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Short communication

The mitotic stability of the minichromosomes of *Trypanosoma brucei*

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The principal immune-evasion strategy of the extracellular protozoan parasite *Trypanosoma brucei* is one of antigenic variation (reviewed in [1–4]). In the mammalian host bloodstream, the parasite adopts a dense cell surface coat composed predominantly of a single immunogenic variable surface glycoprotein (VSG). Periodic switching of the expressed VSG gene results in successive changes in the antigenic identity of cells, prolonging parasitaemia and increasing probability of transmission. To this end, the organism maintains a population of around 100 small linear chromosomes which carry a library of divergent VSG genes at subtelomeric locations (see [5,6]). These minichromosomes (MCs) are between 30 and 150 kb in size and consist of a large palindromic core region made up of 177 bp tandem repeats (Wickstead et al., submitted for publication), subtelomeric VSG genes [7,8], canonical eukaryotic telomeres [9], and smaller portions of other repetitive DNAs (for example, 70 bp repeats [10]). They are also transcriptionally silent—minichromosomal VSG genes only become active upon duplicative transposition to active VSG expression sites (VSG-ESs) occurring on larger chromosomes.

Central to the role of MCs in *T. brucei* is the diversity of VSG genes that they carry. A large diversity within the population of MCs can be maintained only if individual MCs are segregated with fidelity at mitosis—without mitotic fidelity, stochastic impetus will inevitably reduce the variety of VSG genes carried by any one cell. This requires a partitioning mechanism for MCs, and in situ hybridisation analysis of MCs and intermediate-sized chromosomes (ICs) as a population has revealed an association with the mitotic spindle that is required for accurate segregation [11]. Intriguingly, this partitioning mechanism appears not to involve classical pole-to-kinetochore interactions, leading to the proposal of a lateral interaction model for the segregation of MCs (and possibly some larger chromosomes) in *T. brucei* [12].

The intimate association between MCs and antigenic variation and the mechanism of partitioning make the mitotic segregation of small chromosomes (and factors that affect it) an interesting area of trypanosome biology. Moreover, questions as to the precise stability of MCs in *T. brucei* have become sharpened recently by two factors: (1) the demonstration that repeat or *VSG* loci on MCs may be used to improve the regulation of a tetracycline-inducible expression system [13], and (2) the subsequent advent of an MC-integrating RNA interference (RNAi) vector that appears to aid the generation of inducible RNAi cell lines ([13]; G. Rudenko, personal communications).

The evidence available to date are suggestive of a certain degree of fidelity to the segregation of *T. brucei* MCs [9,11,14,15], compatible with the maintenance of a diverse VSG gene library. However, there are few data available which give direct estimates as to the accuracy of MC segregation and those data that do exist often conflict [9,15]. Here, we present quantitative data on the stability of MCs during somatic growth achieved by exogenously marking individual MCs. We also investigate some of the factors which might affect MC stability. We show that three marked MCs are extremely stable with respect to mitosis ($<10^{-3}$ losses cell^{-1} gen^{-1}), and that this stability is independent of the transcriptional state of the marker.

Abbreviations: GFP, green fluorescent protein; IC, intermediate chromosome; MC, minichromosome; rDNA, ribosomal RNA genes; RNAi, RNA interference; TAC, trypanosome artificial chromosome; VSG, variable surface glycoprotein; VSG-ES, VSG expression site

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1. Stability of exogenous minichromosomal markers

We introduced tetracycline-regulated transgenes into the MCs of procyclic form *T. brucei* cells by targeting either the very high copy number 177 bp repeat [16,17] or the single-copy *VSG*-S8 locus [14]. This was achieved by transforming the tetracycline repressor-expressing PTP cell line with the constructs pGad8-177 or pGad8-VSG-S8 as described in [18]. Fig. 1A shows the anatomy of these constructs. We have used these and similar vectors to create high-resolution physical maps of 17 MCs as well as partial maps of 2 ICs, as described elsewhere (Wickstead et al., submitted for publication). We assessed the mitotic stability of three of these mapped MCs: two MCs of 61 and 106 kb tagged at the 177 bp repeat, named MC^{177}-1 and MC^{177}-2; and one 91 kb MC tagged at the *VSG*-S8 locus, named MC^{VSG} (sizes inclusive of ~6 kb vector sequence). The structures of MC^{177}-2 and MC^{VSG} are typical of *T. brucei* MCs generally—exemplifying the canonical tripartite structure of large 177 bp repeat core, non-repetitive subtelomeres, and telomeres (Wickstead et al., submitted for publication). The structure of MC^{177}-1 is less common, in that the 177 bp repeat core is broken by other simple repeat DNA. Fig. 1B shows the structures of the three marked MCs used in this study.

Quantitative assessments of the mitotic stability of the drug resistance marker on marked minichromosomes were made as follows: procyclic cells containing the marked MCs were cultured under selection (1 μg ml^{-1} tetracycline and 20 μg ml^{-1} hygromycin B) for 7 days. Drugs were then washed out and each cell line split into three parallel cultures containing no drug, 1 μg ml^{-1} tetracycline (transcription without selection) or 1 μg ml^{-1} tetracycline and 20 μg ml^{-1} hygromycin B (transcription and selection). Following culturing for 36 days (~100 generations) under these conditions, the three parallel cultures were cloned by limiting dilution in the absence of selection. Positive wells were subcultured into normal medium (no drug) and also medium containing 1 μg ml^{-1} tetracycline and 20 μg ml^{-1} hygromycin B to test for drug resistance. All positive wells from cultures grown in the presence of selection tested positive for drug resistance after cloning ($n = 85$). Positive wells generated from untransformed PTP cells consistently showed sensitivity to drug ($n = 64$).

Table 1 shows the stability of the marked MCs under the conditions described. All three marked MCs were found to be extremely stable with respect to mitosis when maintained in the absence of selection and without transcriptional activation. In each case the marker was lost less than once in every

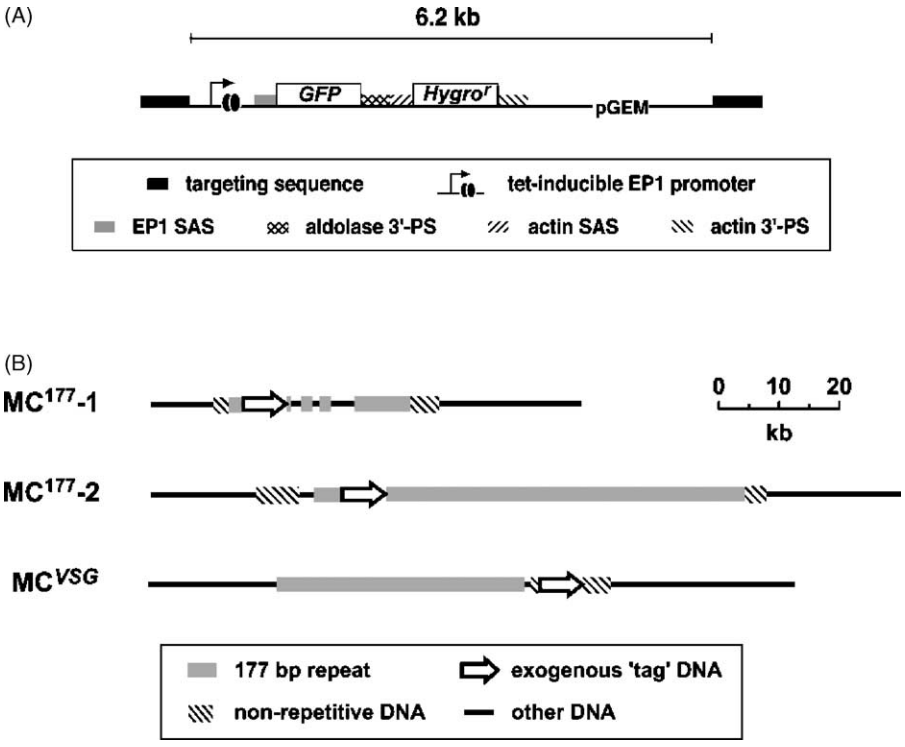

Fig. 1. (A) Anatomy of vectors for insertion of regulated marker genes into minichromosomes. Vectors contain a polycistron encoding a *GFP* and hygromycin B resistance gene (*Hygro^r*) under the control of a tetracycline-inducible procyclin EP1 promoter. Targeting sequences were: the 177 bp repeat (pGad8-177) or the single-copy minichromosomal *VSG*-S8 (pGad8-VSG-S8). See [18] for a full description. (B) The structures of the exogenously marked minichromosomes MC^{177}-1, MC^{177}-2 and MC^{VSG}. Structures were determined by high-resolution physical mapping of chromosomes as described elsewhere (Wickstead et al., submitted for publication). The locations of 177 bp repeats (grey box) and non-repetitive DNA (striped box) are shown. When induced, ectopic transcription runs left to right.

Table 1
Stability of exogenously marked minichromosomes

Marked MC	MC size (kb)	Transcription (tetracycline)	Selection (hygromycin B)	No. sensitive clones	Rate of loss (per 1000 cell generation)
MC^{177}-1	61	−	−	1/33 (3%)	0.3 ± 0.3
		+	−	1/18 (6%)	0.6 ± 0.6
		+	+	0/31 (0%)	<1
MC^{177}-2	106	−	−	0/27 (0%)	<1.1
		+	−	0/32 (0%)	<0.9
		+	+	0/31 (0%)	<1
MC^{VSG}	91	−	−	0/37 (0%)	<0.8
		+	−	1/30 (3%)	0.3 ± 0.3
		+	+	0/23 (0%)	<1.3

Cell lines containing minichromosomes marked with hygromycin B resistance marker gene were maintained for 36 days (∼100 generations) with induction of transcription (1 μg ml^{-1} tetracycline) and selection (20 μg ml^{-1} hygromycin B) where indicated. Cells were cloned and then clones were tested for sensitivity to hygromycin B. Rates of loss are calculated from the number of sensitive clones assuming irreversible first-order kinetics and are shown with estimate of standard error (when $n \geq 1$), or as highest value compatible with the observation of no sensitive clones at 95% confidence level (when $n = 0$).

1000 cell generations (i.e. $<10^{-3}$ losses cell^{-1} gen^{-1}). Considering the transcriptionally silent nature of native minichromosomes, it was possible that the introduction of highly transcriptionally active markers might have a destabilising effect on their mitotic segregation. However, no significant differences were observed in the mitotic stability of the MCs when maintained in the absence of selection but with high-level (RNA polymerase I-mediated) ectopic transcription. Again rates of loss were $<10^{-3}$ losses cell^{-1} gen^{-1} (Table 1).

In the first description of an exogenously marked MC, Zomerdijk et al. [9] made an estimate of MC stability by looking for growth rate lag upon the reapplication of drug selection to cells grown for an extended period in the absence of selection. The lack of detectable growth delay lead to an estimated rate of MC loss of $<10^{-3}$ losses cell^{-1} gen^{-1}. However, when Lee and Axelrod [15] analogously tagged the same rDNA promoter sequence (although not necessarily the same MC) and estimated the rate of loss at the population level on the basis of loss of hybridisation signal, a much greater level of instability was observed (∼0.05 losses cell^{-1} gen^{-1}).

Lee and Axelrod [15] also described the transfection of *T. brucei* with linear plasmids bearing telomeric sequence. They dubbed the resulting DNAs 'trypanosome artificial minichromosomes' (TACs), but given that the input plasmids consistently acquired large quantities of endogenous DNA—most likely of minichromosomal origin—they are perhaps better described as integration events at MC subtelomeres (unlike the relatively unstable, but non-integrated, TACs of Patnaik et al. [19]). The marker on 1 of 2 marked MCs examined was very stable with respect to mitosis (no detectable loss over 60 generations; based on marker hybridisation), but another was lost at ∼0.05 losses cell^{-1} gen^{-1}. Such high loss rates are unlikely to represent the true stability of untagged MCs, since endogenous markers for individual MCs are inherited by all clones (of several examined) taken from non-clonal populations [14].

2. Summary

Although the numbers are still small, the data presented here add substantially to the published data regarding the stability of *T. brucei* minichromosomes. The data strengthen the original assertion made by Zomerdijk et al. [9] that MCs are extremely stable with respect to both replication and mitotic segregation with $<10^{-3}$ losses cell^{-1} gen^{-1}. Since the fate of tagged MCs was assessed using a cell-by-cell approach, we confirm that this stability results from fidelity in marker segregation at mitosis. This is distinct from maintenance of a given mass of marker in a population as a whole [15], which can arise from stochastic segregation if MC replication is still successful. The data shown here also suggest that the mitotic stability of MCs is not dependent on their transcriptional state; despite the usually silent nature of MCs, ectopic markers may be turned on and off with no apparent loss of stability. These data, along with our experience of handling cell lines containing transgenically marked MCs, suggest that problems of mitotic stability are unlikely to greatly hamper the utility of MCs as sites for the integration of tetracycline-regulated expression vectors or RNAi constructs.

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