Telomere length regulation and transcriptional silencing in KU80-deficient *Trypanosoma brucei*

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**ABSTRACT**

KU is a heterodimer, consisting of ~70 and ~80 kDa subunits (KU70 and KU80, respectively), which is involved in a variety of nuclear functions. We generated *tb*KU80-deficient trypanosomes to explore the potential role of the *tb*KU complex in telomere maintenance and transcriptional regulation of variant surface glycoprotein (VSG) genes in *Trypanosoma brucei*. Using real-time PCR, we demonstrated that the expression of several different VSG genes remains tightly regulated in *tb*KU80-deficient bloodstream-form cell lines, suggesting that VSG transcription profiles do not change in these cells. Owing to developmental silencing of the VSG Expression Sites (ES), no VSG is transcribed in the insect procyclic stage. With a green fluorescent protein reporter system, we showed that *tb*KU80-deficient mutants are fully capable of ES silencing after differentiation into procyclic forms. Using T7 RNA polymerase to explore the transcriptional accessibility of ES chromatin *in vivo*, we demonstrated that *tb*KU80-deficient bloodstream-form cell lines were able to generate transcriptionally repressed ES chromatin after differentiation into procyclic cells. Finally, we demonstrated progressive telomere shortening in *tb*KU80-deficient mutants. The possible function of *tb*KU80 in telomere maintenance and regulation of telomerase is discussed.

**INTRODUCTION**

*Trypanosoma brucei* is a unicellular eukaryotic parasite that causes sleeping sickness in humans and nagana in African cattle. In the mammalian host, the bloodstream form of the parasite is enveloped by a dense surface coat consisting of a single member of the variant surface glycoprotein (VSG) family. The periodic switching of VSGs, a process known as antigenic variation, allows the parasite to escape the host’s immune response (1,2). There are several hundred VSG genes encoded at various loci in the parasite’s genome (3). However, the expressed VSG is always located at the end of the chromosome adjacent to a telomere in a polycistronic transcription unit known as an Expression Site (ES). Current estimates suggest that there are approximately twenty potential ESs within the genome, of which only one is transcribed at any given time. All the others are transcriptionally repressed. After parasite ingestion by the vector *Glossina*, the Tsetse, bloodstream forms differentiate into procyclic forms in which all ESs are transcriptionally repressed. Navarro et al. (4) demonstrated that developmental ES repression correlates with a decrease of transcriptional accessibility of chromatin, suggesting that it is mediated by chromatin remodeling. Although there has been considerable progress in understanding the monoallelic VSG transcription (5,6), the molecular mechanisms governing switching and transcriptional repression are still not understood. Since the active ES is always located at a telomere, studies on telomere-binding proteins in trypanosomes might shed light on ES transcriptional regulation. Recently, Conway et al. (7) identified a homologue of the telomeric KU complex in *T. brucei*. KU is a heterodimer, consisting of two tightly associated polypeptides of ~80 and ~70 kDa (KU80 and KU70, respectively). KU proteins have been identified in a variety of organisms, including yeast, flies, humans and mice. They are involved in a variety of nuclear functions, including DNA double strand break repair by the non-homologous end joining pathway (NHEJ), V(D)J recombination of immunoglobulin genes and chromosome integrity (8). Importantly, KU is critical for telomere maintenance. Two KU-dependent phenomena, which have been investigated intensively in *Saccharomyces cerevisiae*, are of particular interest to us: the transcriptional silencing of genes that are adjacent to telomeres, a phenomenon termed telomere position effect (TPE), and telomere length regulation (TLR) (9–12). In yeast, transcriptionally repressed telomeric chromatin is organized in discrete foci in the nuclear periphery (13–15). These telomere clusters co-localize with pools of silent information regulator proteins (Sir2–4), which are essential for TPE (13). Deletion of yeast *KU* genes disrupts the subnuclear organization of telomeres, redistributes Sir proteins and interferes with TPE, suggesting that *KU* is important for establishment or maintenance of silent telomeric chromatin (11,14,16,17). Recently, elegant experiments revealed that the localization of telomeres near the periphery is necessary, albeit not sufficient, for silencing and that other KU-independent pathways...
exist to tether telomeres to the nuclear periphery (18–21). The position-dependent expression of a subtelomeric surface glycoprotein gene has been described in \textit{S. cerevisiae} recently (22). Expression of the \textit{FLO10} gene is varied in several mutant strains and transcriptional repression of \textit{FLO10} is dependent on the subtelomeric location of the gene. Interestingly, both SIR and KU proteins are critical for transcriptional silencing of this locus.

In the last decade, many telomeric proteins that are involved in chromosome stability and TLR have been characterized (23). In \textit{S. cerevisiae}, the KU complex binds to telomeric repeats \textit{in vivo}, where it interacts with a variety of proteins and protects telomeres from nucleolytic and recombinational activities (24–26). The importance of KU for TLR was revealed after discovering that \textit{yku70} and \textit{yku80} mutants possess abnormally short telomeres (9,10). Additional experiments demonstrated that KU interacts with the RNA component of the telomerase complex (27,28), suggesting that KU is involved in the recruitment of telomerase to chromosome ends (29). In mammalian cells, the rate of progressive telomere shortening strongly correlates with the length of a G-rich single stranded overhang (G-overhang), which is found at the end of each telomere throughout the cell cycle (30,31). Interestingly, wild-type \textit{S. cerevisiae} acquires a detectable G-overhang only during late S-phase. However, KU-deficient yeast cells possess a G-overhang throughout the cell cycle, implicating that KU regulates the G-overhang structure (24,32). Experiments in \textit{Schizosaccharomyces pombe} contrast significantly with studies done in \textit{S. cerevisiae}. Although the KU complex is required for maintenance of telomere length in \textit{S. pombe}, KU is dispensable for transcriptional silencing at telomeres and is located throughout the nucleus rather than concentrated at telomeric repeats (33). A similar role for KU has been proposed in \textit{T. brucei}, where the deletion of \textit{tbKU} appeared to affect telomere maintenance but not transcriptional control of telomeric metacyclic ESs in the procyclic (tsetse mid gut) stage of the trypanosome life cycle (7). However, the role of \textit{tbKU} in allelic exclusion and developmental regulation of bloodstream-form ESs has not been addressed and accurate measurements of telomere shortening at silent ESs are needed to pursue the hypotheses concerning telomere structure and maintenance in \textit{T. brucei}.

In this study, we show that the exclusive expression of the active ES is not affected in \textit{tbKU80}-deficient bloodstream forms. Furthermore, we demonstrate that \textit{tbKU80}-deficient cells are fully competent to silence ESs after differentiation into procyclic forms. Finally, we evaluate telomere shortening rates and analyze the G-overhang structure in \textit{tbKU80}-deficient trypanosomes to define more precisely the role of the \textit{tbKU} complex in TLR. We demonstrate that the shortening rates in the absence of \textit{tbKU80} correlate with the predicted telomere shortening due to the end replication problem.

**MATERIALS AND METHODS**

Trypanosome cell lines and plasmid constructions

\textit{T. brucei} bloodstream forms [strain Lister 427, antigenic type MTat 1.2 and clone 221a (34)] were cultured in HMI-9 at 37°C (35). This strain had been manipulated to generate the bloodstream and procyclic cell lines ‘single marker’ and ‘29–13’, respectively (36). Both cell lines constitutively express T7 RNA polymerase. Bloodstream forms were cultured in HMI-9 containing 2.5 μg/ml G418 (Sigma). Procyclic forms were cultured in SDM-37 (37) containing 15 μg/ml G418 and 25 μg/ml hygromycin (Sigma). Bloodstream forms were differentiated into procyclic forms as described previously (38). The green fluorescent protein (GFP)–\textit{tbKU80} fusion protein was expressed from a derivate of the pLew82 vector (39). To generate the expression construct for \textit{TY}-epitope-tagged \textit{tbKU70}, pLew111 [pLew82 without luciferase open reading frame (ORF)] was amplified by inverse PCR introducing a XhoI restriction site at the 5’ end and Stul restriction site at the 3’ end and excluding the bleomycin gene. The blastidicin gene was released from pHD309-bsr (40) by digesting with Smal and Xhol. The two fragments were ligated generating pFlan1. The \textit{tbKU70} ORF was amplified by PCR from genomic DNA using primers that introduced a \textit{TY}-tag (MEVHTNQDPLDA) sequence at the 5’ end. The PCR product was cloned into pFlan1. These T7 promoter driven cassettes were integrated into the rDNA spacer of the single marker cell line. Expression was induced by adding 500 ng/ml of tetracycline to the medium.

The constructs used to generate \textit{tbKU80} deletion mutants contained ~300 bp of 5’ and 3’ \textit{tbKU80} non-coding sequences (UTRs) for targeting, separated by drug resistance cassettes. Both 3’-UTRs and 5’-UTRs were amplified by PCR from genomic DNA and cloned into pBluescript II SK + (Stratagene), generating pCJ10. The hygromycin phosphotransferase ORF, flanked by ~100 bp of 5’-UTRs and 300 bp of 3’-UTRs from the actin gene, was removed from pH3D309 (40) and cloned into Pmel-digested pCJ10. The puromycin N-acetyltransferase ORF, flanked by ~100 bp of 5’-UTRs and 220 of 3’-UTRs from the actin gene, was released from pH3D309. This fragment was treated with DNA polymerase I to generate blunt ends and cloned into Pmel-digested pCJ10. The final construct was cut with NotI to release the targeting cassette for transfections. To generate the GFP reporter constructs, the GFP cassettes were amplified from pCOS57 with two different primer pairs. One primer pair included the T7 promoter upstream of the \textit{GFP} ORF another primer pair excluded the T7 promoter. The two PCR products were each cloned into SpeI/Stul-digested pbRn6 (41). The blastidicin S deaminase ORF was amplified from pHD309-bsd including the 3’-UTRs and 5’-UTRs. The selectable marker was then cloned into the two NheI-digested constructs described above. The final constructs were cut with Sacl and Xhol to release the GFP-reporter cassettes for transfection. All transfections were performed as described previously (40).

**Immunofluorescence**

This protocol was carried out as described previously, with minor modifications (42). Briefly, \(1 \times 10^6\) bloodstream forms, induced with tetracycline, were washed with trypanosome dilution buffer [TDB, (43)] before fixing in 2% formaldehyde for 10 min at 4°C. The fixed cells were attached onto glass cover slips by centrifugation at 2000 \(g\) for 5 min at 4°C and permeablisized with 0.2% NP-40 in phosphate buffered saline (PBS) for 10 min at room temperature. BB2 mouse monoclonal IgG (gift from Keith Gull) and anti-GFP rabbit polyclonal IgG (Molecular Probes) were used to detect \textit{TY}-\textit{tbKU70} or...
GFP-tagged \( t b \)KU80, respectively, and DAPI was used to stain DNA. BB2 and anti-GFP were visualized with tetramethyl rhodamine isothiocyanate-conjugated goat anti-mouse or fluorescein isothiocyanate-conjugated donkey anti-rabbit antibodies (Jackson Laboratories), respectively. Cells were mounted in anti-fade mounting solution (Vectorshied, Vecta Laboratories Inc.) and analyzed with a fluorescence microscope (Nikon Optiphot). Representative images were captured using a DeltaVision image restoration microscopy system (Applied Precision). Deconvolution was performed using the softWoRxTM V 3.0 Software.

Immunoprecipitation
This protocol was carried out with minor modifications according the manufacturer (Molecular Probes). Briefly, \( 1 \times 10^8 \) bloodstream forms were harvested, washed once with ice-cold TDB and resuspended in 1 ml wash buffer A1 (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA and 0.1% NP-40). Cells were sonicated for 30 s using a Micro Ultrasonic Cell Disruptor (Kontes). The lysate was preincubated with 50 \( \mu \)l protein G agarose beads (Sigma) for 1 h at 4°C. The beads were removed by centrifugation and the lysate was transferred to a fresh tube. An aliquot of 10 \( \mu \)g anti-GFP polyclonal rabbit IgG (Molecular Probes) was added and incubated overnight at 4°C. The following day samples were incubated with 30 \( \mu \)l protein G agarose beads (Sigma) for 90 min at 4°C. The beads were then washed for 20 min sequentially with 1 ml buffer A1, A2 (50 mM Tris, pH 7.5, 150 mM NaCl and 0.1% NP-40) and A3 (50 mM Tris, pH 7.5 and 0.1% NP-40). Proteins were eluted from the beads by boiling with 50 \( \mu \)l loading buffer (50 mM Tris–HCl, pH 6.8, 5% 2-mercaptoethanol, 2% SDS, 0.1% bromophenol blue and 10% glycerol), separated by SDS–PAGE electrophoresis, and electroblotted onto a nitrocellulose membrane. \( t b \)TY-KU70 and \( t b \)GFP-KU80 were analyzed by immunoblotting with monoclonal BB2 anti-TY (gift from Keith Gull) or monoclonal anti-GFP (Roche) antibodies, respectively. Primary antibodies were detected with horseradish-peroxidase-conjugated sheep anti-mouse antibodies (Amersham-Pharmacia).

Real-time-PCR
Total RNA from \( 1 \times 10^8 \) bloodstream forms was isolated using RNA Stat-60 (Tel-Test) according to the manufacturer’s instructions. An aliquot of 10 \( \mu \)g of total RNA was incubated with RQ1 RNase-free DNase (Promega) according to the supplier’s recommendations followed by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. The precipitated RNA was resolved in 10 \( \mu \)l DEPC-treated water and used for reverse transcription reaction using the ProStar First Strand RT–PCR Kit (Stratagene) with the supplied random primers. PCR was performed with an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems) according to the manufacturer’s instructions. All values were normalized to an \( \alpha \)-tubulin internal control. Post-amplification melting curves were performed to confirm that a single PCR product was produced in each reaction. Genomic DNA was used to generate a standard curve with which the copy numbers of gene products in each sample were calculated. The sequences of primers used in the real-time PCR can be obtained upon request.

Fluorescence cell analysis
Samples of living parasites were analyzed with a FACS Calibur system following the manufacturer’s recommendations (Becton Dickinson). Briefly, \( 1 \times 10^8 \) bloodstream forms were collected from a log-phase cell culture and GFP-auto-fluorescence was analyzed in medium. The mean fluorescence intensity was determined using CellQuest software (Becton Dickinson).

Telomere blot
This protocol was carried out with minor modifications as described previously (42). Briefly, \( 5 \times 10^8 \) bloodstream forms were harvested, washed once in TDB, resuspended in 1 ml TNE (10 mM Tris, pH 7.4, 100 mM NaCl and 10 mM EDTA) and lysed by adding 1 ml TNES (TNE + 1% SDS) supplemented with 100 \( \mu \)g/ml protease K (Roche). After 3 h incubation at 37°C, the DNA was extracted with phenol/chloroform, precipitated and resuspended in TNE supplemented with 100 \( \mu \)g/ml RNase A. After incubation for 3 h at 37°C, treatment with protein K (100 \( \mu \)g/ml) followed for another hour. DNA was then extracted with phenol/chloroform, isopropanol precipitated, and resuspended in TE (10 mM Tris, pH 7.4 and 10 mM EDTA). DNA was digested overnight with a mixture of AluI, Hinfl and KsaI under recommended conditions (New England Biolabs). DNA restriction fragments were separated on an 1% agarose gel and transferred onto a nitrocellulose membrane. Telomere restriction fragments were detected using a probe containing TTAGGG-repeats that was labeled with [\( \alpha \)-\( ^32 \)P]dCTP as described previously (44).

G-strand overhang-assay
G-strand overhang-assays were performed following a protocol described by Wellinger et al. (32) with minor modifications. DNA was extracted from bloodstream forms and HeLa cells (kindly provided by Dr Bibo Li). Briefly, \( 2 \times 10^9 \) cells were harvested, washed twice in ice-cold TDB and resuspended in 1 ml sterile TNE. An aliquot of 1 ml TNES + 100 \( \mu \)g/ml protease K was added and incubated overnight at 37°C. After phenol/chloroform extraction, the DNA was precipitated, gently resuspended in 300 \( \mu \)l TNE + 100 \( \mu \)g/ml RNase A and incubated for 3 h at 37°C. After adding 300 \( \mu \)l TNES + protease K, the DNA was incubated overnight at 37°C followed by phenol/chloroform extraction as described above. An aliquot of 10–20 \( \mu \)g of gDNA was digested overnight with MboI and AluI (New England Biolabs) and separated on a 0.8% agarose gel. The gel was dried and pre-hybridized in 0.5 M NaPO4, pH 7.2, 1 mM EDTA, pH 8.0, 7% SDS and 1% BSA (Church Mix, (45)) for 2 h at 30°C. An aliquot of 50 ng of oligonucleotides (CCCTAA)4 or (TTAGGG)4 were labeled with \( ^{32} \)P-labelled ATP by T4 polynucleotide kinase (New England Biolabs) for 45 min at 37°C. Hybridization was performed overnight at 30°C in Church Mix. After washing three times for 30 min with 4\( \times \) SSC (60 mM Na2C6H5O7 and 600 mM NaCl) and once for 30 min with 4\( \times \) SSC and 0.1% SDS at 20°C, the gel was exposed to a phosphorimager screen (Molecular Dynamics) for at least 72 h. Subsequently, the gel was denatured for 30 min in 1.5 M NaCl and 0.5 M NaOH, and neutralized for 15 min in 3 M NaCl and 0.5 M Tris–HCl, pH 7.0, followed by pre-hybridization in Church Mix at 55°C for 1 h and hybridization overnight with
the same probes. After washing as described above but at 55°C, the gel was exposed to a phosphorimager screen for 3–5 h.

RESULTS

Expression pattern and co-localization of \(tb\)KU80 and \(tb\)KU70

Two homologs of KU proteins, \(tb\)KU70 and \(tb\)KU80, were recently identified in \(T.\)brucei (7). Before proceeding to functional analysis of these proteins, we first wanted to characterize the \(tb\)KU complex in trypanosomes. In particular, we were interested in the nuclear distribution of \(tb\)KU and the interaction between \(tb\)KU80 and \(tb\)KU70. In order to avoid the need for \(tb\)KU-specific antibodies, we fused GFP and a TY peptide tag to \(tb\)KU80 and \(tb\)KU70, respectively. By indirect immunofluorescence (IF), both GFP–\(tb\)KU80 and TY–\(tb\)KU70 were clearly detectable in the nucleus (Figure 1, left panels). The signals in the cytoplasm seem to be unspecific cross reactivity of the antibodies, because they were also detectable in the controls. The distribution of the proteins was not homogeneous, but was manifested as distinguishable foci that were excluded from some areas in the nucleus. Since this distribution was similar to the pattern observed with a telomere probe used in fluorescence \textit{in situ} hybridization assays (FISH, data not shown), we compared both patterns by combining IF and FISH (see Supplementary Material). Specific co-localization of \(tb\)KU and telomeres could not be detected. Partial overlap of GFP–\(tb\)KU80 and telomere signals is considered to be coincidental and would mask any telomere-specific signal. Next, we generated a cell line that expressed both GFP–\(tb\)KU80 and TY-tagged \(tb\)KU70 to investigate if the two proteins form a complex in trypanosomes. Both proteins clearly co-localized in the nucleus of this cell line (Figure 1, right panels). This co-localization was observed in all cells and was not cell cycle dependent, as demonstrated by two examples of cells that are in G1 phase (one nucleus) or mitosis (two nuclei).

\(tb\)KU80 and \(tb\)KU70 interact \textit{in vivo}

Since co-localization does not prove that two proteins physically interact, we carried out co-immunoprecipitation (co-IP) assays with a cell line that co-expressed TY–\(tb\)KU70 and GFP–\(tb\)KU80. As controls, we generated two additional cell lines: one expressing TY–\(tb\)KU70 and GFP and one only TY–\(tb\)KU70. Protein expression levels of all cell lines were analyzed with the corresponding antibodies (Figure 2, input). The parental cell line (single marker) served as negative control (Figure 2, wild type). After IP was performed with a polyclonal antibody directed against GFP, the precipitated

![Figure 1](https://academic.oup.com/nar/article-abstract/32/22/6575/2375655/1)
proteins were analyzed with a monoclonal α-GFP antibody [Figure 2, left panel, IP (α-GFP)] or a monoclonal α-TY antibody [Figure 2, right panel, IP (α-GFP)]. Only in the cell line expressing both fusion proteins it was possible to co-immunoprecipitate TY–tbKU70 with an antibody specific for GFP–tbKU80 (Figure 2, right panel, lane 5). TY–tbKU70 was not detectable in the control precipitates, suggesting that tbKU exists as a heterodimer in T. brucei similar to the complex described in other organisms.

### Transcriptional silencing of ESs is not affected in tbKU80-deficient trypanosomes

To proceed with functional analysis of tbKU, we replaced both alleles of the tbKU80 gene with selectable markers to generate a tbKU80-deficient cell line. The correct integration of the marker genes was confirmed by Southern blotting (data not shown). Consistent with previous publications, tbKU80 knockout mutants were viable and showed no developmental or growth phenotype [data not shown, (7)]. Only one VSG is normally expressed in bloodstream-form trypanosomes. To examine if tbKU80 is involved in this monallelic expression of VSG genes, we compared the expression levels of a variety of known VSG genes in wild-type and tbKU80-deficient trypanosomes. Real-time PCR was performed with specific primer pairs to detect transcripts of VSG 221 (single copy gene, active ES), VSG 118 (single copy gene, inactive ES), VSG B (one telomeric and two internal copies), VSG bR2 (two telomeric and one internal copies) or VSG VO2 (one telomeric and one internal copy) with cDNA from wild-type and ΔtbKU80 parasites. As expected, mRNA of the active VSG221 was highly abundant in both cell lines (Table 1). In contrast, transcripts of other tested VSG genes were not detectable at all or are very close to the threshold which might represent unspecific amplification (Table 1). These results suggest that tbKU80-deficient cells are still able to regulate VSG transcription tightly and that tbKU is not involved in ES regulation in bloodstream-form trypanosomes.

### tbKU80-deficient cells are proficient in establishing developmentally silenced chromatin

Several groups have reported in detail that KU plays a central role in the establishment of transcriptionally repressed telomeric chromatin in yeast. After differentiation of bloodstream forms to the insect procyclic forms in vitro, transcription from the active ES promoter is turned off. This developmental silencing is promoter specific and depends on the telomeric location of the ES (46,47). Navarro et al. (4) used a T7 RNA polymerase-driven reporter system to study the transcriptional accessibility of the ES. In contrast to ESs in bloodstream forms, the T7 promoter was repressed along the entire ES in procyclic parasites, suggesting that the ES chromatin was remodeled to a less accessible, compact chromatin upon differentiation. We employed a similar assay to evaluate whether tbKU80-deficient cells are still able to establish developmentally silenced chromatin. A GFP reporter cassette driven by a T7 promoter was introduced immediately downstream of the active VSG in bloodstream forms (Figure 3B). In addition, we introduced a GFP cassette without a T7 promoter to assess the transcriptional activity of the endogenous ES promoter (Figure 3A). To compare the developmental ES regulation of wild-type trypanosomes with that of ΔtbKU80 parasites, GFP expression levels were monitored by FACS analysis in bloodstream forms (red bars) and in procyclic forms two weeks after differentiation (blue bars). The results of two representative clones from each reporter cell line are shown. As expected, wild-type cells efficiently down regulated the ES promoter (Figure 3A). GFP expression was reduced to a level comparable to control cells lacking the GFP reporter cassette. A similar effect was observed in ΔtbKU80 cells, suggesting that ES-promoter silencing is not influenced by tbKU80. To test whether chromatin remodeling is affected by the deletion of tbKU80, we differentiated parasites in which the GFP cassette was transcribed by the heterologous T7 polymerase. Upon differentiation, both wild-type cells and ΔtbKU80 mutants were able to reduce reporter activity to ~10% of bloodstream form activity (Figure 3B) as described previously (4). Only a slight difference, which was within the range of clonal and experimental variation, was observed between wild-type and tbKU80-deficient parasites, suggesting that any effect of tbKU depletion was very minor. These data suggest that the KU complex is not involved in down regulation of the ES promoter in procyclic forms and that

### Table 1. VSG expression profiles in wild-type and ΔtbKU80 cells

<table>
<thead>
<tr>
<th>VSG</th>
<th>Wild-type copy numbers</th>
<th>ΔtbKU80 copy numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSG 221</td>
<td>1.8 × 10^6</td>
<td>1.4 × 10^6</td>
</tr>
<tr>
<td>VSG 118</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>VSG B</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>VSG bR2</td>
<td>179</td>
<td>325</td>
</tr>
<tr>
<td>VSG VO2</td>
<td>118</td>
<td>454</td>
</tr>
</tbody>
</table>

Values represent calculated copy numbers of gene products in each sample (nd, not detectable). Results were normalized to α-tubulin.
tbKU80-deficient cells are fully capable of establishing developmentally silenced chromatin in trypanosomes. Telomere shortening

A previous study suggested that tbKU80-deficient mutants exhibit shortened or broken telomeres after extensive propagation, but the precise shortening rate was not determined (7). However, this is important to reveal the function of KU in telomerase recruitment and regulation. To evaluate the telomere shortening rates in tbKU80-deficient trypanosomes, we monitored telomere length in wild-type cells, a tbKU80 single knockout cell line (tbKU80+/−), and a tbKU80-deficient cell line (ΔtbKU80) for eight weeks. To be certain that any phenotype was owing to the loss of tbKU80, a cell line in which GFP–tbKU80 was reintroduced into the ΔtbKU80 mutant was also monitored. Genomic DNA was isolated at two-week intervals and digested with frequently cutting restriction enzymes that do not cut within the repetitive telomeric DNA. This preparation of bulk telomeres was analyzed by Southern blot with a telomere-specific probe. As described previously (48,49), wild-type cells displayed a continual elongation of telomeres (Figure 4, lanes 1–5). Similarly, the deletion of one tbKU80 allele had no apparent effect on telomere growth (lanes 6–10). Strikingly, ΔtbKU80 parasites exhibit progressive telomere shortening (lanes 11–15). A clear reduction of telomere length was visible after only two weeks (lane 12). Overall telomere length was reduced by 0.8–1.0 kb after eight weeks. Eight weeks represent ~210 generations, so this translates into a telomere shortening rate of ~4 bp per cell division. Interestingly, identical telomere shortening rates were observed in telomerase-deficient trypanosomes (Oliver Dreesen, unpublished data). Furthermore, we could completely recover the telomere growth defect by reintroduction of GFP–tbKU80 (lanes 16–20), suggesting that this phenotype was directly caused by the loss of tbKU80. Although we cannot exclude a role in end protection, our data suggest that tbKU80 is likely to be important for telomerase activity. Unfortunately, our attempts to make a cell line lacking both telomerase and KU80 were unsuccessful.

G-overhang structure in ΔtbKU80 trypanosomes

The rate of telomere shortening is dependent on the length of single-stranded G-overhang with which telomeres end (50). To examine the role of tbKU80 in telomere length control, we compared the G-overhang structure in wild-type and tbKU80-deficient trypanosomes. Furthermore, we wanted to know if the loss of tbKU80 would alter G-overhang structure, as has been described in yeast (24). For this purpose, DNA was isolated, digested with enzymes that do not cut repetitive telomeric DNA and separated on agarose gels. The dried native gels were hybridized with a radiolabeled (CCCTAA) 4 oligonucleotide probe to detect G-overhangs. Human DNA served as a positive control and G-overhangs were clearly detectable.
in this sample (Figure 5, upper left panel, arrow head). A (TTAGGG)₄ probe, which should not produce a signal, was used to confirm that the DNA was still in its native condition (Figure 5, upper right panel). However, no overhang signal was detectable in DNA derived from either wild-type or tbKU80-deficient trypanosomes with the (CCCTAA)₄ probe, despite loading at least 10-fold more trypanosome telomeric DNA compared to the HeLa cell sample (Figure 4, left panels) as is evident after denaturation, when the gels were re-hybridized with the same probes to confirm equal loading (Figure 5, lower panels). The weak signals in the upper panels in lanes with 1 and 1.5 µg DNA are unspecific, as they appear with both probes. As it is not possible to detect overhangs shorter than ~30 nt with this assay, we therefore conclude that trypanosomes have short overhangs and that, unlike in yeast, tbKU80 has no apparent influence on overhang structure in T.brucei.

**DISCUSSION**

In this study we investigated the role of T.brucei tbKU80 in transcriptional regulation of VSG genes, developmental silencing of ES and telomere maintenance. In order to characterize the KU complex in T.brucei, we first analyzed the localization and distribution pattern of KU in trypanosomes and the interaction between tbKU80 and tbKU70. The localization of tbKU80 is not uniform throughout the nucleus, as it is in some mammalian cells and in S.pombe (33,51). Rather it resembles the distribution of KU in S.cerevisiae, where the KU complex and telomere-repeat-binding protein Rap1p co-localize at telomeric clusters (52). We showed that both tbKU80 and tbKU70 form punctate patterns throughout the nucleus and that they co-localize. With co-IP experiments, we demonstrate that these structures most likely represent heterodimers. In yeast, KU seems to be tightly associated with telomere function and structure. IP experiments showed that telomeric DNA can be co-immunoprecipitated with a tagged yKu80 protein (24). Furthermore, Laroche et al. (16) showed that mutation of KU genes disrupts the subnuclear organization of telomeres in the periphery. Although tbKU distribution patterns are similar to those observed by telomere FISH, the partial co-localization of GFP–tbKU80 and telomeres that we observed by combining IF with FISH might be coincidental. Furthermore, neither the size nor the distribution of telomere clusters was altered in DtbKU80 trypanosomes and telomeric repeats could not be co-immunoprecipitated with GFP–tbKU80 (data not shown). These data suggest that tbKU80 does not play a critical role in the nuclear organization of telomere clusters in trypanosomes, and that interactions between tbKU80 and telomeres might be transient or weak. However, firm conclusions about the role of tbKU80 in ES regulation and telomeric silencing cannot be derived from these observations. Work by several groups revealed that redundant telomere-anchoring pathways exist in other cells, and that localization of telomeres to the nuclear periphery is
forms. We could not detect any differences in the VSG expression profiles of wild-type cells compared with that of ΔbKU80 cells. This is consistent with the model of ES transcriptional regulation proposed by Navarro and Gull (6). This model advocates that a unique location, the Expression Site Body (ESB), is responsible for allelic exclusion by sequestering factors that are essential for full ES transcription. The lack of these factors rather than active repression (for example by the KU complex) might be the cause of transcriptional attenuation of inactive ESs in bloodstream forms. However, repression of inactive ESs by unidentified factors cannot be excluded, based on our data, since KU-independent repression is known to occur at fission yeast telomeres and at budding yeast telomeres in strains lacking Rif1 or Rif2 (17,33).

Trypanosomes encounter a different situation after differentiation from bloodstream to procyclic forms, where all ESs are transcriptionally repressed. Analysis of ES localization by FISH with probes specific for telomeres and subtelomeric ES-specific 50 bp repeats suggests that the majority of ESs are in the nuclear periphery (J.Lowell, unpublished data). Furthermore, Navarro et al. (4) demonstrated that developmental ES-silencing correlated with a decrease in transcriptional accessibility of chromatin to an abundantly expressed single-unit T7 RNA polymerase. Transcription from chromosomally integrated T7 promoters is repressed along the entire ES after differentiation into procyclic forms, but not at chromosome-internal loci. In contrast, a T7 promoter integrated into an inactive ES in bloodstream forms is fully accessible, indicating that distinct mechanisms control ES regulation in bloodstream and procyclic forms and that ES repression upon differentiation is mediated by remodeling chromatin to yield a structure that is no longer permissive for transcription. We used the same experimental approach to elucidate the role of ΔbKU80 in developmental silencing of ES. As shown with GFP reporter cassettes driven by endogenous ES promoter or T7 promoter, ΔbKU80 trypanosomes are fully capable of silencing their ES promoter after differentiation. In addition, there is no detectable difference in T7 promoter accessibility between wild-type and ΔbKU80 parasites. Hence, it seems that ΔbKU80 does not play a significant role in developmental ES regulation and chromatin remodeling. Whether the exclusive telomeric location of ESs is important for developmental transcriptional regulation of the endogenous ES promoter is still not known and is intensively discussed. The spread of transcriptionally repressed heterochromatin from telomeres into subtelomeric regions has been described in other organisms. Since telomeric repression of different promoters has been reported in T.brucei (4,41), it is reasonable to believe that telomeres influence adjacent chromatin structure in trypanosomes. However, it is uncertain whether telomere-induced repression can spread to the ES promoters, which can be as far as 50 kb upstream of the telomere. In order to understand telomeric repression and its role in ES regulation, it is essential to analyze chromatin modifications and modifying enzymes in trypanosomes.

Substantially more is known about trypanosome telomere structure. T.brucei telomeres consist of 10–20 kb duplex TTAGGG repeats that end in T-loop structures (54). In contrast to mammalian or yeast cells, telomeres grow constantly in trypanosomes and, interestingly, the telomere of the active ES is susceptible to large truncations. The KU complex is

Figure 5. G-overhang structure in ΔbKU80 trypanosomes. DNA was extracted from ΔbKU80-deficient cells (ΔbKU80), wild-type cells (wild type) and from HeLa cells (human), and digested with MboI and AluI restriction enzymes. Different amounts of DNA, as indicated in each lane, were separated by gel electrophoresis and analyzed with radiolabeled probes specific for G-overhangs [(CCCTAA)n, left panels] or C-overhangs [(TTAGGG)n, right panels]. To confirm equal loading the gels were denatured and re-hybridized with the same probes as above (lower panels). Different exposure times of the gels are indicated on the right.

Two different phenomena were investigated: the exclusive expression of one ES in bloodstream forms and the developmental silencing of the ES after differentiation into procyclic forms. We could not detect any differences in the VSG expression profiles of wild-type cells compared with that of ΔbKU80 cells. This is consistent with the model of ES transcriptional regulation proposed by Navarro and Gull (6). This model advocates that a unique location, the Expression Site Body (ESB), is responsible for allelic exclusion by sequestering factors that are essential for full ES transcription. The lack of these factors rather than active repression (for example by the KU complex) might be the cause of transcriptional attenuation of inactive ESs in bloodstream forms. However, repression of inactive ESs by unidentified factors cannot be excluded, based on our data, since KU-independent repression is known to occur at fission yeast telomeres and at budding yeast telomeres in strains lacking Rif1 or Rif2 (17,33).

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Supplementary Material

Supplementary Material is available at NAR Online.

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