Subnuclear localization of the active variant surface glycoprotein gene expression site in *Trypanosoma brucei* (antigenic variation/in situ hybridization/nucleolus/RNA polymerase I)

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**ABSTRACT** In *Trypanosoma brucei*, transcription by RNA polymerase II and 5′ capping of messenger RNA are uncoupled: a capped spliced leader is trans spliced to every RNA. This decoupling makes it possible to have protein-coding gene transcription driven by RNA polymerase I. Indeed, indirect evidence suggests that the genes for the major surface glycoproteins, variant surface glycoproteins (VSGs) in bloodstream-form trypanosomes, are transcribed by RNA polymerase I. In a single trypanosome, only one VSG expression site is maximally transcribed at any one time, and it has been speculated that transcription takes place at a unique site within the nucleus, perhaps in the nucleolus. We tested this by using fluorescence in situ hybridization. With probes that cover about 50 kb of the active 221 expression site, we detected nuclear transcripts of this site in a single fluorescent spot, which did not colocalize with the nucleolus. Analysis of marker gene-tagged active expression site DNA by fluorescent DNA in situ hybridization confirmed the absence of association with the nucleolus. Even an active expression site in which the promoter had been replaced by an rDNA promoter did not colocalize with the nucleolus. As expected, marker genes inserted in the rDNA array predominantly colocalize with the nucleolus, whereas the tubulin gene arrays do not. We conclude that transcription of the active VSG expression site does not take place in the nucleolus.

*Trypanosoma brucei*, an extracellular parasite of mammals, uses antigenic variation of its coat to escape complete destruction by the immune system of the host (reviewed in refs. 1–5). The coat consists of a single protein, the variant surface glycoprotein (VSG). There are hundreds of VSG genes (VSGs) spread throughout the genome, and only one of these is expressed at any given time from one of approximately 20 VSG expression sites (ESs) located at the ends of chromosomes. The VSGs present in expression sites can be replaced by others by recombination mechanisms. Each ES is a poly-cistronic transcription unit controlled by a single promoter, located 40 to 60 kb upstream of the telomeric VSG (see Fig. 1A and refs. 6–8). A set of expression site-associated genes (ESAGs), which may meet specific metabolic requirements such as transferrin uptake (2), is cotranscribed with the VSG (9, 10).

Because it has approximately 20 ESs, the trypanosome needs mechanisms to activate and inactivate an ES and to prevent more than one ES from being active at any one time. Until recently, it seemed probable that activation/inactivation was controlled by a form of telomeric silencing (2, 11–16). Our work has failed to confirm this, however, and has indicated that there must be some form of crosstalk between ESs (17). A plausible form of crosstalk would be competition between ESs for a single nuclear site (17, 18).

Ever since Kooter and Borst (19) found that the transcription of ESs is insensitive to high concentrations of α-amanitin, a characteristic property of RNA polymerase (Pol) I, evidence has been accumulating that ESs are transcribed by Pol I (reviewed in refs. 20 and 21) rather than by a modified form of Pol II (22–24). In contrast to the situation in animal cells, Pol I of *T. brucei* can efficiently mediate the synthesis of mRNA (25–27), because trypanosomes mRNAs get their caps by trans splicing from an independently synthesized and capped precursor RNA (reviewed in refs. 28–30). Three other arguments also favor Pol I as the polymerase transcribing the VSGs: transcription of these genes (like the rRNA genes) is completely insensitive to concentrations of Sarkosyl that abolish the transcription of other protein-coding genes (31); the ES remains fully active and can still be switched off and on when the VSG ES promoter is replaced by a ribosomal promoter (14); and VSG/rRNA chimeric promoters are functional (32).

If a VSG ES were transcribed at a single site in the nucleus and if transcription were carried out by Pol I, which is normally restricted to transcription of the major rRNA genes in the nucleolus, then the site of VSG ES transcription may be the nucleolus as well. We have used dual-fluorescence in situ hybridization to test this hypothesis.

**MATERIALS AND METHODS**

*Trypanosome Culture.* The trypanosomes used belong to strain 427 of *T. brucei brucei* (33). Procyclic-form trypanosomes were grown in semisediment medium at 28°C as described (34). Bloodstream-form trypanosomes were cultured in vitro in HMI-9 medium (35). The 221a variant (MiTat 1.2a) of *T. brucei* was used, which expresses the VSG 221 from the 221 ES (33). Transformants of variant 221a used included 3174, which contains a resistance gene for neomycin in the ribosomal array. Transformants of procyclic trypanosomes derived from bloodstream-form variant 221a included r4, which has a resistance gene for neomycin in the ribosomal array (31) and RPhyro. RPhyro and r4 constructs differ in the size of the ribosomal promoter used as the target and in the processing signals for the marker genes tubulin, in the case of

Abbreviations: VSG, variant surface glycoprotein; ES, expression site; ESAG, expression site-associated gene; Pol, RNA polymerase; FITC, fluorescein isothiocyanate; PARP, procyclic acidic repetitive protein; DAPI, 4′,6-diamidino-2-phenylindole; CAT, chloramphenicol acetytransferase.

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RPhyro and PARP (procyclic acidic repetitive protein), in the case of r4 (see Fig. 1C).

The pro.Anv.pTSA.CAT.HYG.NM8.NsiI (proCAT) transformant of EATRO 1125 stock of T. brucei (procyclic form) was used to localize the active PARP A locus (37).

Cell Fixation and Preparation of the Microscope Slides. Midlogarithmic-phase culture-form trypanosomes were harvested, washed in PBS (0.15 M NaCl, 10 mM sodium phosphate, pH 7.2), and resuspended in PBS prior to fixation. In vitro-cultured bloodstream-form trypanosomes were handled in the same way, but the buffer used was phosphate/saline/glucose (60 mM Na₂HPO₄/3 mM NaH₂PO₄/44 mM NaCl/55 mM glucose, pH 8.0). The cells were then diluted 1:2 in 2× fixation solution (1× fixation solution: 4% formaldehyde and 5% acetic acid in PBS) and incubated at room temperature for 20 min on a rotating wheel. Fixed cells were centrifuged for 10 min at 3,000 × g and the fixation medium was replaced by 70% ethanol, followed by two additional washes in 70% ethanol to remove all traces of formaldehyde. At this stage, cells could be stored at 4°C for several weeks without apparent loss of signal quality in in situ hybridization. Microscope slides were prepared by dropping 20 μl of the fixed-cell suspension on glass slides precleaned with ethanol/ether, 1:1 (vol/vol). Slides were allowed to air dry, and then were baked at 80°C for 10 min to improve cell adherence to the glass.

DNA Probes. The probes derived from the VSG ES used in the in situ hybridization shown in Fig. 3 are shown in Fig. 1. pTg221.8 is an 11.3-kb BglII fragment inserted into pAT153 (19); pTg221.4 is an 8.5-kb EcoRI fragment inserted into pAT153; pTg221.14 is a 15-kb BamHI fragment inserted into pAT153; and pTg221.11 is a 21.5-kb BglII fragment inserted into pAT153 (7). The clone pTg221.11 is not derived from the 221 ES, but from a homologous ES, and does not contain any 50-bp repeat sequences. The ES probe mix 4 contained the clones pTg221.8, pTg221.4, pTg221.14, and pTg221.11. The 50-bp repeat probe is a 1-kb fragment of the 221 ES 50-bp repeat array inserted into pBlueScriptSK. The rDNA probe R2 was labeled with biotin-16-dUTP, and all other hybridizations, cells either were not nuclease treated or were subjected to an RNase treatment (200 μg of RNase A per ml in 2× SSC) for 1 h at 37°C, as indicated. Both incubations were followed by three washes in 2× SSC for 5 min each. Finally, cells were dehydrated with ethanol and air-dried. Prehybridization was not necessary. Hybridization mixture (7.5 μl; 37.5 ng of each probe in a double hybridization) was applied

hybridization, cells were treated with DNase I (Promega, 0.2 unit of RQI DNase per μl/40 mM Tris-HCl [pH 7.9]/10 mM NaCl/6 mM MgCl₂/0.1 mM CaCl₂) after pepsin treatment and before hybridization for 1 h at 37°C in a humid chamber. For all other hybridizations, cells either were not nuclease treated or were subjected to an RNase treatment (200 μg of RNase A per ml in 2× SSC) for 1 h at 37°C, as indicated. Both incubations were followed by three washes in 2× SSC for 5 min each. Finally, cells were dehydrated with ethanol and air-dried. Prehybridization was not necessary. Hybridization mixture (7.5 μl; 37.5 ng of each probe in a double hybridization) was applied
to the slides, and probe and target nucleic acids were denatured simultaneously under an 18 × 18 mm coverslip for 5 min on an 80°C plate. Hybridizations were performed at 37°C in a humid chamber for 16 h. After hybridization, slides were rinsed three times for 20 min each in 50% formamide/2× SSC, pH 7.0, at 37°C in a shaking water bath. Finally, slides were washed two times for 5 min each at room temperature in Tris/saline (0.1 M Tris-HCl/0.15 M NaCl, pH 7.4).

**Immunocytochemical Detection.** For the detection of the biotinylated probes (R2 probe only), slides were incubated for 45 min at 37°C with streptavidin-Texas Red (Vector Laboratories) diluted 1:100, then for 30 min at 37°C with biotinylated goat anti-streptavidin (Vector) diluted 1:100, and finally for 30 min at 37°C with streptavidin-Texas Red diluted 1:100. All solutions were diluted with Tris/saline containing 0.5% blocking reagent (Boehringer Mannheim), and incubations were carried out in a humid chamber and followed by a 5-min wash at room temperature to remove coverslips, and three additional 5-min washes at room temperature in Tris/saline. For detection of the digoxigenin-labeled probes, slides were incubated for 45 min at 37°C with 1:200-diluted fluorescein isothiocyanate (FITC)-conjugated (Sigma) mouse anti-digoxigenin and then for 30 min at 37°C with (1:500-diluted FITC)-conjugated rabbit anti-mouse digoxigenin. For simultaneous detection of biotin- and digoxigenin-labeled probes, the first and second layers of antibodies were mixed in blocking solution. After the last wash, slides were dehydrated through an ethanol series, air dried, and mounted in antifading solution {10:1 glycerol:0.2 M Tris-Cl, pH 7.5/2% diazabicyclo[2.2.2]-octane (DABCO)/0.02% NaCN} containing 75 ng of 4,6-diamidino-2-phenylindole (DAPI) per ml.

**Microscopy.** A Zeiss Axiovert 100 TV microscope equipped with a ×100 objective (numerical aperture 1.3), single bandpass filters for Texas Red, FITC, and DAPI fluorescence, and a double band-pass filter for simultaneous detection of red and green fluorescence was used for visual analysis. For photographic purposes, digital images were acquired with a Photometrics charge-coupled device Series 200 camera with a KAF 1400 chip. Weak signals from small targets were intensified to allow their visualization in the images. Images of RNase-treated trypanosomes are difficult to interpret for readers, because without the outline of the trypanosome delineated by cytoplasmic RNA hybridization, only (weak) fluorescent spots are visible, as shown in Figs. 3G and 4E. As nuclear localization studies with and without RNase pretreatment gave the same results, most of the pictures in Figs. 3–5 show DNA-RNA hybridization. However, all the data presented in Table 1 are based on RNase A-treated cells.

**RESULTS**

**Preserving Nuclear Substructure.** In initial experiments we used noncross-linking fixatives (38), resulting in high sensitivity but also distortion and flattening of the nucleus, making the subnuclear localization of sequences questionable. Therefore, we turned to fixatives containing cross-linking agents that appear to preserve the cell (Fig. 2A) and its nuclear morphology (Fig. 2B; see also Fig. 5A). A spherical region of low DNA density or condensation can be seen in some cases. This region corresponds to the transcription domain of rRNA genes in the nucleus (see below), i.e., the nucleolus. Because of the small diameter of the nuclei (2 μm) and the limited z-plane resolution of the confocal laser scanning microscopy (0.7 μm; ref. 39), we opted for conventional fluorescence microscopy for analysis of colocalization. The images are two-dimensional projections of three-dimensional objects. Consequently, fluorescence in situ hybridization signals resulting from DNA or RNA may accidentally colocalize by superimposition. We define colocalization here as total or partial overlap of signals.

**Localization of the Inactive Expression Sites in the Nucleus.** Previous work by Chung et al. (40) has shown that the long telomeric repeats of the approximately 100 minichromosomes and 25 larger chromosomes of T. brucei are clustered in 10–20 spots that tend to be localized in the periphery of the nucleus. To test whether the inactive expression sites also are clustered, we use probes hybridizing to the ES promoter or to the arrays of 50-bp repeats located upstream of each ES (Fig. 1A; refs. 41 and 42). No specific nuclear localization or clustering of expression sites was detected (Fig. 2 C and E). Although in some cases the fluorescent signals appeared to be in the periphery of the nucleus, in the majority of the cells analyzed these signals were randomly distributed in two dimensions within the nucleus.

**Nuclear Localization of VSG Expression Site Nuclear Transcripts.** Studies on RNA synthesis in isolated nuclei indicate a high density of engaged RNA polymerase exists on the DNA template (7). We detected nuclear RNA transcripts at the active ES in cells treated with DNase I before hybridization (this procedure turned out to be more successful in eliminating crossreaction with silent ESs than doing the hybridization in trypanosomes that had not been denatured). The set of probes used (mix 4; Fig. 1A and Materials and Methods) covers a region of about 50 kb but does not extend into the VSG gene, as the highly abundant VSG mRNA results in a strong cytoplasmic signal that obscures the nuclear signal. Despite the large region probed by mix 4, the green hybridization signal in Fig. 3A appears as a single intense fluorescent spot in the nucleus. The cytoplasmic signal reflects the mRNA derived from the ES4Gs in the expression site. Both the nuclear and cytoplasmic signals were sensitive to RNase A (data not shown).

The ribosomal probe R2 (red) hybridizes to the 185 rRNA gene and RNA (see Materials and Methods), giving a fluorescent signal corresponding to the nucleolus, and also giving a cytoplasmic signal (Fig. 3C). Simultaneous analysis of both the ES and nucleolus signals (Fig. 3B) shows that the active ES colocalizes with the nucleolus in only 20% of the cases (Table 1). We attribute this colocalization to accidental two-dimensional overlap in the x-y plane of two signals from different z planes (43). The same result was obtained in a minimum of three independent experiments with 50–150 trypanosomes per analysis, as is the case for all of the obser-

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**Table 1. Nuclear localization in Trypanosoma brucei**

<table>
<thead>
<tr>
<th>Trypanosome clone</th>
<th>Markers inserted</th>
<th>Probe</th>
<th>Nucleic acid analyzed</th>
<th>Nuclease treatment</th>
<th>Overlap with nucleolus, % overlap (range)</th>
<th>Scoreable cells %</th>
<th>Cells analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>221a</td>
<td>None</td>
<td>Mix 4</td>
<td>RNA</td>
<td>DNase</td>
<td>20 (18–22)</td>
<td>&gt;80</td>
<td>&gt;450</td>
</tr>
<tr>
<td>3174</td>
<td>neo+hyg (VSG ES)</td>
<td>neo+hyg DNA</td>
<td></td>
<td>DNA</td>
<td>25 (18–32)</td>
<td>60</td>
<td>&gt;450</td>
</tr>
<tr>
<td>RP2</td>
<td>hyg (VSG ES)</td>
<td>hyg</td>
<td>DNA</td>
<td>RNase</td>
<td>23 (20–31)</td>
<td>60</td>
<td>&gt;450</td>
</tr>
<tr>
<td>RPhygro</td>
<td>hyg (rDNA)</td>
<td>hyg</td>
<td>DNA</td>
<td>RNase</td>
<td>75 (65–85)</td>
<td>80</td>
<td>&gt;450</td>
</tr>
<tr>
<td>r4</td>
<td>neo (rDNA)</td>
<td>neo</td>
<td>DAPI</td>
<td>RNase</td>
<td>100</td>
<td>60</td>
<td>350</td>
</tr>
<tr>
<td>221a</td>
<td>None</td>
<td>Tubulin DNA</td>
<td></td>
<td>RNase</td>
<td>36 (32–40)</td>
<td>&gt;90</td>
<td>450</td>
</tr>
<tr>
<td>proCAT</td>
<td>cat+hyg (PARP A locus)</td>
<td>cat+hyg DNA</td>
<td></td>
<td>RNase</td>
<td>26 (23–30)</td>
<td>60</td>
<td>450</td>
</tr>
</tbody>
</table>
To test whether marker genes inserted into an rDNA array localize at the nucleolus as expected, we analyzed two additional transfectants, each containing a marker gene in an rDNA array: RPhygro and r4 (Fig. 1C). In the r4 trypanosomes only 60% of the nuclei gave a spot with the neo probe (green), but there was 100% overlap between the neo and the nucleolar signals as previously reported by Rudenko et al. (31) for this transformant (Fig. 1B; Table 1). In the RPhygro trypanosomes, we found an overlap with the nucleolus in 75% of the cases, and ~80% of the cells stained positive (Fig. 1D–F; Table 1). Similar results were obtained with bloodstream-form and with procyclic-form trypanosomes. We attribute the difference in the results obtained with the two marker genes to the use of different ribosomal promoter fragments as targeting sequences in the two constructs. Nevertheless, these results show that a marker gene integrated in the rDNA is predominantly located at the nucleolus, as expected.

As another control, we used a probe for the tubulin transcription unit, which is transcribed by Pol II. Fig. 4 G and H show photographs of T. brucei variant 221a hybridized with a probe for tubulin (green) and the ribosomal probe R2 (red). Tubulin genes are present in the trypanosome genome as two multicopy clusters (45, 46). These clusters appear as two discrete spots in the nucleus (Fig. 4G). As the two tubulin clusters often are not in the same focal plane, the two signals
ative to by an invariant coat consisting of procyclin or PARP. The trypanosome is taken up by a tsetse fly, it replaces its VSG coat 

Incidental overlap of any non-nucleolar signal with the large nucleolus in the trypanosome nucleus is high, as is also shown by our results with the tubulin gene probe: the average diameter of the nucleus is about 2 μm and that of the nucleolus is almost one-third of that. The value of approximately 20% overlap with the nucleolus, which we found for both the active ES and for one of the tubulin clusters, is within the theoretical range of accidental colocalization (43).

The combination of a large nucleolus and weak signals from single-copy genes may also be responsible for the conclusion of Chung (50) that a marker gene under control of a PARP promoter is transcribed in the nucleolus. As PARP genes and VSG genes are transcribed by an RNA polymerase with the same characteristics, possibly Pol I, it seemed unlikely that the active PARP locus would be, and that the active VSG ES would not be, in the nucleolus. We now find that a transcribed marker gene in the PARP A locus does not colocalize with the nucleolus, the same result that we obtained for the active VSG ES.

Although our results show that the active VSG ES is not in the nucleolus, this does not imply that it could not be transcribed by Pol I. It would be of interest to test whether antibodies against Pol I colocalize with nuclear ES transcripts. The gene for the largest subunit of T. brucei Pol I has been cloned (23, 54), but so far polyclonal antisera (kindly provided by A. W. C. A. Cornelissen, University of Utrecht, The Netherlands) raised against a fusion protein, which were polymerase class-specific on Western blots, did not react with nuclei of fixed trypanosomes.

Transcription outside the nucleolus also does not mean that the ES could not be always in the same unique position attached to a special nuclear substructure. Such a unique location accommodating only a single active VSG ES would help to explain why only one VSG ES can be active at a time (17, 18). At present we cannot verify this, as we lack reference points in the nucleus. However, the alternative possibility that all inactive expression sites are bundled together in a single subcompartment is ruled out by our observation that expression sites are distributed throughout the nucleus. Attachment to the nuclear envelope cannot be excluded, as confocal laser scanning microscopy analysis of trypanosome nuclei is inconclusive because of their small size.

Formation of a nucleolus is directed by rDNA (51, 55) but requires transcription by Pol I because rDNA transcribed by Pol II does not result in normal nucleoli in yeast (56). A high rate of transcription by Pol I from an rDNA promoter is not sufficient, however, to obtain nucleolar localization in trypanosomes. Nor is an active VSG ES in which the endogenous promoter is replaced by an rDNA promoter (14) transcribed in the nucleolus (Table 1). The convenient ability of the trypanosome to generate mRNA from genes transcribed by Pol I (25,
26) should allow a further dissection of the requirements for nucleolar and nucleoplasmic localization of such transcription units.

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