Sequence homology and microhomology dominate chromosomal double-strand break repair in African trypanosomes

Lucy Glover1, Richard McCulloch2 and David Horn1,*

1London School of Hygiene & Tropical Medicine, Keppel Street, London, WC1E 7HT and 2Glasgow Biomedical Research Centre, 120 University Place, Glasgow G12 8TA, Scotland, UK

Received January 10, 2008; Revised February 20, 2008; Accepted February 25, 2008

ABSTRACT
Genetic diversity in fungi and mammals is generated through mitotic double-strand break-repair (DSBR), typically involving homologous recombination (HR) or non-homologous end joining (NHEJ). Microhomology-mediated joining appears to serve a subsidiary function. The African trypanosome, a divergent protozoan parasite, relies upon rearrangement of subtelomeric variant surface glycoprotein (VSG) genes to achieve antigenic variation. Evidence suggests an absence of NHEJ but chromosomal repair remains largely unexplored. We used a system based on I-SceI meganuclease and monitored temporally constrained DSBR at a specific chromosomal site in bloodstream form Trypanosoma brucei. In response to the lesion, adjacent single-stranded DNA was generated; the homologous strand-exchange factor, Rad51, accumulated into foci; a G2M checkpoint was activated and >50% of cells displayed successful repair. Quantitative analysis of DSBR pathways employed indicated that inter-chromosomal HR dominated. HR displayed a strong preference for the allelic template but also the capacity to interact with homologous sequence on heterologous chromosomes. Intra-chromosomal joining was predominantly, and possibly exclusively, microhomology mediated, a situation unique among organisms examined to date. These DSBR pathways available to T. brucei likely underlie patterns of antigenic variation and the evolution of the vast VSG gene family.

INTRODUCTION
Trypanosomatids are protozoa that branched early from the eukaryotic lineage (1) and several are parasites of substantial medical and veterinary importance (http://www.who.int/tdr/diseases/). The African trypanosome, Trypanosoma brucei, is spread among mammalian hosts by the tsetse fly and causes Human African Trypanosomiasis, which is fatal if untreated, and represents the leading cause of mortality in some areas. Trypanosoma brucei also causes Nagana disease in cattle, rendering 10 million square kilometres of land unsuitable for livestock.

Trypanosoma brucei circulates free in the host bloodstream, escaping immune responses by a process of antigenic variation, which involves monoallelic expression of variant surface glycoprotein (VSG) genes (2). The expressed VSG is always adjacent to a telomere and homologous recombination (HR) is thought to be the major mechanism contributing to antigenic variation, occurring in up to 1% of cells per population doubling (3,4). Analysis of genome sequence data revealed a vast reservoir (>1500) of VSG genes clustered at subtelomeres (5). Most are pseudogenes that may be used to assemble intact genes using short, possibly imperfect stretches of sequence homology (6).

DNA double-strand-breaks (DSBs) typically occur during DNA replication and can also be brought about by other chemical and physical forces (7,8). Non-homologous end-joining (NHEJ) and HR are the major DSBR pathways in mammals and unicellular eukaryotes, respectively and NHEJ also operates in many prokaryotes that encode a two-component, Ku/DNA ligase apparatus (9). HR-repair requires an undamaged homologous sequence in the same cell. When multiple potential templates are available, the choice may be governed by chromosome disposition prior to damage or, alternatively, damage may induce a homology search (10). Chromosome disposition likely leads to post-replicative preference for template sequences on sister chromatids (11,12), a process that requires cohesion (13). Other repair templates may be sequences nearby on the same chromosome (14), allelic sequence on a homologous
chromosome (15) or homologous sequences on hetero-
logous chromosomes (16). DSBs not repaired by HR or
NHEJ may be repaired by microhomology-mediated
joining (MMJ) which appears to serve as a back-up
or salvage pathway (17–20,21). The DSBR pathways
described above have been co-opted in several instances
for ’programmed’ DNA rearrangements. Prominent
examples are immunoglobulin and T-cell receptor gene
rearrangement (22) and mating-type switching (23) in
vertebrates and fungi, respectively. The response to DNA
damage is also the basis for experimental genetic
manipulation.

Much of our current thinking regarding DSBR in
*Trypanosoma brucei* comes from the analysis of rare recombinants
that integrate transfected linear DNA. This has revealed
efficient HR (24) and MMJ (25). MMJ has also been
reported using in vitro extracts while NHEJ has not been
reported (26). In addition, several proteins have been
shown to play a role in DSBR in *Trypanosoma brucei*,
including Mre11 (27,28), Rad51 (25) and related proteins (29)
and a sirtuin (30). Antigenic variation can operate via
Rad51-dependent or independent HR pathways (25,31)
and a Rad51-related protein has also been shown to play a
role (29).

DSBR takes place in the context of chromatin. Little is
known about the DNA-damage response or chromosomal
DSBR in trypanosomes, or how different pathways con-
tribute to repair. Specific telomere removal was reported
recently in *Trypanosoma brucei* but this did not trigger a classical
DNA-damage response (32). Rather, the terminally
deleted chromosome was replicated and segregated without
being repaired. We have now used conditional expression of the meganuclease, I-SceI, to generate a
DSBR and represents the first report of
HR occurs between homologous and heterologous
chromosomes while, in contrast to the situation in other
vertebrates and fungi, respectively. The response to DNA
damage is also the basis for experimental genetic
manipulation.

Much of our current thinking regarding DSBR in
*Trypanosoma brucei* comes from the analysis of rare recombinants
that integrate transfected linear DNA. This has revealed
efficient HR (24) and MMJ (25). MMJ has also been
reported using in vitro extracts while NHEJ has not been
reported (26). In addition, several proteins have been
shown to play a role in DSBR in *Trypanosoma brucei*,
including Mre11 (27,28), Rad51 (25) and related proteins (29)
and a sirtuin (30). Antigenic variation can operate via
Rad51-dependent or independent HR pathways (25,31)
and a Rad51-related protein has also been shown to play a
role (29).

DSBR takes place in the context of chromatin. Little is
known about the DNA-damage response or chromosomal
DSBR in trypanosomes, or how different pathways con-
tribute to repair. Specific telomere removal was reported
recently in *Trypanosoma brucei* but this did not trigger a classical
DNA-damage response (32). Rather, the terminally
deleted chromosome was replicated and segregated without
being repaired. We have now used conditional expression of the meganuclease, I-SceI, to generate a
DSBR and represents the first report of
HR occurs between homologous and heterologous
chromosomes while, in contrast to the situation in other
vertebrates and fungi, respectively. The response to DNA
damage is also the basis for experimental genetic
manipulation.

**MATERIALS AND METHODS**

*Trypanosoma brucei* growth and manipulation

Lister 427, MITat1.2 (clone 221a), bloodstream form cells
were grown in HMI-1. Transformation was performed as
described previously (33), cell density was determined
using a haemocytometer and tetracycline (Tet) was from
Sigma and was used at 1 μg/ml.

Plasmid constructs

Plasmid constructs for expression of the Tet repressor
from the *TUB* locus (TetR-BLE) and for tet-on expression
of I-SceI with an N-terminal SV40 nuclear-localization-
signal from a *RNA* spacer locus (I-SceI-HYG) were
described previously (32).

To generate pR"P"1100- primers for an I-SceI site (lower
case), ScfB (GGGCGCGGATagccggataacgggtaatA)
and ScfB (GGCCCGCGGATagccggataacgggtaatA),
and ScfR (GGCCTattacagtcttccgTCCCGGG), were
annealed and ligated at the NotI site in pESP RF-PAC.
(32). The entire R"P" cassette, including TUB processing
signals, was then amplified (Phusion DNA Pol, Finzymes
Diagnositcs) using the TUBIR5xcm (AGCTcacaGTCCCTT GTGtggGTCCCTATGGGCTT) and TUBIR3xcm
(GATCacaCACAAGGACcggCCCCTGACTATTTC
TTTG) primers, digested with XcmI (lower case) and
ligated to similarly digested pARD (33). pR"P"1100 was
digested with BamHI/Bsp120I prior to introduction into
*Trypanosoma brucei*.

DNA analysis

For Southern blot analysis of DSBR, purified genomic
DNA was digested with HindIII and processed according
to standard protocols. Gels were washed in 0.25 M HCl
for 15 min followed by two washes in H2O. The RFP
probe was a 687-bp HindIII/NotI fragment encompassing
the full ORF; the 2110.1 probe was a 699-bp SacI
fragment from pARD (33); the αTUB probe was a 516-
bp XcmI/Stul fragment and the 7240 probe was a 731-bp
HindIII/XhoI coding region fragment.

For slot-blot analysis, 3 μg of each DNA sample was
added to 200 μl of 10× SSC. Twenty micro litres were
removed, added to 200 μl of 0.4 M NaOH and denatured
at RT for 5 min. Hybond N (Amersham), supported by
one layer of Whatman 3MM paper, was soaked in 20×
SSC followed by H2O and placed in a slot-blot manifold.
DNA samples were then loaded into the slots and drawn
onto the membrane using a vacuum pump followed by
washing each well with ~250 μl of 10× SSC. Membranes
were processed as for Southern blotting. The RFP and
7240 probes were the same as used for Southern blotting;
the 2110.2 probe was a 680-bp NarI/XcmI fragment from
pARD (33) and the 2100 probe was a 681-bp PstI/Neo1
fragment from pARD.

A Typhoon TRIO phosphorimagier (Amersham) was
used to quantify signals. For slot-blot analysis, controls
for background (signal from cells lacking DSBs), ssDNA versus dsDNA (the probe was
double stranded), loading (90% of the DNA was native)
and the presence of additional alleles for the 2110.2 and
2100 probes. Thus, percentage values were derived as
follows: adjusted native value divided by the denatured
value multiplied by 22.2 (×2.9 × 100) for RFP and by 44.4
(×2.9 × 100 × 2) for 2110.2 and 2100.

A series of RFP and PAC-specific primers were used to
amplify and sequence repair junctions.

**Microscopy and protein analysis**

Western blotting and fluorescence microscopy were
conducted out using standard protocols as previously
described (30). Briefly, for immunofluorescence analysis,
cells were labelled using rabbit-anti-Rad51 (29) primary
antibody at 1:500 and fluorescein-conjugated goat- anti-
rabbit secondary antibody (Pierce) at 1:2000. Samples
were mounted in Vectashield (Vector Laboratories)
containing 4, 6-diamidino-2-phenylindole (DAPI). Cells
were scored for Rad51 foci and cell cycle phase by two
of us to generate mean values ± standard deviation.
Images were captured using an Eclipse E600 microscope with digital camera (Nikon). We employed Metamorph software for image processing and deconvolution; all settings were identical for each data set. For western blotting, rabbit- anti-Rad51 was used at 1/200 and blots were developed using an ECL kit (Amersham).

RESULTS

An experimental system to explore pathways of DSBR in *T. brucei*

We have used the I-SceI meganuclease that cleaves a specific 18-bp sequence to produce single DSBs in *T. brucei* chromosomes. Theoretically, a site of this length would occur only once in $7 \times 10^{10}$ bp, equivalent to a genome >2000 times the size of that in *T. brucei*. Formation of single chromosomal DSBs was temporally and spatially controlled by placing the I-SceI gene under the control of an inducible promoter and by introducing a single chromosomal I-SceI cleavage site (32). For this study, we introduced DSBs at a locus >1 Mbp from the nearest telomere on chromosome 11, the largest *T. brucei* chromosome at ~5.7 Mbp in length. Most genes are tightly clustered and co-transcribed on *T. brucei* chromosomes and the locus chosen is within the 452-bp segment between the Tb11.02.2110 and 2120 genes (Figure 1A and see www.genedb.org). We previously sequenced the region flanking the 2110, $\alpha$-acetyltransferase gene in the Lister 427 strain, showed that the gene is essential for growth and identified a polymorphic HindIII site nearby (33). The sequenced allele was designated ‘a’ and we mapped the additional HindIII site at allele ‘b’ (Figure 1A and data not shown). This HindIII site was important to allow us to distinguish between alleles on Southern blots (Figure 1B).

We embedded an I-SceI site within a red fluorescent protein (RFP)–puromycin N-acetyltransferase (PAC) fusion gene and added the appropriate 2110/2120 targeting sequences to generate the pRSP2110 construct. The RFP/I-SceI/PAC (RSP) cassette was flanked by (TUB) sequences that served a dual-purpose, to promote pre-mRNA trans-splicing and polyadenylation and as templates for HR with chromosome 1 (see below). When the pRSP2110 construct was introduced into *T. brucei*, PAC served as a selectable marker and recombinant ‘Sce2110’ cells were resistant to puromycin, but RFP-fluorescence was not detected. This is likely due to insufficient expression at the pol II-transcribed locus since we saw only weak red fluorescence in cells where the RSP cassette was inserted at a highly transcribed pol I locus (data not shown). Genomic DNA was extracted for Southern analysis and this indicated that the cassette had integrated at allele ‘a’ (Figure 1B).
An I-SceI-induced lesion in the hemizygous $R^2P$ cassette may be repaired via a number of pathways (Figure 1C). HR is known to be efficient in *T. brucei* and can repair a break by gene conversion using genetic information from an undamaged sister chromatid, the homologous chromosome 11b or up to 40 ectopic copies of the *TUB* sequence from the tandem arrays on chromosomes 1a or b. Since we use continuous expression of I-SceI, a site regenerated by identical sister chromatid repair can be cleaved again until repair mutates the recognition site and hence, this repair route would not be observed using our assay. In contrast, use of the 11b template would remove $R^2P$, while *TUB* recombination would replace $R^2P$ with a $\alpha$TUB gene, changes that can be detected. NHEJ or MMJ are error-prone and are characterized by deletions at the break-site. In such cases, analysis of the junction sequences allowed us to distinguish between these alternative end-joining pathways (see below). Thus, the approach was designed to allow the analysis of competition between repair pathways that use homologous templates on different chromosomes and NHEJ or MMJ pathways.

**Physical monitoring of DNA resection and repair**

Introduction of a DSB on *T. brucei* chromosome 11 was controlled by placing the I-SceI gene downstream of a Tet-inducible promoter. Upon addition of Tet to the medium, the enzyme generated a DSB at the $R^2P$ locus. To monitor the kinetics of repair, we extracted DNA from cells at different time points following I-SceI-induction. Prior to initiation of mitotic HR, a DSB must be processed by degradation of the 5' strand in a process known as resection to generate single-stranded DNA (ssDNA) with a 3' end. The ssDNA is thought to generate the signal for the DNA damage checkpoint, and is the substrate for Rad51 binding to initiate a search for a suitable homologous repair template (7). To physically monitor ssDNA adjacent to the lesion on chromosome 11, hybridization analysis was applied to native chromosomal DNA samples (Figure 2A and B); hybridization to native DNA will occur only if ssDNA is present. Denatured samples were analysed in parallel to control for loading. To increase sensitivity, we loaded nine times more native DNA relative to denatured DNA for each time point.

To increase sensitivity, we loaded nine times more native DNA relative to denatured DNA for each time point. We used an RFP probe and a series of probes at different locations on chromosome 11. The RFP probe allowed resection to be monitored immediately adjacent to the lesion, while the 2110.2 and 2100 probes monitored resection within 1 kb and 2.4 kb. The 7240 probe served as a control to monitor DNA $\sim 2.8$ Mbp from the lesion. The analysis indicated DNA resection across all three regions tested within 3 kb of the lesion (Figure 2A) while the distal, control probe showed no evidence of resection. Thus, resection was sufficiently extensive to facilitate ectopic HR or HR with chromosome 11b (see Figure 1C). After correction for loading, background, DNA conformation and gene copy number (see Materials and methods section), we saw that $\sim 10\%$ of the DNA at each of the three sites adjacent to the lesion was in the single-stranded conformation 9 h after I-SceI induction (Figure 2B). This effect was transient with little ssDNA detected after 24 h. Single-stranded RFP was detected at 12 h however, after adjacent single-stranded sequences were apparently sequenced, presumably reflecting the formation of Holliday junction intermediates (34).

To physically monitor DNA repair, we used Southern blot analysis (Figure 2C). First, loss of the RFP signal indicated that few cells escaped the action of I-SceI (Figure 2C and D). With regard to repair, the predominant pathway involved use of chromosome 11b as HR template to regenerate the ‘7.6 kb’ allele. We also saw evidence for ectopic recombination with the *TUB* array on chromosome 1. These events generated a fragment of 9.5 kb (7.6 kb + 1.9 kb of $\alpha$TUB sequence) and, though the signal was weak, we were able to independently confirm these ectopic recombination events (see below and Figure 5B, lane 9). Thus, although the 2110/2120 sequence was more distal to the break relative to the *TUB* sequence, it was favourised in a competition between the two sequences. NHEJ or MMJ were expected to generate RFP fragments of $<4.7$ kb. Such events may not have been detected using this assay due to size heterogeneity and/or loss of RFP sequence (but see below). Since HR with chromosome 11b predominated, we plotted RFP-loss and 11b-mediated repair against time (Figure 2D). The 2110 signal, representing 11b repair, was normalized to the ratio of 2110 signals representing each allele in wild-type cells. The analysis indicated that chromosome 11b-mediated HR represented $\sim 85\%$ of the repair events.

**Rad51 assembles into foci in response to a single DSB**

Rad51 (RecA in bacteria and RadA in archaeabacteria) forms helical filaments on ssDNA and catalyses homologous strand exchange (35). Several recombination proteins, including Rad51, show diffuse localization in undamaged cells, but localize to sites of DNA damage forming sub-nuclear foci detectable by microscopy. Since we demonstrated the processing of single DSBs to generate ssDNA, we also wanted to determine whether these DSBs could trigger the assembly of Rad51 foci. We carried out immunofluorescence analysis using anti-Rad51 to compare wild-type cells to cells with I-SceI-induced lesions. Figure 3A shows that Rad51 was enriched in the nuclei of wild-type cells but the signal was typically diffuse (left-hand panels) in contrast to the situation in cells with a lesion, where a substantial proportion displayed Rad51 foci (right-hand panels). We used deconvolution to enhance foci and these images more clearly show nuclear foci in many induced cells and the absence of foci in the majority of wild-type cells. Consistent with previous work (29), foci were detected in only $\sim 1\%$ of wild-type nuclei, likely reflecting the recruitment of repair proteins to sites of spontaneous DNA damage. We then counted Rad51 foci in cells induced for different periods of time (Figure 3B). In cells with a lesion on chromosome 11, there was a rapid increase in the proportion of nuclear foci, peaking at $\sim 30\%$ 9 h after induction. A high proportion of cells with foci was still detected after 24 h but had diminished to background after 48 h (Figure 3B).
In the related trypanosomatids, *Trypanosoma cruzi* and *Leishmania major*, Rad51 expression is increased following chemical or radiation-induced DNA damage (36,37). To determine whether I-SceI-induced lesions lead to increased expression of Rad51, we carried out western blotting using anti-Rad51 and a series of whole-cell protein extracts representing different times following I-SceI-induction (Figure 3C). We saw no evidence for an increase in Rad51 expression indicating that the pre-existing cellular pool of Rad51 was redistributed into foci in response to DNA damage.

**Rad51 foci and the G2/M cell cycle checkpoint**

A single DNA-DSB can trigger a DNA damage checkpoint that arrests the cell cycle and allows time for repair prior to further progression thereby suppressing deleterious genome rearrangements (7). Efficient and temporally
constrained introduction of DSBs in *T. brucei* provided an opportunity to explore DNA damage checkpoint control. No method is available to synchronize bloodstream form *T. brucei*, but nuclear and mitochondrial (kinetoplast) DNA, stained with DAPI, provide excellent cytological markers that define position in the cell cycle (38). In bloodstream form cells, ~80% of cells display a single nucleus and a single kinetoplast (1N1K) indicating earlier phases of the cell cycle (G1/S). A single nucleus and two kinetoplasts (1N2K) indicate late nuclear G2 and two nuclei and two kinetoplasts (2N2K) indicate completion of mitosis. First, we examined the proportion of cells with Rad51 foci in these three categories after 12 h of I-SceI induction. We had also noted that some cells had two Rad51 foci, so we subdivided each category into cells with zero, one or two foci (Figure 4A).

Approximately 30% of cells in G1/S phases (1N1K) had a single focus and ~75% of cells in G2 phase (1N2K) had foci, with about half of these displaying two. Very few post-mitotic cells (2N2K) had foci and this category of cells gave similar results 24 h following I-SceI-induction indicating that the paucity of foci is not simply because cells with a lesion had insufficient time to progress to mitosis. Rad51 foci were of varying degrees of brightness with the brightest foci predominantly observed in G2 phase nuclei. This could reflect sister chromatids with lesions in close juxtaposition due to cohesion or differences in resection or Rad51-loading. Cells with two foci were exclusively in the G2/M phase of the cell cycle consistent with the idea that many cells do indeed bear lesions on both sister chromatids and also that cohesion can be lost in the absence of repair.

To determine whether a G2/M cell cycle checkpoint was triggered by the lesions on chromosome 11, we counted the proportion of cells in the late G2 phase of the cell cycle consistent with the idea that many cells do indeed bear lesions on both sister chromatids and also that cohesion can be lost in the absence of repair.

To determine whether a G2/M cell cycle checkpoint was triggered by the lesions on chromosome 11, we counted the proportion of cells in the late G2 phase of the cell cycle consistent with the idea that many cells do indeed bear lesions on both sister chromatids and also that cohesion can be lost in the absence of repair.

To determine whether a G2/M cell cycle checkpoint was triggered by the lesions on chromosome 11, we counted the proportion of cells in the late G2 phase of the cell cycle consistent with the idea that many cells do indeed bear lesions on both sister chromatids and also that cohesion can be lost in the absence of repair.

To determine whether a G2/M cell cycle checkpoint was triggered by the lesions on chromosome 11, we counted the proportion of cells in the late G2 phase of the cell cycle consistent with the idea that many cells do indeed bear lesions on both sister chromatids and also that cohesion can be lost in the absence of repair.

To determine whether a G2/M cell cycle checkpoint was triggered by the lesions on chromosome 11, we counted the proportion of cells in the late G2 phase of the cell cycle consistent with the idea that many cells do indeed bear lesions on both sister chromatids and also that cohesion can be lost in the absence of repair.

To determine whether a G2/M cell cycle checkpoint was triggered by the lesions on chromosome 11, we counted the proportion of cells in the late G2 phase of the cell cycle consistent with the idea that many cells do indeed bear lesions on both sister chromatids and also that cohesion can be lost in the absence of repair.
The lower images indicate a 2N2K cell that had completed mitosis. Rad51 foci were rarely detected in these cells.

**HR dominates DSB repair**

We employed ‘clonogenic’ assays to determine the proportion of cells that survive I-SceI-induced lesions and also to further explore the quantitative contribution of different chromosomal DSBR pathways in *T. brucei*. Clonogenic analysis under standard or I-SceI-inducing conditions indicated 57±11% survival following I-SceI induction (Figure 5A). We expected most of these survivors to revert to puromycin sensitivity due to loss, or error-prone repair, of the RsP cassette (see Figures 1C and 2C) and screening with puromycin confirmed this prediction. Twenty four of the 93 uninduced sub-clones were tested and were all puromycin resistant, while all but three of 24 I-SceI-survivors were sensitive.

To distinguish between different repair mechanisms, 22 survivors were analysed by Southern blotting; half of the samples are shown in Figure 5B. The analysis shown in Figure 2C and D indicates that HR with chromosome 11b was the predominant repair mechanism and, consistent with this, 19 survivors reflected this mechanism (nine shown in Figure 5B). Figure 2C also indicates ectopic HR with the *TUB* locus on chromosome 1 and use of this pathway was confirmed by the survivor in lane nine (Figure 5B, band at 9.5 kb in the blot) and two survivors analysed on a second blot (data not shown).

**Figure 4.** Rad51 foci and the G2M cell cycle checkpoint. Cells were processed for Rad51 immunofluorescence microscopy and DNA was counter-stained with DAPI. (A) The bar-chart shows the proportion of Sce2110 cells at different phases of the cell cycle with zero, one or two Rad51 foci 12 h after I-SceI-induction. Cell cycle phase was defined by the number of nuclei (N) and kinetoplasts (K) as determined by DAPI staining. *n* = 50 at each cell cycle phase. Error bars, SD. (B) G2M phase (1N2K) kinetics. The proportion of 1N2K cells was counted at different times after I-SceI-induction. *n* = 200 at each time point. Error bars, SD. (C) Immunofluorescence analysis of Rad51 in Sce2110 cells after I-SceI-induction. Rad51 signals are shown after deconvolution (d). N, nucleus; K, kinetoplast. Scale bar, 5 μm.
Figure 1A). One survivor (clone 15, not shown) may have arisen due to a longer gene conversion tract, but another possible explanation is NHEJ or MMJ with loss of RFP. Indeed, we saw three examples of apparent end joining with retention of RFP sequence (Figure 5B). Finally, we quantified the chromosome 11 signals, relative to chromosome 1, and found no evidence for chromosome loss. Thus, HR dominated DSBR while, in a competition between allelic and ectopic inter-chromosomal recombination, allelic recombination was strongly favoured.

Microhomology-mediated joining contributes to chromosomal DSBR

Of 22 I-SceI-survivors analysed by Southern blotting, three appeared to reflect NHEJ or MMJ based on the presence of residual RFP signal on truncated HindIII fragments (Figure 5B, lanes 4, 6 and 9). Two of these were ‘mixed’ and also reflected HR with chromosome 11 (lane 6) or with chromosome 1 (lane 9, band at 9.5 kb). We saw two additional ‘mixed’ survivors on the second blot that reflected HR with both chromosomes 11 and 1 (data not shown). Detection of four survivors that exhibit evidence of multiple repair pathways was explained by I-SceI-induction in cells that had already replicated their nuclear genome prior to DSBR. To examine repair junctions in examples of end joining, we amplified junction fragments from genomic DNA using polymerase chain reaction (PCR) and then sequenced the products. Among samples shown in Figure 5B (lanes 6 and 9) and additional samples from earlier preliminary clonogenic analysis, we identified two distinct junctions from four independent clones. Sequence alignment clearly revealed that end joining was microhomology mediated (Figure 5C). We then used a
PCR assay designed to survey large numbers (ca. ≥ 10 000) of end-joining events. This yielded only two major, reproducible products (Figure 5C) that were directly sequenced. As expected, one of the junctions was identical to the one identified in three independent clones while the other was unique (Figure 5C). The three junctions identified (six independent sequences in total) reflected microhomologies of 11–13 bp with one or two mismatches and loss of 72–278 bp (Figure 5C). Interestingly, junction 1 retains an intact RFP/PAC ORF explaining why three clonogenic survivors, including clones 6 and 9, displayed puromycin resistance (see above).

**DISCUSSION**

The introduction of chromosomal DSBs in a spatially and temporally co-ordinated manner has been an extremely powerful approach for investigating DSBR (39). We have taken this approach in *T. brucei* and demonstrate a number of major advantages over previous approaches used to examine DSBR pathways, which have relied upon the introduction of linear constructs and analysis of rare recombinants. First, the method allows introduction of single DSBs at specific loci. Second, temporal control and the efficiency of cleavage and repair allows for physical monitoring of DSBR. Third, we are able to compare allelic and ectopic recombination and chromosomal MMJ.

The specific induction of a single DNA DSB in *T. brucei* reveals DNA resection and accumulation of Rad51 foci, the timing of which is consistent with the idea that the former triggers the latter. Rad51 foci are sites of active DNA recombination as demonstrated in yeast (40) and are seen in the nuclei of *T. brucei* treated with the DNA-damaging agent, phleomycin (29). The current results now suggest that a single DNA lesion can also be visualized using Rad51 as a molecular marker in *T. brucei*. Rad51 may not accumulate into foci in the G1 phase of the cell cycle, however, reminiscent of the situation in yeast (41). We also show that a single lesion activates a G2/M DNA damage checkpoint in *T. brucei*. This checkpoint arrests cell cycle progression prior to mitosis and is particularly important to allow time to repair stalled or broken replication forks, the major source of lesions requiring recombinational repair.

Following the induction of a DSB, we monitored chromosomal DSBR and assessed the contribution of different mechanisms (summarized in Figure 6). Lesions are generated in most, if not all, cells and successful repair generated viable cells in ~60% of cases. HR clearly dominated DSBR with the allelic sequence on the homologous chromosome favoured over a homologous sequence at an ectopic location. Monitoring of the entire population or clonogenic survivors suggested that ~85% or 75% of cells used allelic recombination, respectively. Under-representation of allelic recombination in the survivor assay is explained by repair after nuclear genome replication in some cases. Thus, allelic recombination was responsible for ~85% of repair. If we calculate the number of repair events as a function of total cells instead of survivors, we see ~50% allelic HR and 5% ectopic HR and MMJ (Figure 6).

HR repair requires that the lesion associates with undamaged homologous sequence. Our results indicated that allelic recombination is preferred over ectopic recombination, even when the allelic-HR-substrate is >1 kb from the break and beyond the ectopic-HR substrate. A similar search for chromosomal break-distal homology has been reported in *Saccharomyces cerevisiae* (42) which, interestingly, contrasts with inefficient recombination with non-terminal homologous sequences on DNA constructs introduced into *T. brucei* (24). This remarkable preference for allelic recombination raised the question of how homologous sequences are ‘found’ and ‘selected’ in a chromosomal context. First, the length of homologous segments may impact on donor choice. In our analysis, the ectopic HR substrates are 240 bp and 323 bp while, in a *T. brucei* transformation assay, maximal transformation was observed when using substrates of 200 bp or longer (24). This argues against the idea that the ectopic homologous sequence is insufficient to compete with the allelic sequence. Second, donor sequence copy number may have an impact, as demonstrated in *S. cerevisiae* (43), but this would have favoured ectopic recombination since there are up to 40 potential donor templates on chromosome 1. Homologous chromosome were reported to be co-aligned along their lengths via multiple interstitial interactions during G1 and G2 in *S. cerevisiae* and *Drosophila* (44) but this view has been disputed (45). Thus, favoured allelic recombination in *T. brucei* may reflect either chromosome disposition prior to damage or a damage-induced homology search (10).

Homologues of factors required for NHEJ are absent or diverged in trypanosomatids (26) and Ku and Rad51-independent MMJ has been reported in *T. brucei*.
following the introduction of linear constructs (25) and using cell extracts (26). Disruption of factors required for NHEJ reveal MMJ as a subsidiary repair pathway in mammalian cells (46) and the genetic requirements for MMJ but no evidence for NHEJ. Thus, HR and MMJ dominate chromosomal DSBR in T. brucei, but, since NHEJ can occur in as few as 0.33% of S. cerevisiae MMJ but no evidence for NHEJ. Thus, HR and MMJ, and found several examples or we looked for evidence of intra-chromosomal end joining, either NHEJ or MMJ, and we are unable to exclude the possibility that NHEJ also operates at a low level in T. brucei.

Recombination plays a specialized role in VSG gene rearrangement and host immune evasion in T. brucei and can bring about antigenic variation in 1% of cells per population doubling. The telomeric VSG expression sites may select from among a vast variety of subtelomeric donors for translocation of a new VSG or formation of VSG mosaics (6). Repetitive flanking sequences are important for this switching and diversification and these rearrangements can operate via Rad51-dependent or independent pathways (25,31). Below, we briefly consider our results in relation to antigenic variation, the potential role of microhomology and the choice of recombination partners.

NHEJ is important for immunoglobulin class switch recombination in mammalian B cells. When the NHEJ pathway is eliminated, however, by Ku disruption for example, the programmed DSBs are channelled into an end-joining pathway that uses stretches of microhomology and often involves chromosomal translocation (17,19–21). Antigenic variation appears to be unaffected in Ku-deficient T. brucei, consistent with the idea that NHEJ is not required to generate VSG diversity (48). We now show that NHEJ plays, at most, a minor role in chromosomal DSBR in T. brucei. In the case of VSG recombination, flanking, imperfect, so-called 70-bp repeats, present an abundance of microhomologous recombination substrates and assembly of mosaic VSG genes (6) may also be driven by microhomology. We suggest that, with a deficiency in NHEJ, MMJ could play an important role in VSG rearrangement and expression in T. brucei.

We previously reported use of I-SceI to remove the telomere at a silent VSG expression site, but this failed to trigger a DNA damage response or, in most cases, to trigger recombination (32). This dramatic difference, relative to a lesion in the core of the chromosome reported here, may reflect the suppression of subtelomeric recombination by telomere-binding proteins, as demonstrated in mammalian cells (49) or, more likely, by the phylogenetically restricted DNA base modification, β-glucosyl-hydroxymethyluracil, found throughout silent VSG expression sites (50,51). It will now be important to analyse active expression sites that may display enhanced recombination due to transcription (52) and the absence of DNA base modification.

In addition to the avenues outlined above, the system reported here will allow for a genetic dissection of the chromosomal DSBR pathways available to T. brucei.

This will lead to a better understanding of chromosomal recombination and repair and of the mechanisms underlying antigenic variation in this divergent eukaryote and important pathogen.

ACKNOWLEDGEMENTS

This work was funded by The Wellcome Trust (069909). We thank Sam Alsfeld, Martin Taylor and John Kelly (LSHTM) for comments on the draft manuscript and Mark Mitchell (Bristol, UK) for help with graphics. Funding to pay the Open Access publication charges for this article was provided by The Wellcome Trust.

Conflict of interest statement. None declared.

REFERENCES

1. Simpson, A.G., Stevens, J.R. and Lukes, J. (2006) The evolution and diversity of kinetoplastid flagellates. Trends Parasitol., 22, 168–174.
2. Horn, D. (2004) The molecular control of antigenic variation in Trypanosoma brucei.Curr. Mol. Med., 4, 563–576.
3. Morrison, L.J., Majiwa, P., Read, A.F. and Barry, J.D. (2005) Probabilistic order in antigenic variation of Trypanosoma brucei. Int. J. Parasitol., 35, 961–972.
4. Robinson, N.P., Burman, N., Melville, S.E. and Barry, J.D. (1999) Predominance of duplicative VSG gene conversion in antigenic variation in African trypanosomes. Mol. Cell Biol., 19, 5839–5846.
5. Callejas, S., Leech, V., Reitter, C. and Melville, S. (2006) Hemizygous subtelomeres of an African trypanosome chromosome may account for over 75% of chromosome length. Genome Res., 16, 1109–1118.
6. Marcello, L. and Barry, J.D. (2007) Analysis of the VSG gene silent archive in Trypanosoma brucei reveals that mosaic gene expression is prominent in antigenic variation and is favored by an archive substructure. Genome Res., 17, 1344–1352.
7. Harrison, J.C. and Haber, J.E. (2006) Surviving the breakup: the DNA damage. Annu. Rev. Genet., 40, 209–235.
8. Symington, L.S. (2002) Role of RAD52 epistasis group genes in homologous recombination and double-strand break repair. Microbiol. Mol. Biol. Rev., 66, 630–670.
9. Shuman, S. and Glickman, M.S. (2007) Bacterial DNA repair by non-homologous end joining. Nat. Rev. Microbiol., 5, 852–861.
10. Barzel, A. and Kupiec, M. (2008) Finding a match: how do homologous sequences get together for recombination? Nat. Rev. Genet., 9, 27–37.
11. Johnson, R.D. and Jasim, M. (2000) Sister chromatid gene conversion is a prominent double-strand break repair pathway in mammalian cells. EMBO J., 19, 3398–3407.
12. Kadyk, L.C. and Hartwell, L.H. (1992) Sister chromatids are preferred over homologs as substrates for recombilatory repair in Saccharomyces cerevisiae. Genetics, 132, 387–402.
13. Sjogren, C. and Nasmyth, K. (2001) Sister chromatid cohesion is required for postreplicative double-strand break repair in Saccharomyces cerevisiae. Curr. Biol., 11, 991–995.
14. Smith, J.A., Bannister, L.A., Bhattacharjee, V., Wang, Y., Waldman, B.C. and Waldman, A.S. (2007) Accurate homologous recombination is a prominent double-strand break repair pathway in mammalian chromosomes and is modulated by mismatch repair protein Msh2. Mol. Cell Biol., 27, 7816–7827.
15. Moynahan, M.E. and Jasim, M. (1997) Loss of heterozygosity induced by a chromosomal double-strand break. Proc. Natl Acad. Sci. USA, 94, 8988–8993.
16. Richardson, C., Moynahan, M.E. and Jasim, M. (1998) Double-strand break repair by interchromosomal recombination: suppression of chromosomal translocations. Genes Dev., 12, 3831–3842.
17. Corneo, B., Wendland, R.L., Deriano, L., Cui, X., Klein, I.A., Wong, S.Y., Arnal, S., Holub, A.J., Weller, G.R., Pancake, B.A. et al, (2007) Rag mutations reveal robust alternative end joining. Nature, 449, 483–486.
