872–880 *Nucleic Acids Research, 2007, Vol. 35, No. 3* *Published online 23 January 2007*
doi:10.1093/nar/gkl1100

Deletion of a trypanosome telomere leads to loss of silencing and progressive loss of terminal DNA in the absence of cell cycle arrest

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Eukaryotic chromosomes are capped with telomeres which allow complete chromosome replication and prevent the ends from being recognized by the repair machinery. The African trypanosome, *Trypanosoma brucei*, is a protozoan parasite where antigenic variation requires reversible silencing of a repository of telomere-adjacent variant surface glycoprotein (*VSG*) genes. We have investigated the role of the telomere adjacent to a repressed *VSG*. In cells lacking telomerase, the rate of telomere-repeat loss appeared to be inversely proportional to telomere length. We therefore constructed strains in which a single telomere could be immediately removed by conditional I-*Sce*I meganuclease cleavage. Following telomere deletion, cells maintain and segregate the damaged chromosome without repairing it. These cells continue to proliferate at the normal rate but progressively lose terminal DNA at the broken end. Although sirtuin-dependent repression is lost along with the telomere, *VSG*-silencing is preserved. The results provide direct evidence for telomere-dependent repression but suggest a telomere-independent mode of *VSG*-silencing. They also indicate the absence of a telomere-loss checkpoint in *T. brucei*.

INTRODUCTION

Telomeres are nucleoprotein complexes that form protective caps at the natural DNA double strand breaks (DSBs) found at chromosome ends (1). Telomere loss or DSBs elsewhere in the genome are potent DNA damage response (DDR) stimuli (2). In proliferating cells, even a single DSB triggers a DDR (3). The classical DDR involves cell-cycle arrest and repair and ensures the maintenance of genome integrity. G_2/M arrest can last for the length of several cell cycles (4) and, if damage is extensive and/or not repaired, can be followed by senescence or apoptosis. Consequently, if DDR pathways are defective, DSBs can have profound consequences including chromosome rearrangement and oncogenesis. Telomeres also influence sister chromosome separation (5) and recruit a histone deacetylase, sirtuin complex that represses the expression of adjacent genes (6). Thus, telomeres protect chromosome ends, influence chromosome dynamics, repress local gene expression and mask the DNA end from damage surveillance (7).

In the mammalian host bloodstream, *Trypanosoma brucei*, the African trypanosome, escapes the immune response through mono-telomeric expression and reversible silencing of variant surface glycoprotein (*VSG*) genes. Antigenic variation can occur via rare epigenetic switching to alternative telomeric expression sites (ESs) (8). Unusually, RNA polymerase I, a polymerase generally confined to ribosomal RNA genes in eukarya, transcribes the active *VSG* towards a telomere. A typical ES is polycistronic with the promoter ~50 kbp from the telomere-adjacent *VSG* which produces approximately 10% of total cellular protein. *Trypanosoma brucei* telomeres repress RNA polymerase I transcription within a few thousand base pairs of the telomere in a sirtuin-dependent manner but ES silencing is sirtuin-independent (9,10).

In total, there are probably more than 200 telomeres in *T. brucei*; the majority cap the minichromosomes (11). The specialized reverse-transcriptase known as telomerase extends *T. brucei* telomeres by adding T_2AG_3 repeats (10,11). Human chromosome ends are protected by a six subunit complex known as shelterin (12) and an apparent homologue of one of the subunits, TRF2, has been characterized in *T. brucei* (13). t-loops are another feature shared by human and *T. brucei* telomeres (14). Unlike the situation in many organisms however, there is no rigorous telomere length monitoring in *T. brucei*. Telomerase-dependent extension is minimally 6 bp/population doubling (PD) but is increased by transcription at the chromosome end (10,15,16), a feature that may only affect the single active ES telomere in bloodstream form cells. Growth and occasional deletions are responsible for highly variable telomere lengths ranging from less than one to more than 15 kbp in each cell (11).

Telomeres play key roles in regulating virulence gene expression and recombination in a range of pathogens and are thought to play important roles in *T. brucei* (17). We established tetracycline-inducible I-*Sce*I expression and used this system to delete a specific *T. brucei* telomere. The resulting cells allowed us to explore the role of the

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telomere in gene expression and in protecting the chromosome end from nucleolytic degradation and the DDR. We show that telomeric DNA represses the expression of adjacent genes and protects terminal DNA from degradation. Telomeres do not appear to be required for *VSG* silencing however and, remarkably, *T. brucei* telomere deletion does not trigger a DDR.

MATERIALS AND METHODS

Trypanosoma brucei growth and manipulation

All bloodstream form cells were derived from Lister 427 MITat1.2 (clone 221a) and grown in HMI-11. Cells were transformed with linear DNA constructs as previously described (18). Drug selection was applied as described (19) and cell density was determined using a haemocytometer. Tetracycline (Tet) was from Sigma and was removed from the medium by washing the cells twice in HMI-11. To estimate *VSG221* reactivation frequency, VSGX-expressing cells were pre-grown in Tet (1 μg/ml) for 24 h and then 1.5×10^6 cells were distributed into each well of a 24-well plate in medium containing Tet and puromycin (5 μg/ml). Wells were inspected for growth after 6 days and positive cultures were examined for VSG221 expression.

Plasmid constructs

pHD1313 (20) was used to introduce the tetracycline-repressor (*TetR:BLE*) into *T. brucei*. pESP^i*PAC* and pESP^i*RFP:PAC* were used to introduce a Tet operator (*TetO*) followed by a *PAC* gene or an *RFP:PAC* fusion downstream of the *VSG221* ES promoter respectively. The upstream and downstream targeting fragments in these two constructs were amplified by PCR from pRK8(+) (21) using the VTet5U (GATC*GAGCTC*GCA TTTACAACTGATGCTATA), VTOp3U (GATC*GGAT CC*ACTCTATCATTGATAGGGTGaGTCTGATATC CTAGATTTC) primer pair and from BAC TBH25N7 (22, Acc:AL671259) using the VTet5D (GATC*GGGC CC*GGTGTGGCGGACGTCTCG), VTet3D (GATC*GG TACC*GGCTGGATCAATTCCTGCAG) primer pair, respectively. The VTOp3U primer encodes the *TetO* sequence (underlined) and the 'a' represents the transcription start site (21). Relevant restriction sites encoded by the primers are in italics. The fragments were cloned either side of a *PAC* gene flanked by *T. brucei* mRNA processing signals. The *RFP:PAC* fusion was assembled by ligating *RFP* and *PAC* genes PCR amplified using the RFP5Fu (GATC*AAGCTT*atgGTGCGCTCCTCCAAG), RFP3Fu (GATC*GCGGCCGC*ACAGGAACAGGTGGTGGCG) primer pair with pDsRed1-C1 (Clontech) and the PAC5Fu (GATC*GCGGCCGC*ACCGAGTACAAGCCC ACG), TUBIR3Fu (GATC*GGGCCC*CCCCTCGACT) primer pair respectively. The *RFP* start codon is indicated in lower-case. *BLA* and *HYG* constructs were used to disrupt the telomerase gene (GeneDB ID: Tb11.01.1950) generating homozygous *telomerase*$^{\triangle 222\text{-}954}$ null strains (see 10). The I-*Sce*I cleavage site was added to the telomere-mediated fragmentation construct, pTMF (16), by annealing the SceF (GGCC*TAGGGATAACAGGGTAAT*) and SceR (GGCC *ATTACCCTGTTATCCCTA*) primers and ligating the product to *Not*I-digested pTMF. The I-*Sce*I ORF was amplified by PCR from pSCM525 using the SceNLS (GATC*AAGCTT*atgCCAAAGAAGAAGC GAAAGGTA – I-*Sce*I codons 2–9) and Sce3 (GATC*GGATCC*tta - I-*Sce*I codons 231–237) primers such that an SV40 nuclear localization signal (PKKKRKV, codons underlined) was encoded at the *N*-terminus. Start and stop codons are indicated in lower-case. The recombinant I-*Sce*I gene was ligated to a construct for Tet-on inducible expression from a *RRNA* promoter. This vector is used to integrate the expression cassette at *T. brucei RRNA* loci in cells expressing TetR (19). *BLA* and *HYG* constructs were used to disrupt the *SIR2rp1* gene (GeneDB ID: Tb927.7.1690) generating homozygous *sir2rp1*$^{\triangle 1\text{-}285}$ null strains (9).

DNA and RNA analysis

Southern and northern analyses were carried out according to standard protocols. Telomere healing locations were mapped using genomic DNA from *T. brucei* clones that survived *PAC* selection (0.2 μg/ml puromycin) after I-*Sce*I induction; *PAC* at a repressed ES was sufficient to confer resistance to this concentration of puromycin. Briefly, the 221B (GGCGTTACCAAGCTTGTTGA) and Tel2 $(ACCCTA)_5$ primers were used to amplify the healed end by PCR and the 221Uxho primer (N_{10}TTTCCCC CCTCAAATTTCCCC) was used for sequencing.

Protein analysis

Western blotting and fluorescence microscopy were carried out using standard protocols as previously described (9). Briefly, extracts of total cell protein were separated on SDS-polyacrylamide gels and stained with coomassie blue (Sigma) or subjected to western blotting using rabbit-αVSG221 and an ECL kit (Amersham). For immunofluorescence analysis, cells were labelled using rabbit αNPT-II (Europa Bioproducts) primary antibody and fluorescein-conjugated goat-αrabbit secondary antibody (Pierce). Cells were mounted in Vectashield (Vector Laboratories) containing 4,6-diamidino-2-phenylindole (DAPI) and images were captured using an Eclipse E600 microscope with digital camera (Nikon) and Metamorph software.

RESULTS

Derivation of isogenic strains with active and repressed *VSG* expression sites

Approximately twenty telomeric loci are occupied by similar polycistronic *VSG* expression sites (ESs) in *T. brucei*. In bloodstream form cells, all but one are reversibly repressed and the frequency of switching is very low, typically $<10^{-6}$/PD (1 PD ~7.5 h). For most studies, we use cells expressing *VSG221*, a single copy *VSG* gene on chromosome VIa (23). To facilitate studies on this well characterized ES in its repressed state, we expressed the tetracycline repressor (TetR) and, in the presence of

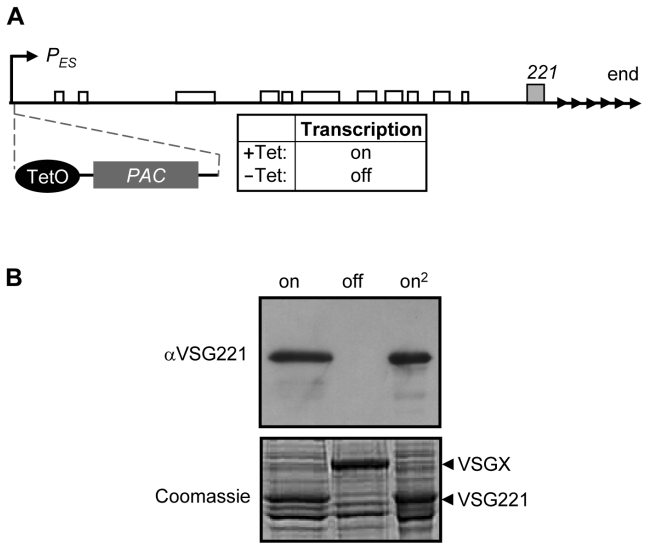

Figure 1. Inactivating the *VSG221* ES. **(A)** The *VSG221* ES was rendered tetracycline (Tet)-dependent by expressing the Tet-repressor and inserting a Tet-operator immediately downstream of the ES promoter. *PAC*, puromycin *N*-acetylase. **(B)** VSG analysis. western blotting indicates that the modified *VSG221* ES is active in the presence of Tet (1 μg/ml, on). Following Tet removal, cloned survivors were found to have switched to an alternative *VSG(X)* ES (off). *VSG221* could be reactivated (on^2) in the presence of Tet and puromycin (see Materials and Methods) indicating that repression is reversible. Arrowheads indicate bands representing the abundant VSGs in the coomassie-stained gel.

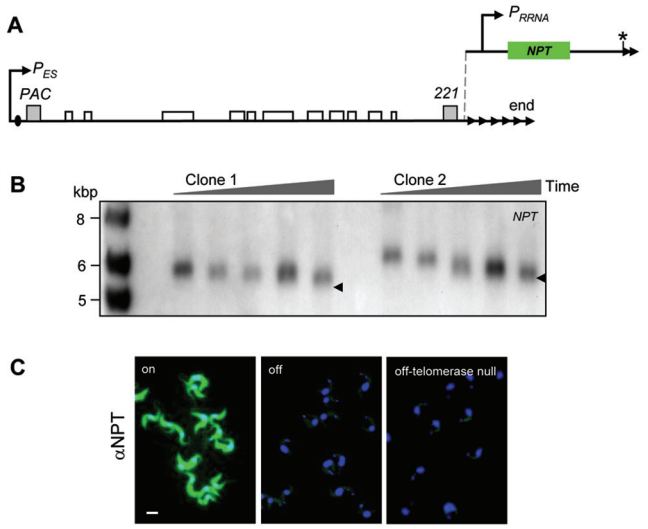

Figure 2. Telomerase is not required for *VSG*-associated repression. **(A)** A telomere-mediated fragmentation construct, pTMF was used to insert a reporter gene (*NPT*, neomycin phosphotransferase) driven by a *RRNA* promoter (P_{RRNA}) 5 kbp from a *de novo* telomere at a repressed ES in telomerase heterozygotes. Transformation using this vector results in deletion of the native telomere and formation of a new telomere by elongation of a $(T_2AG_3)_{33}$ 'seed'. **(B)** Following disruption of the second allele of telomerase, Southern blotting indicates the rate of telomere-repeat loss in the telomerase null strains. DNA extracted at ten-day intervals was digested with *Hpa*I, an enzyme that cleaves immediately upstream of *NPT* and 5.1 kbp from the telomere, non-telomere junction (asterisk). The arrowheads indicate the expected size if DNA were lost at 4 bp/PD. **(C)** Immunofluorescence analysis indicates that the *NPT* reporter remains repressed when adjacent to a short, shrinking telomere. NPT signals are green; blue, DAPI-stained DNA. Scale bar, 5 μm.

Tet, inserted a Tet operator (see 24) adjacent to the *VSG221* ES promoter (Figure 1A). TetR-binding in this position is expected to interfere with ES transcription. The resulting cells continued to express *VSG221* in the presence of Tet but only cells that switched to an alternative *VSG* expression site survived when Tet was removed from the growth medium (see Figure 1B). This is explained by the blockage of *VSG221* ES transcription that occurs when TetR binds *TetO* and because *VSG* expression is essential for viability (25). *VSG221* was reversibly repressed in the switched clones and, under permissive conditions (+Tet), yielded five puromycin resistant clones from 3.6×10^7 cells (equivalent to $<2 \times 10^{-7}$/PD). All four puromycin resistant clones tested had also reactivated VSG221 and one of these clones is shown in Figure 1B. Thus, the approach allowed the generation of isogenic strains with active and repressed *VSG221* ESs with unique markers at each end, *PAC* adjacent to the promoter and *VSG221* adjacent to the telomere (Figure 1A). In the experiments described below, the *VSG221*-associated telomere was manipulated in '221-repressed' strains while '221-active' strains served as controls when assessing loss of repression.

Short telomeres shrink at a diminished rate in telomerase null mutants

To explore the role of telomeric repeats at a repressed ES we began by engineering a short telomere in telomerase null mutants, where terminal DNA is lost at a rate of ~4 bp/PD (10,11). To establish these short and shrinking telomeres we disrupted one allele of telomerase, used a telomere-mediated fragmentation (pTMF) construct to seed a telomere with 33 T_2AG_3 repeats at the repressed *VSG221* locus (Figure 2A) and then disrupted the second allele of telomerase. The TMF construct also integrates an *NPT* reporter adjacent to the telomere (Figure 2). The reporter is subject to position effect repression but generates sufficient antibiotic resistance to allow recombinant clone selection. Clones 1 and 2, generated by this approach, initially had *VSG221*-associated T_2AG_3-repeat tracts of ~800 bp and ~1.2 kbp respectively (Figure 2B). If the rate of DNA loss remained constant at ~4 bp/PD, we calculated that clone 1 repeats would be completely lost following 200 PDs (~60 days). We monitored telomere length using a Southern blot assay over a 40-day period (Figure 2B). Repeat loss in clone 2 was only marginally lower than expected but clone 1 lost only ~300 bp, consistent with an average rate of <2.5 bp/PD. The results suggest that the rate of DNA loss may be inversely proportional to telomere-length. This rate of DNA loss is too slow to be explained by the inability of the conventional DNA replication machinery to fully replicate chromosome termini and indicates a telomerase-independent, possibly replication-slippage mechanism for telomeric DNA replication. These findings are entirely consistent with a recent report of

telomerase-independent stabilization of short *T. brucei* telomeres (15). Telomere-proximal *NPT* reporter repression was maintained in these telomerase null cells with short and shrinking telomeres (Figure 2C).

Telomere cleavage with I-*Sce*I

Our attempts at complete telomere removal using telomerase disruption were complicated by the diminished rate of DNA loss at a short telomere. We therefore decided to use a different approach involving complete and immediate *in vivo* telomere removal. I-*Sce*I is a site specific double strand homing endonuclease encoded by a mitochondrial group I intron of *Saccharomyces cerevisiae* (26). The sequence recognized and cleaved by the enzyme is 18 bp in length and is expected to appear only once in every 6.87×10^{10} bp. This exceeds the size of the *T. brucei* genome by >2000-fold. We introduced an I-*Sce*I cleavage site at the telomere – non-telomere junction in the pTMF construct (see asterisk in Figure 2A). Specific I-*Sce*I cleavage of this construct was verified *in vitro* (data not shown) using recombinant I-*Sce*I (New England BioLabs) and the DNA was targeted to the repressed *VSG221* telomere. The I-*Sce*I ORF was then fused to an SV40 nuclear localization signal and cloned in a Tet-on inducible expression construct (see Materials and Methods). This construct was targeted to a transcriptionally silent locus in cells with the engineered I-*Sce*I site.

To determine whether I-*Sce*I would cleave the repressed *VSG221*-associated telomere, genomic DNA samples were prepared at a number of time points up to 72 h following induced I-*Sce*I expression. The samples were digested with *Hpa*I, which generates a terminal fragment encompassing the *NPT* gene. Southern blotting using an *NPT* probe revealed the expected terminal fragment at ~7 kbp which was diminished in intensity over the time-course of the experiment concomitant with the appearance of the expected 5.1 kbp cleavage product (Figure 3). Significant I-*Sce*I cleavage had occurred only 3 h after induction. Importantly, phosphorimager analysis indicated almost complete (>95%) cleavage 24 h after induction (Figures 3 and 4B) showing that I-*Sce*I expression was tightly regulated. Thus, I-*Sce*I accesses and specifically cleaves *in vivo* chromatin templates in *T. brucei*. Using inducible I-*Sce*I, endonuclease activity can be rapidly delivered to the entire population of cells in a synchronous manner.

A DNA double-strand break (DSB) triggers cell cycle arrest and DNA repair in other cell-types (reviewed in 3). Despite DSBs in >95% of cells, we detected no significant impact on cell growth during the time-course of this experiment which spanned ~10 PD or >1000-fold increase in cell number (Figure 3). Indeed, we saw no evidence for cell cycle arrest or delay following telomere deletion in several independent strains (data not shown).

A minority of cells heal the broken chromosome following telomere deletion

Chromosome healing at a cleaved telomere is very efficient in mammalian cells (27) and terminal G-rich or T_2AG_3-repeat-like sequences can serve as substrates for

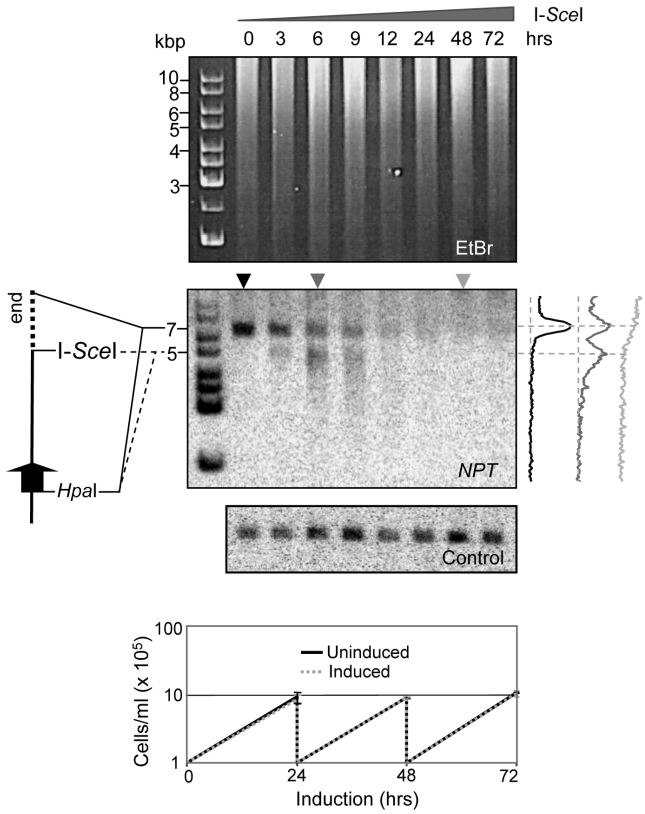

Figure 3. Induction of I-*Sce*I expression results in specific telomere cleavage. Strains with a Tet-inducible copy of I-*Sce*I and a unique I-*Sce*I cleavage site adjacent to the *VSG221* telomere were grown in Tet (1 μg/ml) for three days. DNA samples extracted at various intervals were digested with *Hpa*I (top panel; EtBr, ethidium bromide stain) and analysed by Southern blotting. The map to the left indicates the expected 7 kbp terminal fragment and the 5.1 kbp, I-*Sce*I cleaved product. The phosphorimager scans to the right (0, 6 and 48 h) further illustrate the presence of the expected fragments. Blots hybridized with a control (GeneDB ID: Tb927.7.1690) probe indicate specific cleavage at the I-*Sce*I site. The growth curve below the blots indicates no significant impact on cell growth during the time-course of the experiment. Error bars: one standard deviation. $n = 2$.

de novo telomere seeding in *T. brucei* (16). In addition, telomere-repeat-like sequences adjacent to DSBs act as 'anti-checkpoints' in *S. cerevisiae* by seeding *de novo* telomeres in the absence of a conventional DNA damage response (28).

To determine the fate of terminal sequences following telomere deletion, we carried out further Southern analysis with the same samples used in Figure 3, but now using a restriction enzyme that generates discrete *NPT* and *VSG221* fragments (indicated in Figure 4A). More than 95% of the *NPT* signal and ~90% of the *VSG221* signal is lost after 48 h under inducing conditions (Figure 4A and B). Almost complete loss of the *NPT* signal and persistence of a residual and undiminished *VSG221* signal between 48 and 72 h (Figure 4B) suggests significant healing between the *NPT* and *VSG221* genes. The only T_2AG_3-like tract in the *VSG221* ES, that might be expected to serve as a substrate for *de novo* telomere seeding (16), lies between the *NPT* and *VSG221* genes

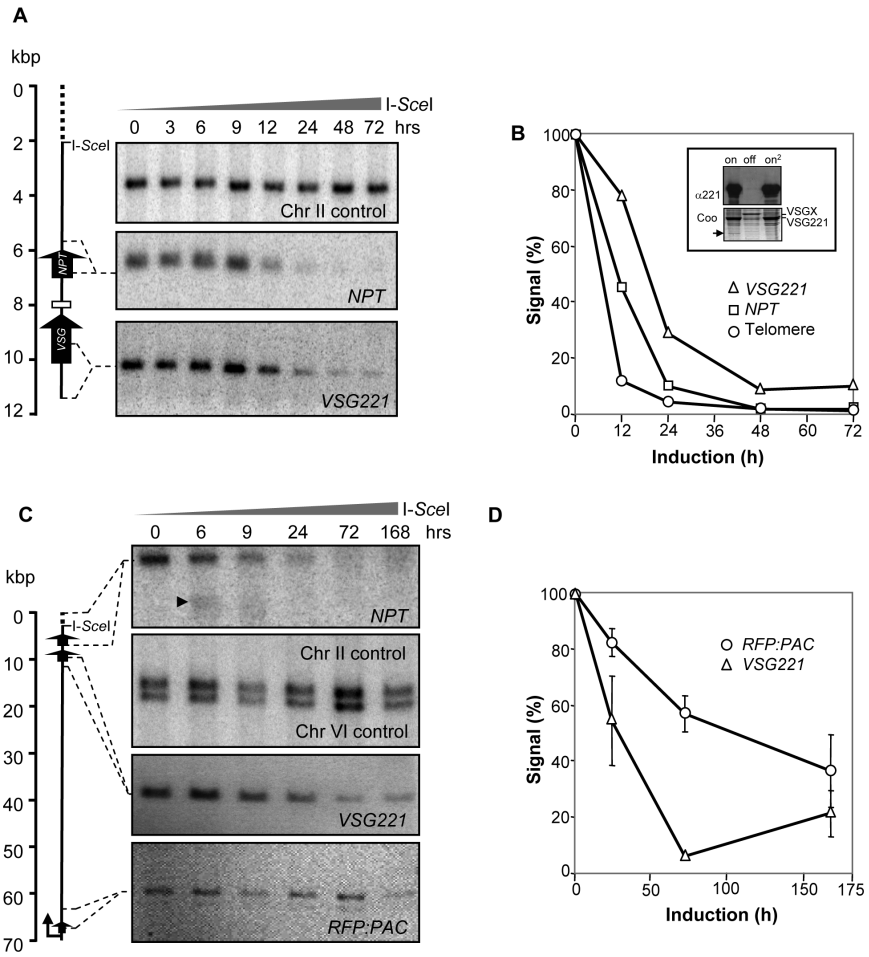

Figure 4. A damaged chromosome is maintained but loses terminal DNA. Cells with Tet-inducible I-*Sce*I and a unique I-*Sce*I cleavage site adjacent to the *VSG221* telomere were grown in Tet (1 μg/ml) for up to one week. DNA samples extracted at various intervals were digested with *Pst*I (**A** and **C**) or *Hpa*I (top panel in **C**) and analysed by Southern blotting. Data for the plots shown in **B** and **D** were derived by phosphorimager analysis of the blots in Figures 3 and 4A, and 4C respectively. The maps to the left indicate the location of relevant probes and the I-*Sce*I site. **(A)** Location of a T_2AG_3-repeat-like tract is indicated by an open box. **(B)** Inset, VSG analysis in switched cells. Labels and clone derivation as in Figure 1B. Coo, coomassie-stained gel. Arrow, NPT band, not seen in 'off' or 'on^2' cells. **(C)** Arrowhead indicates 5.1 kbp cleavage-product. The chromosome II (GeneDB ID: Tb927.2.2190) and chromosome VI control probes (GeneDB ID: Tb927.6.910) were sequentially hybridized. **(D)** Error bars: one standard deviation. $n = 2$.

(see Figure 4A and accession number AJ271641, nt 211–322; 46 bp of $T_{1\text{-}2}AG_{1\text{-}6}$ followed by 10.5 degenerate repeats; T_2AG G/A G/A/C, 106 bp in total). Several independent healing events were mapped (data not shown, see Materials and Methods) and were all found to occur within this tract indicating that the T_2AG_3-like sequence serves as a substrate for *de novo* telomere seeding in ~10% of cells. *VSG221* remained reversibly repressed in these 'healed' clones as demonstrated by the emergence of puromycin-resistant cells that also reactivated *VSG221* (Figure 4B inset). Consistent with loss of the terminal DNA segment, the NPT band is not detected in these reactivated, 'on^2' cells.

Progressive loss of sub-telomeric sequences following telomere deletion

The results described above indicate that only approximately 10% of cells heal the chromosome end between the *NPT* and *VSG221* sequences. Thus, telomere deletion leads to the loss of *NPT* and *VSG221* DNA in the majority of cells. From the data shown in Figure 3, we noted that the 5.1 kbp terminal fragment from which the telomere had been cleaved became relatively less intense by 12 h and also the presence of a 'smear' of smaller *NPT* fragments. This suggested that, rather than being repaired, the DNA may be degraded from the cleaved end by native exonuclease(s). The data shown in Figure 4A and B indicates that telomere deletion is indeed followed by diminishing *NPT* signal which is then followed by diminishing *VSG221* signal. Twelve hours after I-*Sce*I induction, for example, ~90% of the cells have a cleaved telomere, ~50% of cells have lost the *NPT* gene and ~20% have lost the *VSG221* gene (Figure 4B). The relative rates of loss of the *NPT* and *VSG221* signals indicate that DNA is progressively degraded from the DSB. The interval between approximately 50% telomere

cleavage (data derived from blot in Figure 3) and 50% diminished signals for the *NPT* gene located at 4 kbp from the cleavage site is ~5 h (Figure 4B). Although variable within the population, this roughly translates to a rate of DNA loss at a broken end of 100 bp every 7.5 min or 6 kbp/PD.

Polycistronic *VSG* ESs extend ~50 kbp from the promoter to the telomere (see 29) and we predict a length of ~65 kbp for the *VSG221* expression site following addition of the *NPT* and *PAC* cassettes (see Figure 2A). To facilitate expression monitoring at both ends of the *VSG221* ES (see below), we replaced the promoter-adjacent *PAC* gene (see Figure 1A) with a red fluorescent protein (*RFP:*)*PAC* fusion gene. Using two independent clones with reversibly repressed *RFP:PAC* and *VSG221* genes, we asked whether progressive DNA loss continues through the ES towards the core of the chromosome following telomere deletion. Both clones were monitored for one week after I-*Sce*I induction, equivalent to >20 PD or $>10^6$-fold increase in cell number. Southern blots showed specific and inducible telomere cleavage (Figure 4C, top panel). The *VSG221* ES is on chromosome VIa (23) and signals for genes within the core of chromosomes VI and II were stable during the course of this analysis (Figure 4C). *VSG221* and *RFP:PAC* analysis indicated loss of the *VSG221* signal prior to the *RFP:PAC* signal (Figure 4C and D) and DNA loss consistent with a rate of ~6 kbp/PD (see above). The analysis confirms that terminally deleted *T. brucei* chromosomes are replicated and segregated without being repaired.

Telomere deletion leads to loss of sirtuin-dependent repression without ES de-repression

In bloodstream-form *T. brucei*, ectopic promoters adjacent to telomeres are subject to sirtuin-dependent repression while a sirtuin-independent mechanism maintains *VSG* ES silencing (9). The *NPT* signal in the *sir2rp1* null strain (Figure 5C) confirmed sirtuin-dependent repression of the current *NPT* reporter but phosphorimager analysis revealed only 5% NPT mRNA in *SIR2rp1*-null mutants relative to the active ES reflecting the quantitatively more significant, sirtuin-independent mode of *VSG* ES silencing.

Replication and segregation of a chromosome lacking a telomere provided an opportunity to directly test the impact of the telomere (and potentially other associated factors) on local gene expression. We estimated the mean DNA loss over time following I-*Sce*I induction and this is illustrated in Figure 5A. Then, using individual cell, (immuno)fluorescence analysis, we looked for de-repression over a three-day time-course. This analysis revealed transient and non-synchronous de-repression of *NPT* at 6 and 9 h but no de-repression of *VSG221* and no de-repression of *RFP* (Figure 5B, the fields shown are representative of the population and the results were confirmed with an independent clone). Transient de-repression of *NPT* is consistent with the fact that it is adjacent to a 'broken' end for only a short time before being lost itself (see Figure 5A). These results suggest

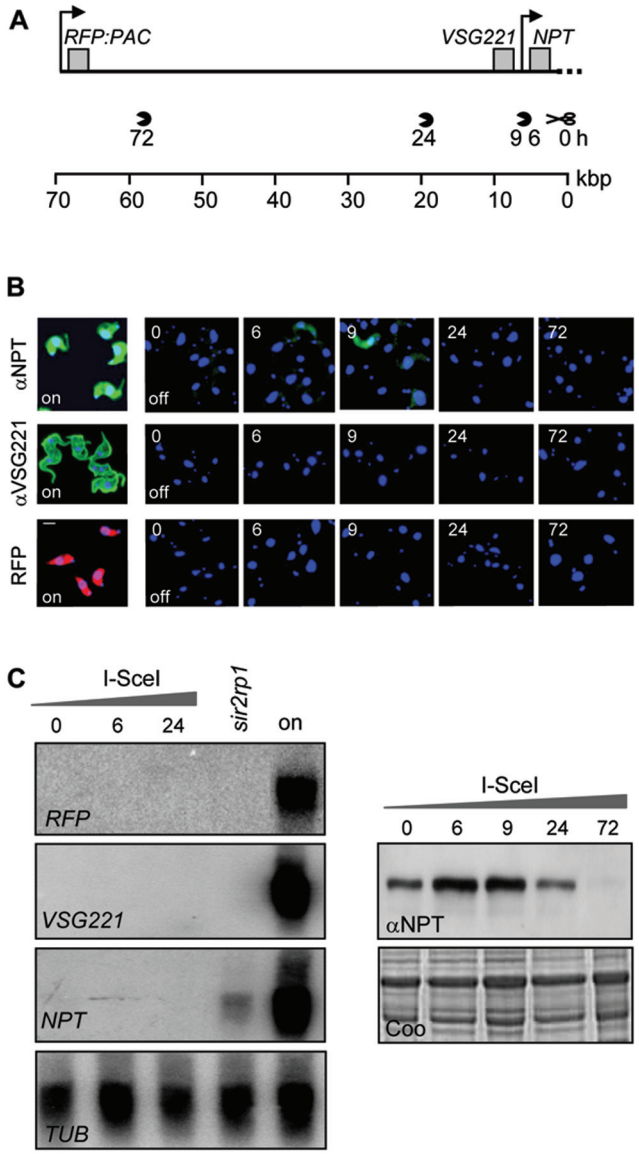

Figure 5. The effect of telomere deletion on local gene expression. (**A**) The schematic map of the *VSG221* ES illustrates the reporter genes and the mean extent of DNA loss at different time points following I-*Sce*I induction (based on DNA loss at 6 kbp/PD). Reporter expression was assessed following I-*Sce*I induced (+Tet) telomere deletion. The *VSG221* ES is permissive for activation under these conditions (see Figure 1). (**B**) Immunofluorescence (NPT, VSG221) and fluorescence (RFP) analysis reveals transient, non-synchronous loss of telomeric silencing (NPT) but otherwise maintained ES repression (VSG221, RFP). Numbers indicate hours following I-*Sce*I induction. NPT and VSG signals are green; RFP signals are red; blue, DAPI-stained DNA. Scale bar, 5 μm. (**C**) Northern (left-hand side) and western (right-hand side) blot analysis. Numbers indicate hours following I-*Sce*I induction. mRNA expression was also analysed in *sir2rp1* null mutants and in cells with an active *VSG221* ES (on). For northern analysis, tubulin (*TUB*) is used as a loading control and for western analysis, an equivalent coomassie (Coo) stained gel is shown as a loading control.

that the sirtuin effect spreads from within telomeric repeats and that telomere deletion leads to loss of sirtuin-dependent repression. Since transcription from the ES promoter remains repressed following telomere

deletion, the results also suggest the absence of any additional repressive effect spreading from the telomere. In fact, despite the longer time frame between telomere deletion and *RFP* loss (Figure 5A), *RFP* de-repression is not seen at any point during progressive loss of ES sequences. This suggests that the *VSG* and associated sequences are also not required for maintained ES silencing.

Maintained silencing of *RFP* and *VSG221* was confirmed by northern blotting with RNA extracted at a number of time points following I-*Sce*I induction (Figure 5C). Although the low-abundance *NPT* mRNA is not detected by northern blot following I-*Sce*I induction, we were able to confirm NPT de-repression by western blot (Figure 5C). The results are consistent with sirtuin-dependent telomeric gene silencing but suggest that ES silencing is sirtuin and telomere-independent.

DISCUSSION

We removed a *T. brucei* telomere *in vivo* to explore its role in gene repression and in protecting the chromosome end from endonucleolytic degradation and the DDR. Initially, we demonstrated terminal DNA loss at <2.5 bp/PD in telomerase null strains, consistent with a recent report showing that short *T. brucei* telomeres are stabilized in a telomerase-independent manner (15). Telomere removal was then achieved using meganuclease cleavage, and subsequent experiments using unique sub-terminal reporters indicated telomere-dependent gene-repression and telomere-dependent protection of adjacent DNA from rapid degradation. Telomere deletion has little impact on *VSG* ES silencing however and, surprisingly, does not trigger a DDR in *T. brucei*.

Telomeres provide substrates for telomerase and are essential for maintaining chromosome stability (1). Whereas *T. brucei* telomerase null strains lose telomeric DNA at a rate of ~4 bp/PD or less, a broken chromosome end progressively loses terminal DNA at a rate averaging 6000 bp/PD. This >1000-fold higher rate of DNA-loss at an uncapped chromosome end illustrates the protective effect of T_2AG_3 repeats, even in the absence of telomerase. The data reveal nucleolytic activity that is normally excluded from the chromosome end, likely due to the formation of t-loops (14) stabilized by telomere-associated factors (13). Loss of these structures exposes substrates for nucleolytic degradation.

The two major pathways of DNA repair are non-homologous end-joining (NHEJ) and homologous recombination (HR). Single-celled organisms rely more heavily on HR (30) and, in haploid yeast, sub-telomeric DSB-repair more commonly involves HR while NHEJ efficiency correlates with distance from the chromosome end (31). Trypanosomatid genome sequence analysis revealed components of most repair pathways and also a number of NHEJ components although H2AX is notably absent (32). Many DNA repair factors are associated with telomeres (2) and this also appears to be the case in *T. brucei* where the Ku70/80 heterodimer (33,34) and a sirtuin (9) have been shown to influence telomere extension and silencing respectively. *Trypanosoma brucei* cells lacking RAD51 (35,36), Mre11 (37,38) or the sirtuin (9) do not display delay in progression through the cell cycle but they are all more sensitive to DNA damage induced by ionizing radiation or radiomimetic drugs.

In striking contrast to the situation in most other eukaryotes, a terminally truncated and un-repaired *T. brucei* chromosome is maintained and segregated with no significant impact on growth. In other proliferating cells, DSBs induce a DDR (39). In *S. cerevisiae*, for example, chromosome ends with either few or no telomeric repeats (1), and even a DSB in a dispensable plasmid, trigger cell-cycle arrest and DNA repair (40). Some cells do recover but go on to lose the affected chromosome (1). Similarly, only fused or repaired chromosomes are recovered when a DSB is introduced near a telomere in mammalian cells (41). The absence of a conventional telomere checkpoint and consequent failure to repair a truncated chromosome may eventually lead to chromosome loss and cell death. Although a large body of evidence suggests that telomere loss elicits the same response as a DSB elsewhere in the genome (2), it has been proposed that the telomere checkpoint is distinct from the DNA damage checkpoint in *S. cerevisiae* (42). In addition, terminally truncated telomeres can be protected in a sequence-independent manner in *Drosophila* (43). Thus, I-*Sce*I-cleaved *T. brucei* telomeres may recruit or retain factors that protect the terminus from the DDR. It will be important to introduce DSBs elsewhere in the *T. brucei* genome to assess how the DDR machinery responds to damage elsewhere.

Our data show that, before being lost, a reporter 5 kbp from the deleted telomere is de-repressed and this provides direct evidence for telomere-dependent gene repression in *T. brucei*. We show that this same reporter is subject to sirtuin-dependent silencing. In *S. cerevisiae*, telomeric silencing is dependent upon recruitment of a histone deacetylase, sirtuin-complex (6) and we speculate that it is the sirtuin effect that is lost along with the telomere in *T. brucei*. Our results suggest however that *VSG* ES silencing, which relies upon transcription attenuation (44), is not only sirtuin-independent (9) but is also telomere-independent. Thus, telomeric repression and ES silencing appear to be mechanistically distinct.

Although telomeres are not required to maintain ES silencing, they do repress genes in the vicinity of the *VSG* so that they may act in a cooperative manner to fully silence the large *VSG* repertoire. This includes the monocistronic, 'metacyclic' *VSG* ESs that are transcribed from promoters in close proximity to the telomere during the first week of a mammalian infection (8). In addition, we cannot rule out a role for the telomere in establishment of ES silencing since telomere deletion may not reverse telomere-mediated DNA or chromatin modification, for example. The telomere may also play a role in active ES transcription and/or recombination.

Trypanosoma brucei telomeres protect chromosome ends from degradation and repress local gene expression but appear to have little impact on ES silencing or chromosome segregation and fail to trigger a DDR when

deleted. Using meganuclease manipulation, it should now be possible to examine the consequences that follow active ES deletion and the introduction of DSBs at non-telomeric loci.

ACKNOWLEDGMENTS

We thank Bernard Dujon (Pasteur Institute, Paris) for the I-*Sce*I plasmid, pSCM525, Gloria Rudenko (Oxford, UK) for the BAC (TBH25N7) and John Kelly (LSHTM) for critical reading of the manuscript. This work was supported by a Research Career Development Fellowship to DH (052323) and a Project Grant (069909), both from The Wellcome Trust.

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