scale bar, 5 μm.

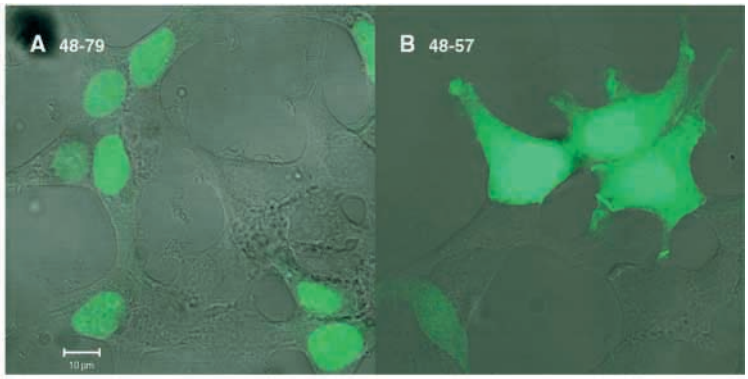

Fig. 7. (A) Localization of GFP-ESAG8 48-79 in transiently transfected human HEK-293T cells examined 24 hours after transfection. (B) Localization of GFP-ESAG8 48-57. Scale bar, 10 μm.

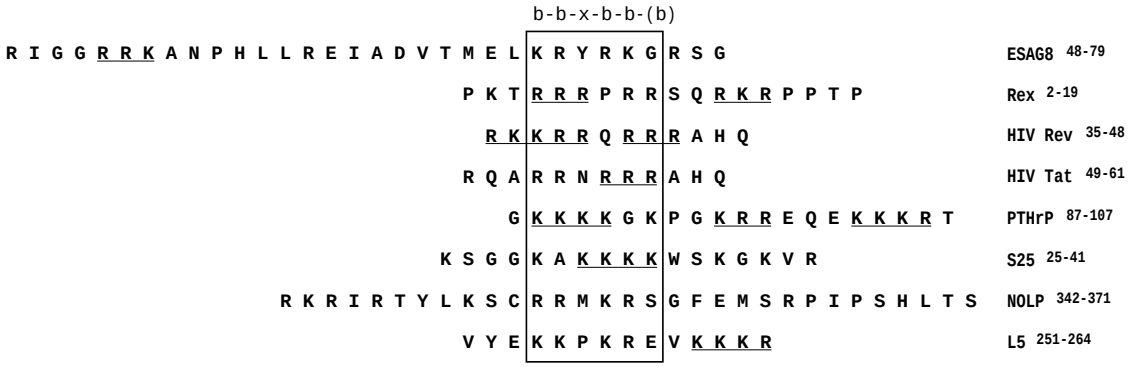

Fig. 8. Comparison of the ESAG8 NOS with a selection of compact NOS sequences from different proteins. All sequences shown are sufficient to target reporter proteins to the nucleolus. The basic residue cluster shown to be essential for nucleolar targeting in some of these sequences is boxed. Contiguous clusters of more than two basic amino acids are underlined. Sequences were compiled from the following references: HTLV-1 Rex (Hofer et al., 1991), HIV-1 Rev and Tat (Kubota et al., 1999), PTHrP (Aarts et al., 1999), S25 (Kubota et al., 1999), NOLP (Ueki et al., 1998), L5 (Rosorius et al., 2000).

We believe this localization is significant, because we have been able to isolate a small linear sequence of ESAG8 that is both necessary and sufficient to mediate nucleolar targeting of GFP, and essential for targeting the full-length GFP-ESAG8 fusion. While the eukaryotic nucleolus has traditionally been seen as the seat of ribosome biogenesis, recent studies have suggested that this organelle is involved in processes as diverse as mRNA splicing and trafficking (Pederson, 1998; Scheer and Hock, 1999). This transformation of our view of the nucleolus means that interpreting the reason for the predominant location of ESAG8 has become more complex.

The nucleolus is the dominant RNA polymerase I transcriptional domain in the nucleus. Since the ES has been shown to be transcribed by a highly α-amanitin resistant polymerase that behaves very similarly to PolI (Lee and van der Ploeg, 1997), and the ES promoter can be functionally replaced with rRNA promoter without disrupting ES regulation in the bloodstream form (Rudenko et al., 1995), it is tempting to speculate that the nucleolar localization of ESAG8 is not coincidental. Although the exclusiveness of ES transcription might be due to a special subnuclear localization, current evidence does not support the idea that the active ES is transcribed in the nucleolus (Chaves et al., 1998; Chaves et al., 1999). If ESAG8 were regulating ES transcription, it might be through an indirect mechanism, perhaps by modulating the activity of the pool of PolI, which appears to be present in very low and highly regulated quantities in *T. brucei* (E. Nogoceke, S. Leal and GAMC, unpublished experiments), or some other essential component required for ES transcription.

Several studies have suggested that the nucleolus also functions in the maturation and trafficking of specific mRNAs. Mutations in several proteins required for mRNA transport in yeast result in mRNA accumulation in the nucleolus (Schneiter et al., 1995). There is also evidence that *NEO* transcripts can accumulate within the trypanosome nucleolus (Rudenko et al., 1991; Kohler, 1999). Some structures that contain splicing components, such as coiled bodies and perinuclear compartments, are also closely associated with the nucleolus in mammalian cells (Pederson, 1998). Thus, ESAG8 may be involved in regulating a step downstream of transcription. Post-transcriptional regulation of ES transcripts is likely to be extremely important, because every cotranscribed RNA is present at very different steady-state levels (Vanhamme et al., 1999). We were unable to overexpress *ESAG8* mRNA or protein, even when the untranslated sequences were optimized and the gene was highly transcribed by T7 RNA polymerase, further implying that post-transcriptional processing is important in regulating the levels of this and other ESAGs. We have good evidence, from two-hybrid and immunoprecipitation experiments (M. H. and G. A. M. C., unpublished data), that ESAG8 interacts with an RNA-binding protein of the *puf* family, (Zhang et al., 1997), supporting the idea that the protein might regulate mRNA processing in some way. There is one other report of a protein, PIE8, that may interact with ESAG8

(Perez-Morga and Pays, 1999). PIE8 may be involved in the differentiation from bloodstream to procyclic forms, since, in late bloodstream-stage trypanosomes, it migrates to the mitochondrion in the early stages in mitochondrial activation. It has not been possible to demonstrate that ESAG8 truly interacts with PIE8 in vivo, or whether the interaction was an artifact of the yeast two-hybrid screen.

Studies from both yeast and mammalian systems have demonstrated that the nucleolus can be used by cells to sequester regulatory components until they are required. Upon release from the nucleolus, these factors are able to effect a cellular response. The yeast RENT complex, for example, is released prior to telophase in order to activate degradation of cyclins and prepare for the next round of cell division (Shou et al., 1999). The Sir2 silencing protein is similarly sequestered in the nucleolus (Straight et al., 1999). In mammalian cells, p19ARF has been shown to sequester the p53 negative regulator Mdm2 within the nucleolus, leading to the activation of p53. Release of Mdm2 from the nucleolus leads to the inactivation of p53 and the resumption of cell division (Weber et al., 1999). In the HIV-1 Tat protein, low levels of a Tat-GFP fusion were found in the nucleus whereas overexpression resulted in nucleolar accumulation, which was not required for activity (Stauber and Pavlakis, 1998). Analogously, the active pool of ESAG8 may exist outside of the nucleolus, where approximately 20% of the GFP-ESAG8 signal was found. None of the non-nucleolar mutant forms of ESAG8 resulted in any overt phenotype, however, perhaps because they were not overexpressed.

It is unclear how nucleolar accumulation is mediated in most eukaryotes, although the lack of clear consensus signals has led to the supposition that nucleolar targeting requires specific interactions of nucleolar components. Specific domains have been identified that are able to target proteins to the nucleolus in mammalian cells, but many of the signals are complex and some are context-dependent. There are short highly basic regions in some viral proteins that robustly confer nucleolar targeting to reporter proteins, independent of context (Kubota et al., 1989). Targeting signals have also been identified in a variety of ribosomal proteins, which are similar to the viral sequences in being highly basic (Annilo et al., 1998; Kubota et al., 1999). In several cases, such as that of nucleolin, accumulation in the nucleolus is mediated by interaction with other nucleolar proteins or RNAs, lending support to the hypothesis that specific interactions are important for nucleolar targeting and/or retention (Schmidt-Zachmann and Nigg, 1993).

The ESAG8 nucleolar targeting signal that we have identified is unusual among eukaryotic nucleolar targeting signals because it is quite small and essentially conforms to the consensus for a bipartite NLS. Indeed, we found that this sequence would target GFP to the nucleus in mammalian cells. The ESAG8 NOS contains several basic amino acids, but they are much less concentrated than the residues in the other compact targeting signals (Fig. 8). Instead, the ESAG8 sequence is richer in hydrophobic amino acids and even contains some acidic residues. That a sequence that is sufficient for nucleolar targeting in trypanosomes could function merely as an NLS in a heterologous system lends further credence to the idea that nucleolar targeting depends on specific interactions that take place after proteins have been translocated across the nuclear pore.

In this context, it is interesting to examine the recently published results of Marchetti and colleagues, who described a cryptic nucleolar localization signal in the *Trypanosoma cruxi* H2B protein (Marchetti et al., 2000). Transfection of *T. brucei* with the first 40 amino acids of TcH2B, fused to the amino terminus of GFP, resulted in a nucleolar concentration of the signal. Nucleolar targeting was not seen when the full-length H2B-GFP fusion protein was expressed, however, or when the H2B40-GFP chimera was fused to β-galactosidase. The authors reasoned that a serendipitous interaction was occurring between the H2B-GFP truncation and a nucleolar component. However, since the β-galactosidase reporter is fairly acidic, an alternative possibility is that addition of this moiety to the very basic H2B truncation may have masked a true nucleolar interaction domain. In support of this perspective, the human ribosomal protein L5 NOS is able to target GFP, but not β-galactosidase, to the nucleolus (Rosorius et al., 2000). There are reports of specific histone variants that accumulate specifically within the nucleolus (Allis et al., 1982; Bhatnagar et al., 1984; Tanaka et al., 1999), so it is also possible that the cryptic targeting signal in the TcH2B amino-terminal domain is not causing a serendipitous accumulation of the GFP reporter, but that nucleolar accumulation is the default pathway for this signal in the absence of the domain that is necessary for H2B to interact with its normal partner, H2A, which may be necessary for its normal localization.

In our experiments, GFP-ESAG8 nucleolar targeting worked in the context of the full-length protein and in truncations. The compact nature of the ESAG8 NLS/NOS, as well as the putative TcH2B NLS/NOS, is quite interesting, since both targeting functions have been built into a relatively small sequence and we have been unable to uncouple nuclear and nucleolar targeting. To address this issue, it might be fruitful to mutate amino acids in the region between the two clusters of basic amino acids that form the bipartite NLS, but we have not undertaken a more comprehensive analysis. Mutations in this region generally do not affect bipartite NLS function, so it might be possible to separate the two localization functions by concentrating on this region. It is interesting to consider that the bipartite NLS may provide a useful scaffold of basic residues on which to assemble domains that can interact with nucleolar components (Robbins et al., 1991).

Finally, we are left with the question of where the functional pool of ESAG8 is located. Unfortunately, mutants that might have disrupted normal ESAG8 function were too poorly expressed to explore this question. None of the truncations or deletions we designed showed any overt phenotypes. Thus, a dominant-negative approach to the analysis of ESAG8 is not a fruitful one at present, since we were unable to overload the cell with truncated forms of the protein. We are currently attempting to establish a conditional knockout of *ESAG8* and are pursuing the identification of interacting proteins to enhance our understanding of the potential role of this gene in ES regulation, or other aspects of trypanosome gene expression.

This work was supported by the National Institutes of Health by grants AI21729 (G.A.M.C.) and training grant GM07982 (M.H.). Special thanks to T. Zanders for excellent technical assistance, to K. Ersfeld and K. Gull for the generous supply of antibody and advice on microscopy and to T. Worley and A. Monteiro for advice on transfecting mammalian cell lines and for providing HEK-293T cells.

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