A role for RAD51 and homologous recombination in *Trypanosoma brucei* antigenic variation

Richard McCulloch[1] and J. David Barry

The Wellcome Centre for Molecular Parasitology, The Anderson College, University of Glasgow, Glasgow G11 6NU, U.K.

Antigenic variation is an immune evasion strategy used by African trypanosomes, in which the parasites periodically switch the expression of *VSG* genes that encode their protective variant surface glycoprotein coat. Two main routes exist for *VSG* switching: changing the transcriptional status between an active and an inactive copy of the site of *VSG* expression, called the bloodstream *VSG* expression site, or recombination reactions that move silent *VSG*s or *VSG* copies into the actively transcribed expression site. Nothing is known about the proteins that control and catalyze these switching reactions. This study describes the cloning of a trypanosome gene encoding RAD51, an enzyme involved in DNA break repair and genetic exchange, and analysis of the role of the enzyme in antigenic variation. Trypanosomes genetically inactivated in the *RAD51* gene were shown to be viable, and had phenotypes consistent with lacking functional expression of an enzyme of homologous recombination. The mutants had an impaired ability to undergo *VSG* switching, and it appeared that both recombinational and transcriptional switching reactions were down-regulated, indicating that RAD51 either catalyzes or regulates antigenic variation. Switching events were still detectable, however, so it appears that trypanosome factors other than RAD51 can also provide for antigenic variation.

African trypanosomes, such as *Trypanosoma brucei*, are single-celled eukaryotic parasites of mammals that reside in the bloodstream and tissue fluids and cause the disease trypanosomiasis, or sleeping sickness in humans. To avoid being eliminated by the host's immune response, trypanosomes have evolved an evasion strategy termed antigenic variation. It is now recognized that antigenic variation is a general survival strategy used by a diverse array of pathogenic microorganisms, and a wide spectrum of molecular mechanisms are used to this end (Borst and Greaves 1987; Deitsch et al. 1997). In African trypanosomes, this consists of spontaneous, periodic changes in the Variant Surface Glycoprotein (VSG) species that acts as a protective coat on the surface of the parasite (Cross 1975). A change of VSG coat by a subpopulation of infecting trypanosomes allows a portion of the trypanosome population to escape the host's immune response directed against the previous VSG species, thereby prolonging the infection. For recent reviews of trypanosome antigenic variation see Cross (1996), Barry (1997), Borst et al. (1998), Cross et al. (1998), Pays and Nolan (1998).

Each trypanosome normally expresses a VSG coat composed of only one VSG species at a given time. It can select that single VSG species from ~1000 *VSG* genes present within its genome (van der Ploeg et al. 1982). To achieve exclusive expression, a *VSG* can only be transcribed if it resides within a specialized telomeric transcription unit called the expression site (Johnson et al. 1987; Kooter et al. 1987; Pays et al. 1989). The expression sites used in the bloodstream are very large (40–60 kb), and contain a number of expression site-associated genes (*ESAG*s) in addition to the *VSG* itself (Cully et al. 1985; Xong et al. 1998). Each cell contains ~20 bloodstream expression sites, but only one is transcribed at a time (Navarro and Cross 1996; Chaves et al. 1998). The remainder of the 1000 *VSG*s act as a reservoir of silent genes, and must be moved into the expression sites before they can be expressed.

Because of the complex arrangement of *VSG* expression described above, a switch from one VSG coat to another can be accomplished in a number of ways. One way is by activating transcription from one of the silent *VSG* expression sites and silencing transcription from the active site. This reaction is termed an in situ switch, and recent work has suggested that it may involve epigenetic mechanisms of transcriptional control (Horn and Cross 1995; Rudenko et al. 1995). The other way to ex-

ecute a switch is by DNA recombination reactions that move a silent *VSG* into the active expression site and remove the resident *VSG*. A number of such reactions have been described. The most commonly observed reaction is a gene conversion process, termed duplicative transposition, in which the *VSG* in the expression site is deleted and replaced by a copy made from a silent *VSG* (Hoeijmakers et al. 1980; Bernards et al. 1981; Pays et al. 1981, 1983a). Silent *VSG*s in a number of genomic locations can act as gene donors in this reaction: transposition of genes from silent expression sites, from silent chromosome-internal arrays of *VSG*s, and from the telomeres of minichromosomes (of which ~100 are present) have been described (for review, see Borst et al. 1996; Barry 1997). The amount of *VSG* sequence copied during duplicative transposition is variable and normally extends beyond the boundaries of the *VSG* ORF. A number of sequence motifs around the *VSG* might provide the sequence homology that drives the recombination reaction. 3′ of the gene, and within the carboxyl terminus of the ORF, short stretches of sequence homology have been found in most *VSG*s (Liu et al. 1983; Michels et al. 1984); further downstream, telomere (and other) repeats could provide sequence homology if the *VSG* donor is also telomeric (de Lange et al. 1983; Aline et al. 1989; Scholler et al. 1989). 5′ of almost all *VSG*s are runs of imperfect 70-bp repeats, and this normally provides the only upstream homology for transposition of chromosome-internal genes (Liu et al. 1983; Campbell et al. 1984); if the recombination occurs between two expression sites, however, the *ESAG*s and surrounding expression site sequence can provide many kilobases of upstream homology (Laurent et al. 1983; Pays et al. 1983b; Myler et al. 1984). Another form of recombination is also possible for *VSG* switching, but has been less commonly observed. This is a reciprocal genetic exchange that occurs upstream of the *VSG* and moves the active gene into a silent telomere, and moves a silent telomeric *VSG* into the active expression site (Pays et al. 1985; Rudenko et al. 1996).

Nothing is known about the trypanosome genes that regulate antigenic variation, or encode the enzymes that catalyze the switching reactions. Moreover, it is not known what pathways of recombination are involved. For example, we do not know whether a classical double-strand break repair mechanism (Shinohara and Ogawa 1995; Eggleston and West 1996) is involved, or whether more specialized means of genetic exchange are utilized (perhaps analogous to intron mobility or yeast mating-type switching; Grivell 1996; Haber 1998). There has been considerable debate over the event(s) that initiates recombinogenic *VSG* switching: Is it due to random DNA breaks around the *VSG* (Borst et al. 1996), or is it a result of specific breaks catalyzed by a discrete enzyme (Barry 1997)? To address these questions, this study describes the identification of a *T. brucei* gene putatively encoding a homolog of eukaryotic RAD51 and analysis of the role that this enzyme plays in antigenic variation.

RAD51 is a eukaryotic homolog of bacterial RecA, and is highly conserved in a diverse array of eukaryotes (Ba-sile et al. 1992; Shinohara et al. 1992, 1993). Like RecA, RAD51 has a pivotal role in the repair of double-strand DNA breaks and in homologous recombination. Comparison of the catalytic properties of purified *Escherichia coli* RecA with RAD51 from *Saccharomyces cerevisiae* and *Homo sapiens* has shown that the enzymes are functionally related. All of the enzymes bind single- or double-stranded DNA in the presence of ATP and form nucleoprotein filaments in which the DNA is extended and underwound relative to its normal structure (Ogawa et al. 1993b; Benson et al. 1994; Sung and Robberson 1995). On relaxed double-stranded DNA, the three filaments have a highly similar structure. All of the enzymes display ATPase activity in the presence of single-stranded DNA and, on binding, can catalyze the transfer of a single-stranded DNA end into duplex DNA. By use of ATP-hydrolysis, the enzymes then generate homology-dependent strand exchange products in which the single-stranded DNA is basepaired with the complementary strand of the duplex and displaces the noncomplementary strand (Sung 1994; Baumann et al. 1996; Baumann and West 1997). In performing these strand transfer and exchange reactions, RAD51 and RecA initiate the process of genetic exchange and ensure that it occurs between homologous DNA sequences. By examining the role of RAD51 in trypanosome antigenic variation, we assess whether *VSG* switching is effected by homologous recombination or some other pathway of genetic exchange, and begin to examine the pathways of recombination that are available to this ancient eukaryotic parasite.

Results

Cloning the trypanosome RAD51 gene

The amino acid sequences of RAD51 proteins from a diverse range of eukaryotes have been shown to be highly conserved, particularly in a central domain of ~200 residues that has sequence similarities with bacterial RecA (Ogawa et al. 1993a; Shinohara et al. 1993; Dunderdale and West 1994). This high level of conservation allowed us to isolate a gene from *T. brucei* putatively encoding a homolog of RAD51. A comparison of the predicted *T. brucei* polypeptide with RAD51 sequences from a number of eukaryotes is shown in Figure 1. Southern mapping (Fig. 2a) suggested that the putative *RAD51* gene is present in single copy within the *T. brucei* genome, and probing of poly(A)$^+$ RNA from bloodstream and procyclic (tsetse fly midgut) *T. brucei* suggested that *RAD51* is expressed at a low level in both stages as a 2.4-kb mRNA (data not shown).

The overall level of sequence identity between the putative trypanosome RAD51 polypeptide and other RAD51s is extremely high: 55% when compared with the *S. cerevisiae* enzyme, ranging to 67% for the *Xenopus laevis* protein. In database searches with the trypanosome polypeptide sequence, RAD51 proteins were identified ahead of other RecA homologs that have been discovered in eukaryotes. Therefore, it seems likely that the
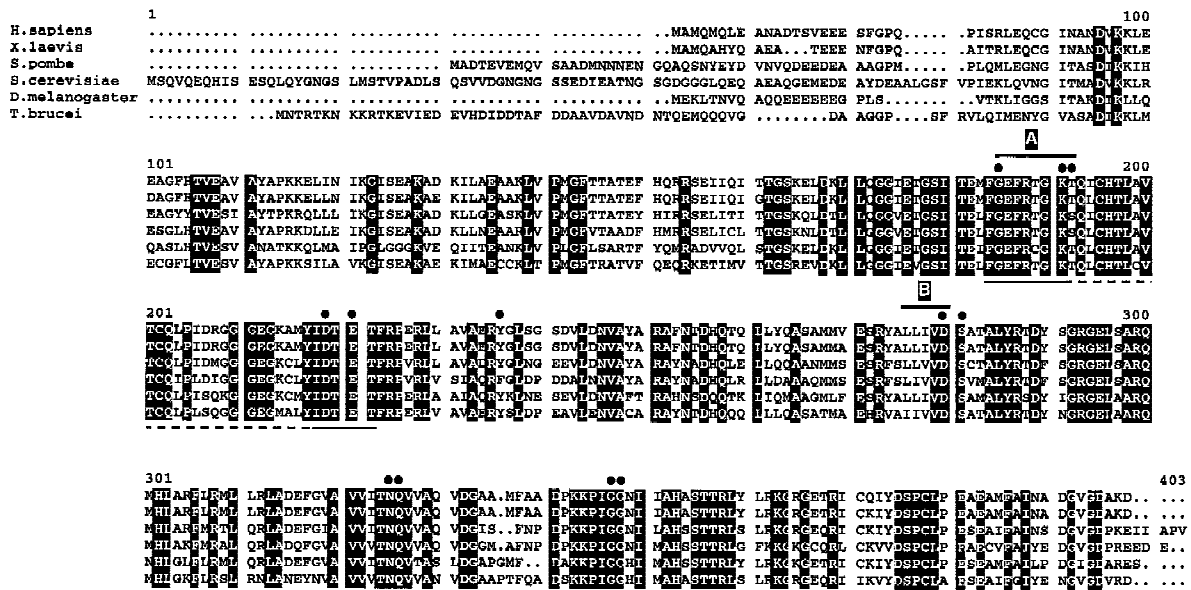
Figure 1. Alignment of the amino acid sequences of some eukaryotic RAD51 proteins. Amino acid residues are shown in single letter code, and the predicted *T. brucei* polypeptide (*bottom*) is compared with RAD51 polypeptide sequences from *H. sapiens* (EMBL accession no. D13804), *X. laevis* (D38488), *Schizosaccharomyces pombe* (D13805), *S. cerevisiae* (M88470), and *Drosophila melanogaster* (L41342). Numbers correspond to the largest polypeptide (from *S. cerevisiae*), and residues that are identical in all the sequences are in a black background. Black dots identify amino acid residues shown to be invariant or highly conserved in the active site of *E. coli* RecA, and the peptides identified as A and B are putative conserved nucleotide-binding motifs. The region identified under the *T. brucei* sequence corresponds to the region of the *RAD51* gene that was PCR amplified for cloning the complete gene; the solid lines show the conserved regions used for generating primers, and the dotted line corresponds to the region that was amplified.

gene cloned encodes a bonafide RAD51 homolog, rather than one of a number of related eukaryotic proteins, which appear to have no role in general homologous exchange (e.g., DMC1; Bishop et al. 1992; Pittman et al. 1998; Yoshida et al. 1998), or have accessory functions in RAD51 recombination (e.g., RAD55 and 57; Sung 1997). Two nucleotide-binding consensus motifs have been identified within the published RAD51-like sequences (Bishop et al. 1992; Shinohara et al. 1992), and both of these are conserved within the *T. brucei* polypeptide. An exhaustive comparison by Story et al. (1993) of 23 bacterial RecA sequences, the DMC1 protein of *S. cerevisiae* and the bacteriophage T4 UVSX protein identified 12 invariant or highly conserved residues. From the three-dimensional structure of *E. coli* RecA, these residues are believed to be involved in the enzyme's active site, and all are conserved in the putative *T. brucei* enzyme. This suggests that the trypanosome will encode a polypeptide with RecA/RAD51 enzymatic properties.

Generation of T. brucei RAD51 *knockout mutants*

The strategy that we used to examine the function of RAD51 was to create transgenic trypanosomes that no longer express active RAD51. To make inactive derivatives of the *RAD51* gene, we generated constructs that deleted the central 391 bp of the ORF and replaced them with cassettes for bleomycin phosphotransferase (providing phleomycin resistance; *BLE*) and puromycin N-acet-

yltransferase (puromycin resistance; *PUR*) expression (Fig. 2a). After transforming these constructs into *T. brucei*, the *RAD51* sequences flanking the expression cassettes provide sequence homology, enabling them to integrate into, and replace, the endogenous *RAD51* locus. The *RAD51* deletion encompasses 8 of the 12 conserved RecA catalytic residues, and both of the putative ATP-binding motifs, so we predicted that any residual RAD51 peptide expressed should be inactive. A similar strategy for disruption of the mouse *RAD51* gene deleted a smaller region of the ORF than described here, and created a lethal phenotype (Lim and Hasty 1996; Tsuzuki et al. 1996).

Trypanosomes are predominantly diploid, and so we disrupted both *RAD51* alleles by performing two rounds of transformation; first, the *RAD51::BLE* construct was electroporated and then, once these transformants were established, the *RAD51::PUR* construct was transformed. Figure 2b shows PCR analysis of bloodstream transformants with primers that flank the *RAD51* ORF. In the first round transformants, a 1.10-kb PCR product from the *RAD51* wild-type allele and a 1.57-kb product corresponding to the integrated *BLE* cassette were detected, suggesting that the construct had integrated into one allele to generate putative heterozygous mutants (*RAD51*⁺/⁻). A 1.80-kb product corresponding to the integrated *PUR* cassette was seen in the second round transformants, and there was no longer any sign of the PCR product from the wild-type allele, suggesting that

Figure 2. Generation of trypanosome *RAD51* knockout mutants. (*A*) A restriction enzyme map of the genomic environment of the *RAD51* ORF and, below, the constructs generated to disrupt RAD51 expression. Highly conserved residues in the RAD51 amino acid sequence are shown as black dots, and the 300-bp *RAD51* 5′ and 3′ sequences that allow for integration of the constructs are indicated with crosses. An amount of 391 bp of the *RAD51* ORF is replaced by expression cassettes that give phleomycin (BLE) or puromycin (PUR) resistance; these contain 5′ and 3′ sequences from the *PARP* locus (gray shading) that allow transcripts of the resistance genes to be processed into mature mRNAs. Restriction enzyme recognition sites, (A) *Acc*I; (B) *Bam*HI; (Bg) *Bgl*II; (C) *Cla*I; (E) *Eco*RV; (H) *Hin*dIII; (Hc) *Hin*cII; (K) *Kpn*I; (P) *Pst*I; (S) *Sal*I; (R) *Eco*RI; (Xb) *Xba*I; (Xh) *Xho*I. (*B*) PCR analysis of bloodstream *T. brucei* transformed with *RAD51* knockout constructs. PCR used the primers ko5′ and ko3′. Size markers (in kilobases) are indicated at left of the agarose gel. (wt) Untransformed cells; (+/−) cells transformed with the construct containing the BLE cassette; (−/−) cells transformed with both the BLE and PUR cassette constructs. The origins of the PCR products are indicated at right of the gel photograph.
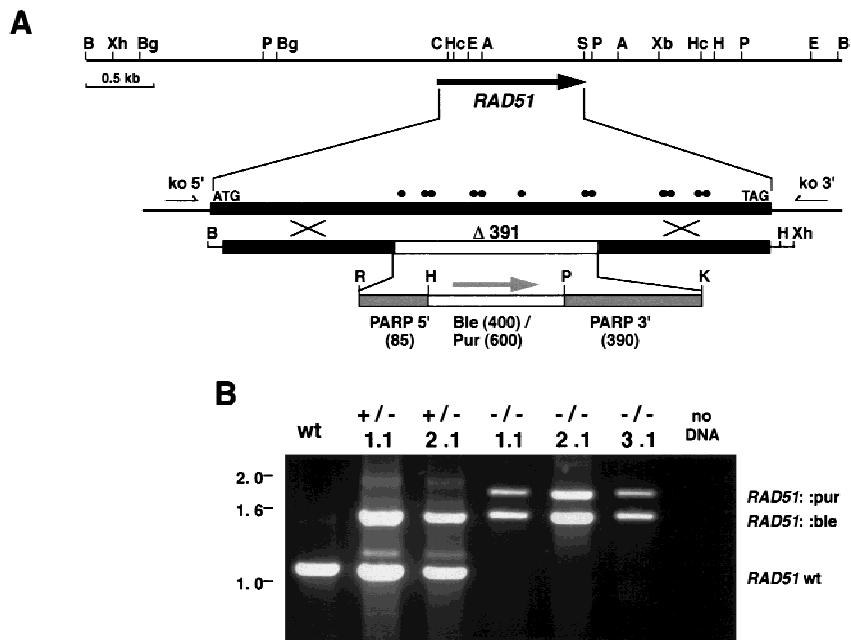
integration of both cassettes had removed *RAD51* wild type from this locus. Note that the two second round transformants shown here (and a third discussed below) were generated completely independently; each was created in a separate experiment, from different starting populations of untransformed cells, involving two rounds of transformation and cloning. Southern blots of genomic DNA from the transformants, when probed with *RAD51* wild type sequence (data not shown), confirmed that no intact *RAD51* alleles were present in the putative homozygous mutants (*RAD51*−/−). Identical results were obtained for procyclic transformants (data not shown), suggesting that the *RAD51* gene can be disrupted in both life-cycle stages and the cells remain viable.

To check that there was no wild-type *RAD51* mRNA being made in the putative homozygous mutants, we isolated total RNA from the various transformants, generated cDNA by reverse transcription, and performed PCR (Fig. 3). The PCR used primers complementary to *RAD51* ORF sequences outside of the deleted region, and so even a small amount of residual wild-type mRNA should be detected. For both life-cycle stages a PCR product of the expected size was readily amplified from both the *RAD51* wild-type and *RAD51*+/− cells, but none could be detected in the *RAD51*−/− cells. We therefore conclude that it is possible to remove all transcripts that can express more than the first third of the RAD51 protein and trypanosomes remain viable.

Trypanosome RAD51−/− *cells are more sensitive to DNA damage than* RAD51 *wild-type or* RAD51+/− *cells*

Homologous recombination is one of a number of DNA repair pathways that are available to cells to reverse the

Figure 3. Reverse transcription PCR of trypanosome *RAD51* transformants. cDNA representing the RNA from bloodstream form (BSF; these are the transgenic line 3174) and procyclic (PC) transformants was tested by PCR for the presence of either *RAD51* or, as a control for the integrity of the substrate, *RNA polymerase I* (*RNA pol I*) mRNA. The PCR reactions were separated by agarose gel electrophoresis, Southern blotted, and probed with either the *RAD51* or *RNA Pol I* ORF. (wt) Untransformed cells; heterozygous or homozygous *RAD51* transformants are indicated by +/− and −/−, respectively.
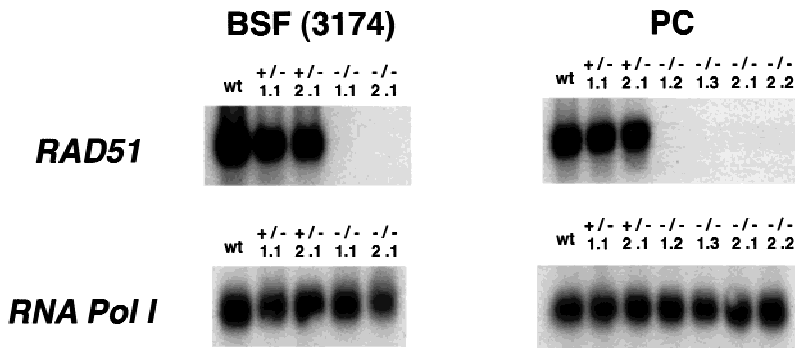
potentially lethal effects of damage to their genomes. Therefore, it has been observed that *RAD51* mutants in yeast, and *recA* mutants in bacteria, display increased sensitivity to DNA damaging agents such as ultraviolet (UV) light or the radiomimetic alkylating agent methyl methanesulphonate (MMS; Ogawa et al. 1993a). To test whether or not our putative trypanosome *RAD51* mutants display a phenotype consistent with the loss of active RAD51, we assayed the transformants for their sensitivity to such agents. Figure 4 shows growth curves of *RAD51* wild-type cells and two independently generated *RAD51*$^{+/-}$ and *RAD51*$^{-/-}$ bloodstream cell lines in the presence of increasing concentrations of MMS.

It is apparent that, in the absence of mutagen (in this case MMS), the *RAD51*$^{-/-}$ cells have a reduced growth rate relative to the *RAD51* wild-type or *RAD51*$^{+/-}$ cells, and reach slightly lower maximal densities in culture medium. The average population doubling time during exponential growth in vitro was 9.6 hr for the *RAD51* wild-type cells and 8.1 and 8.7 hr for the *RAD51*$^{+/-}$ cell lines, compared with 10.6 and 11.1 hr for the *RAD51*$^{-/-}$ cell lines. In other words, the *RAD51*$^{-/-}$ mutation increased the generation time of the cells in vitro by 12% to 30% (relative to wild-type or $^{+/-}$). Next, we checked whether the *RAD51*$^{-/-}$ cells are capable of establishing an infection in mice, where they are exposed to host defenses (Table 1). The reduction in growth rate was not as severe during in vivo growth as was seen in vitro, but was still apparent: The *RAD51*$^{-/-}$ cells had an increased doubling time of 10% to 14% relative to the wild-type or $^{+/-}$ cells in either immunosuppressed or immunocompetent hosts. Although significant, this is a modest effect on growth when compared with either yeast *RAD51* or *E. coli recA* mutants (which show 60% and 40% increased generation times, respectively; Capaldo et al. 1974; Fingerhut et al. 1984). We assume that this is a population phenomenon, whereby a larger portion of the *RAD51*$^{-/-}$ than the *RAD51* wild-type or *RAD51*$^{+/-}$ cells fails to complete cell division, although we have not excluded that individual *RAD51*$^{-/-}$ cells simply take longer to undergo their cell cycle. Clearly, however, a *RAD51* mutation in trypanosomes is not lethal, as it is mice (Lim and Hasty 1996; Tsuzuki et al. 1996), because the cells are capable of replicating efficiently in vivo.

Comparing the growth curves in increasing MMS concentrations (Fig. 4) shows that the *RAD51*$^{-/-}$ cells are more sensitive to the drug than either *RAD51* wild-type or *RAD51*$^{+/-}$ cells. Growth of the wild-type and $^{+/-}$ cells was barely affected by 0.0001% MMS, but 0.0002% and 0.0003% caused some growth retardation. In contrast, growth of the *RAD51*$^{-/-}$ cells was substantially retarded even at 0.0001%, and severely affected at higher concen-

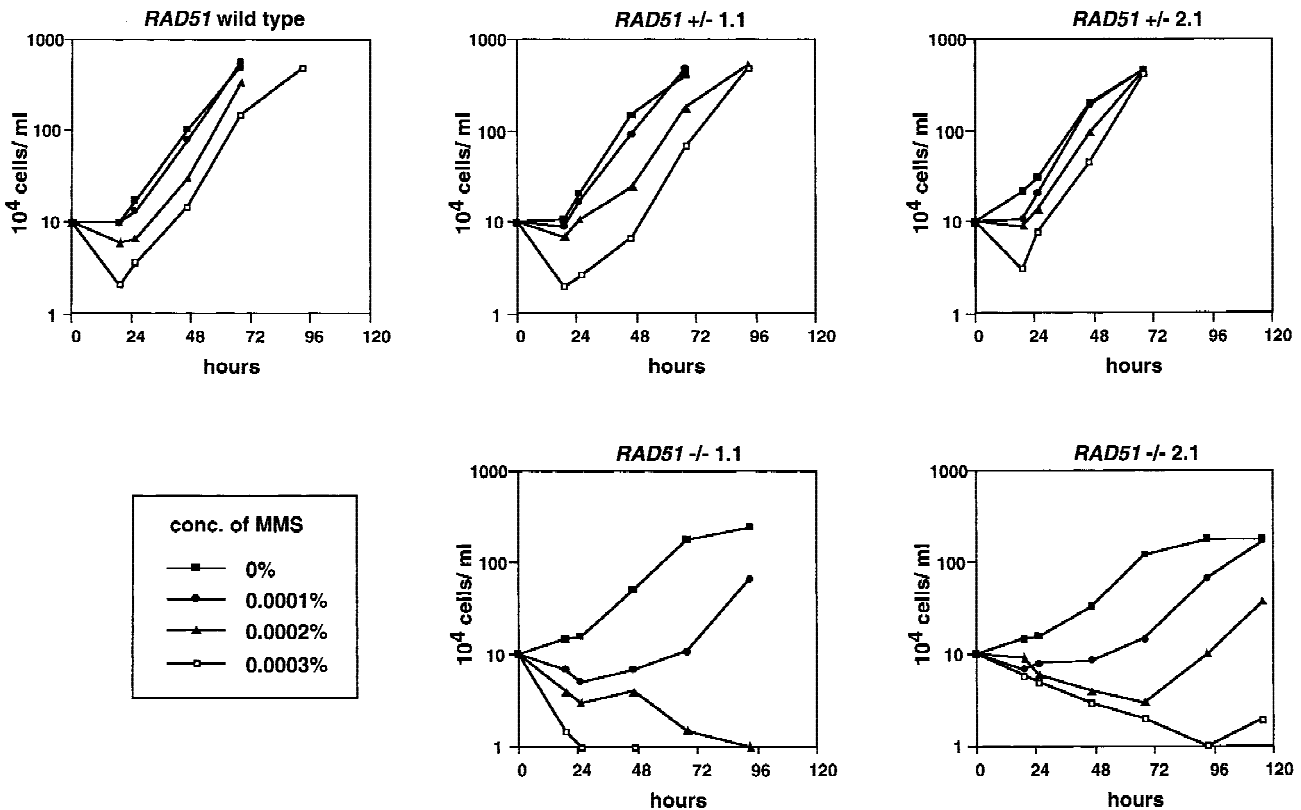**Figure 4.** Growth of *RAD51* knockout trypanosomes in the presence of MMS. Bloodstream trypanosomes that had been unaltered in their *RAD51* gene (*RAD51* wild type) had one allele disrupted (*RAD51*$^{+/-}$) or had both alleles disrupted (*RAD51*$^{-/-}$) were grown in medium containing increasing concentrations of MMS (as shown). The concentration of trypanosomes in the medium was counted at the given times.

Table 1. *Population doubling times (hr) of bloodstream trypanosome* RAD51 *knockouts in mice*

Strain	Immunocompromised	Immunocompetent
RAD51 wild type	7.8 ± 0.3	6.6 ± 0.2
RAD51⁺/⁻ 1.1	7.7 ± 0.2	6.6 ± 0.2
RAD51⁻/⁻ 2.1	8.8 ± 0.7	7.45 ± 0.25
RAD51⁻/⁻ 3.1	8.6 ± 0.5	7.3 ± 0.2

trations. These results are consistent with the $RAD51^{-/-}$ cells being less able to repair DNA damage caused by MMS. To test this hypothesis further, we compared the cells' sensitivity with a second compound, 3-aminobenzamide. This is a competitive inhibitor of the nuclear enzyme poly (ADP-ribose) polymerase (ADPRT), which is responsible for recognizing single-strand breaks in DNA and coordinating DNA repair (Jeggo 1998). Chemical inhibition of ADPRT causes increased levels of sister chromatid exchange. This has been interpreted as meaning that inhibition prevents normal repair of single-strand breaks, and allows them to be processed to double-strand breaks, which are then the target for homologous recombination (Lindahl et al. 1995). As with MMS, we found that the $RAD51^{-/-}$ cells are more sensitive to 3-aminobenzamide (5–15 mM) than the $RAD51$ wild-type or $RAD51^{+/-}$ cells during growth (R. McCulloch, unpubl.). Together, these data suggest that the $RAD51^{-/-}$ mutation we have made impairs the ability of the trypanosomes to repair damage to their DNA, which is most simply explained by an absence of functional RAD51 in the cells.
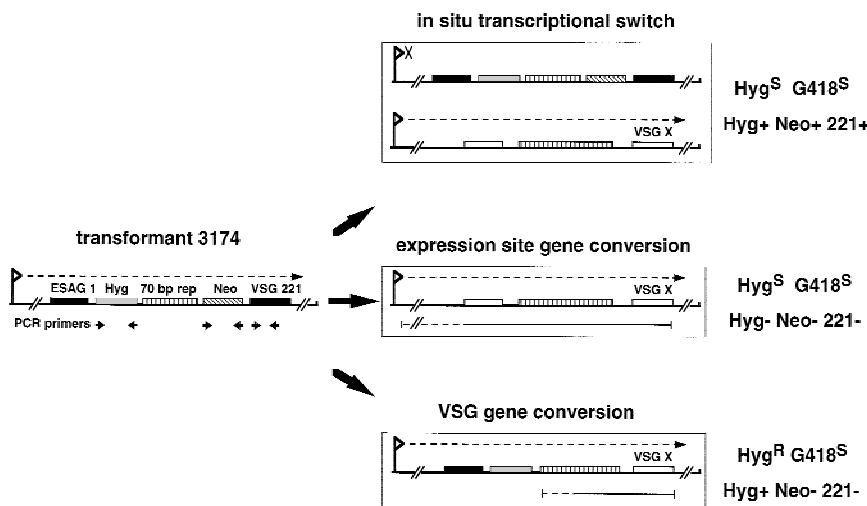
If $RAD51$ is the trypanosome enzyme responsible for DNA strand transfer and exchange, we would expect that the $RAD51^{-/-}$ cells would be deficient in general homologous recombination. Unlike in bacteria and yeast, in which a plethora of genetic assays have revealed a number of different pathways of genetic exchange, very little is known about the recombination mechanisms used by trypanosomes. However, it appears that integration of DNA into the trypanosome genome occurs only into sites with homologous sequences, as no examples of nonhomologous insertions have been described (Lee and van der Ploeg 1990; Eid and Sollner-Webb 1991; Blundell et al. 1996). Therefore, we sought to determine whether the $RAD51^{-/-}$ mutation results in a deficiency in the integration of transformed DNA. This was first assayed by measuring the transformation efficiencies of procyclic cell lines electroporated with the construct pUCtubneo3 (Ten Asbroek et al. 1990), which integrates a neomycin phosphotransferase cassette into the tubulin gene array. For both $RAD51$ wild-type and $RAD51^{+/-}$ cells the measured transformation efficiencies were between 1×10^{-5} and 0.3×10^{-5}. In contrast, the maximum transformation efficiency obtained for the $RAD51^{-/-}$ cells was 1×10^{-6}, indicating a reduction of at least 10-fold. The bloodstream cells used here already contain an integrated neomycin resistance cassette (see below), making pUCtubneo3 transformation impossible. Instead, we used clonal growth on semisolid agarose plates

to measure the transformation efficiency of a construct (*tub::RAD51–BSR*; see below) designed to integrate the complete $RAD51$ gene into the tubulin locus, allowing its re-expression in the knockout mutants. The $RAD51$ wild-type cells had an average transformation frequency of 8×10^{-7}. In three independent experiments with the $RAD51^{-/-}2.1$ transformant, each time selecting 1×10^{7} cells, we recovered no transformants, indicating a reduction in transformation frequency of at least 24-fold. Bloodstream transformants could, however, be recovered by selecting in liquid medium rather than on agarose plates (see below). Together, these data show that in both life-cycle stages the absence of RAD51 impairs, but does not eliminate, the trypanosome's ability to integrate transformed DNA.

Mutating RAD51 *reduces the frequency of trypanosome antigenic variation*

To assess the effect that the $RAD51^{-/-}$ mutation has on trypanosome antigenic variation, we made the gene knockouts in a transgenic strain of trypanosomes that allows us to assay for the frequency of VSG switching as well as score the relative contributions of in situ and gene conversion switching reactions. This strain has been described previously (McCulloch et al. 1997); it contains antibiotic resistance markers for hygromycin and G418 within its active expression site (Fig. 5). By immunizing mice against the VSG coat that this strain expresses (called VSG221) and then injecting measured numbers of trypanosomes into the immunized mice, we can estimate the number of trypanosomes that survive the immune selection, and thereby calculate the frequency with which the cells switch to another VSG coat. Furthermore, having isolated and cloned the surviving switched trypanosomes, we can use the resistance markers within the expression site to determine the switching reaction they have undergone (see Fig. 5).

A comparison of the switching frequencies measured for the $RAD51$ transformants is shown in Table 2. The $RAD51$ wild-type cells underwent antigenic variation at a frequency of $\sim0.9 \times 10^{-6}$ (±0.2) switches/cell/generation. Disruption of one $RAD51$ allele appeared to have no effect on this switching frequency. In contrast, disruption of both alleles appeared to impair the ability of the cells to undergo switching, because a reduction in the switching frequency of the $RAD51^{-/-}$ cells was apparent in all the measurements made. The extent of this reduction was highly variable, however, and could be as little as 2-fold or as great as 130-fold. The reason for this variability is not clear, but is not due to genetic changes in one particular $RAD51^{-/-}$ transformant: Three independently generated $RAD51^{-/-}$ cell lines were tested, and all displayed the same variable phenotype. A likely explanation is experimental fluctuation in the assay, because switched cells can arise at any cell division during expansion of the population prior to injection into the immunized mice. If a switching event occurred at an early division, this would result in a greater number of that switched variant than if it had occurred at a late

Figure 5. Use of the transgenic trypanosome line 3174 to characterize *VSG* switching events. The telomeric region of the actively transcribed expression site in 3174 bloodstream cells is indicated. *VSG221* and the most proximal *ESAG* (ESAG1) are separated by resistance markers for hygromycin (Hyg) and G418 (Neo), which are in turn separated by a stretch of 70-bp repeats; transcription of the site is depicted by a dashed arrow, and the position of primers used to assay for the presence of the genes within the expression site are indicated by small arrows. Three different switching events are observed in this trypanosome strain. In an in situ switch, another expression site (containing an unknown *VSG*; *X*) is transcriptionally activated and the *VSG221* site is inactivated. These cells are sensitive to Hygromycin (Hyg^s) and G418 (G418^s), and each marker gene can be amplified by PCR (Hyg+, Neo+, 221+). A long range gene conversion event from another expression site deletes all the marker genes from the *VSG221* site, and replaces them with *VSGX*, these are Hyg^s and G418^s, and the genes cannot be amplified by PCR (Hyg$^-$, Neo$^-$, 221$^-$). In a gene conversion event extending from the 70-bp repeats to the 3′ end of *VSG221*, *VSGX* replaces only the 221 and Neo markers; these switchers are hygromycin resistant (Hyg^R) and G418^s, and are Hyg$^+$, Neo$^-$, 221$^-$ in PCR.

division. For the *RAD51* wild-type and *RAD51*$^{+/-}$ cells such fluctuations are likely to be less obvious, because active RAD51 will cause switchers to arise with greater frequency (and hence consistency) at each cell division.

To test that the reduction in VSG switching frequency is due to the *RAD51* mutation and not incidental changes the arose during selection and cloning of the transformants, we examined the effect of re-expressing RAD51 in the homozygous mutants. To do this, we transformed *RAD51*$^{-/-}$*2.1* cells with the construct *tub::RAD51–BSR*; this should integrate the complete *RAD51* gene into the tubulin locus, with a coexpressed blasticidin resistance cassette allowing selection. Transformation occurred at the expected frequencies in the *RAD51* wild-type cells, but was more inefficient in the *RAD51*$^{-/-}$ cells; no blasticidin-resistant clones were recovered on plate selection (see above), and in nine independent experiments, each selecting 10^7 cells in liquid medium, only five drug-resistant cell lines arose. Southern analysis showed that only one of the five had integrated the construct into the expected genomic location, and RT–PCR showed that it was expressing intact *RAD51* RNA (data not shown). The VSG switching frequency for this clone was ~0.7×10^{-6} (±0.4) switches/cell/generation (Table 2), indicating that it was the absence of functional RAD51, and no other genetic changes, that caused an impairment in VSG switching in the *RAD51*$^{-/-}$ cells.

Next, we took a selection of switched variants derived from the *RAD51* wild-type cells and from one *RAD51*$^{-/-}$ cell line and compared the switching mechanisms that they used (Table 3; variants arising from three independent switching experiments were examined). No significant differences in the ratios of the different reactions were observed. For both cell lines, transcriptional switches accounted for around one-half of the total events analyzed, and the remainder were apparent gene conversions that removed the marker genes from the active expression site. These gene conversions can remove both markers, and hence involve extensive changes to the expression site, or can remove only the neomycin marker and *VSG221* gene downstream of the 70-bp repeats: the relative amounts of these were also unaltered by the *RAD51*$^{-/-}$ mutation. These data suggest that both in situ transcriptional switching and gene conversion switching were down-regulated by the *RAD51* mutation to cause the observed reduction in the frequency of antigenic variation.

Discussion

To begin to determine the genetic requirements of antigenic variation in African trypanosomes, we have cloned a *T. brucei* gene encoding a homolog of eukaryotic RAD51 and have analyzed its role in this immune evasion process. Our contention that the cloned gene encodes a RAD51-like enzyme is based on phenotypes that we observe when we make trypanosomes mutant in this gene, and also on the very high level of conservation in primary amino acid sequence between the putative *T. brucei* polypeptide and those characterized from other eukaryotes.

Analysis of the phenotypes of the *RAD51*$^{-/-}$ mutants indicated that we were able to generate transgenic trypanosomes that lack functional RAD51 enzyme. The *RAD51*$^{-/-}$ mutation described rendered bloodstream-stage cells more sensitive to two DNA damaging agents tested: methylmethane sulfonate and 3-aminobenzamide. This is consistent with an impairment in the mutant cells' ability to repair DNA damage through ho-

Table 2. *Effect of* RAD51 *mutation on the frequency of* VSG *switching in the 3174 trypanosome strain*

Strain	No. of injected tryps	No. of wells growing[a]	Estimated VSG switching frequency $(\times 10^{-6})$[b]
RAD51 wild type	5×10^7	75/96	0.95
	5×10^7	56/96	0.70
	4.5×10^7	76.5/96	1.1
	4.5×10^7	72/96	1.0
	5×10^7	67/96	0.83
RAD51$^{+/-}$ *1.1*	5×10^7	76/96	0.95
	5×10^7	53.5/96	0.66
RAD51$^{-/-}$ *1.1*	5×10^7	1.5/96	0.02
	5×10^7	1.5/96	0.02
	1.6×10^8	41/96	0.18
RAD51$^{-/-}$ 2.1	5×10^7	0.5/96	0.007
	5×10^7	6.5/96	0.09
	1×10^8	2/96	0.01
	1×10^8	19/96	0.13
RAD51$^{-/-}$ 3.1	7.5×10^7	8.5/96	0.08
	7.5×10^7	43.5/96	0.41
RAD51$^{-/-}$ 2.1 tub::RAD51-BSR	5×10^7	88/96	1.1
	5×10^7	82/96	1.0
	5×10^7	24/96	0.3
	5×10^7	25/96	0.31

[a]Counted 7 days after cloning for wild-type and +/– cells, and up to 10 days after cloning for –/– cells.
[b]Events/cell/generation: determined as described in McCulloch et al. (1997), and as discussed in Materials and Methods.

mologous recombination. We could not, however, detect the same increased sensitivity to UV light (data not shown). This may be because repair of UV damage can occur efficiently by nucleotide excision repair (Sancer 1994) in trypanosomes, thereby masking any defects in homologous recombination. Alternatively, the simple growth curves we have used may not be a sensitive enough assay, as some yeast *RAD51* mutants display only slight UV sensitivity (Game 1983). Genetic transformation of *RAD51*$^{-/-}$ cells by DNA electroporation was less efficient than either *RAD51* wild-type or *RAD51*$^{+/-}$ cells. It is likely that this is because the absence of functional RAD51 reduces the cells' ability to recombine transformed DNA into their genomes. It is interesting to note, however, that transformants could still be generated. Many different assays of genetic exchange in bacteria, yeast, and higher eukaryotes have suggested that organisms contain multiple pathways of homologous and nonhomologous recombination (Haber 1995; Shinohara and Ogawa 1995; Baumann and West 1998), some of which involve RAD51 or RecA and some of which do not (see below). Most of the trypanosome *RAD51*$^{-/-}$ transformants analyzed here had integrated the electroporated constructs into unexpected genomic locations (data not shown), indicating that the normal pathway of homologous integration had been altered by the absence of RAD51. This suggests that RAD51-inde-

pendent pathways of genetic exchange are also present in trypanosomes. The viability of trypanosome *RAD51* homozygous mutants is comparable with the same mutants in yeast (and *recA* in bacteria), and contrasts with the lethality of *RAD51* mutations in mice (Lim and Hasty 1996; Tsuzuki et al. 1996). Whether this reflects RAD51 functions beyond DNA strand transfer and exchange that are present (or critical) in mice but not in single-cell organisms, or is a reflection of the greater necessity for homologous recombination during multicellular development as opposed to clonal outgrowth of a single-cell population, remains to be determined (Edelmann and Kucherlapati 1996; Baumann and West 1998).

Homozygous knockout of *RAD51* reduced the efficiency with which trypanosomes switch from one VSG coat to another. This suggests either that *VSG* switching is, at least in part, catalyzed by RAD51 and involves homologous recombination, or that RAD51 has a regulatory role in VSG switching. The absence of functional RAD51 did not render the trypanosomes completely unable to undergo antigenic variation. In all the experiments, some *RAD51*$^{-/-}$ trypanosomes that had succeeded in switching their VSG coat were present; in some experiments, *VSG* switchers arose nearly as efficiently as in *RAD51* wild-type cells. Nevertheless, this is the first time that disruption of a specific gene in trypanosomes has been shown to have an effect on antigenic variation. Previous attempts to assess the mechanisms involved in VSG switching have relied on indirect experimental approaches. Cornelissen et al. (1985) used 3-aminobenzamide to inhibit trypanosome poly (ADP-ribose) polymerase in vivo, and showed that this caused a reduction in VSG switching frequency. These experiments were later repeated in vitro, with conflicting results (Mhlanga 1994; Lamont and Cross 1987). van Leeuwen et al. (1998) showed that growth of trypanosomes in the presence of the nucleoside analog hydroxymethyl-deoxyuridine caused a 15-fold increase in the amount of the modified base J in the genome, and a reduction in the incidence of chromosome breakage events that have been associated with expression site transcriptional switching in vitro (M. Cross et al. 1998; Rudenko et al. 1998). Whether these treatments affect the RAD51 pathway of switching, or whether they disrupt independent facets of the process, remains to be determined.

Antigenic variation occurs by two main routes,

Table 3. *VSG switching mechanisms used by* RAD51 *wild-type and* RAD51 *homozygous mutant trypanosomes*

Strain (no. of switched variants analyzed)	In situ switch	Expression site gene conversion	*VSG* gene conversion
RAD51 wild type (34)	20/34 59%	9/34 26%	5/34 15%
RAD51$^{-/-}$ 1.1 (45)	23/45 51%	14/45 31%	8/45 18%

Types of switching event were assayed as described in Fig. 5 and McCulloch et al. (1997).

switching of transcription between the single active bloodstream expression site and an inactive site, or movement of silent *VSG*s into the active expression site. Because only the latter of these routes has been shown to involve DNA recombination, we expected that disruption of *RAD51* would affect only this form of *VSG* switching. What we saw, however, was that the reduction in the frequency of antigenic variation was due to a uniform decrease in both routes of switching. This inference is complicated somewhat by the variability in the measured level of this reduction but, even in those experiments in which only very small numbers of switched variants were recovered from the *RAD51*$^{-/-}$ cells, we found examples of both types of switching event (data not shown). Switched variants in the 3174 *RAD51* wild-type trypanosomes are generated by transcriptional switching events ~50% of the time. If disruption of *RAD51* affected only the recombination-based pathways, the maximum reduction we should have seen in the frequency of antigenic variation would be twofold. Because we saw reductions of at least fivefold in all but one experiment, it appears that in situ transcriptional switching is also affected by the *RAD51* mutation. It should be noted, however, that our assay would not distinguish between transcriptional switching and reciprocal recombination events in which the active and inactive expression site exchange ends upstream of the markers. It seems unlikely, though, that this recombination reaction would be unaffected by *RAD51* inactivation, whereas all other reactions are.

An alternative explanation for these results is that no *VSG* switching pathway is affected by the *RAD51*$^{-/-}$ mutation, and the apparent reduction in switching frequency is an indirect consequence of the absence of functional RAD51 (e.g., due to a generalized growth defect). This hypothesis is not supported by any phenotypes we observed, however. The *RAD51*$^{-/-}$ cells retain infectivity in mice, and the observed reduction in their growth rate was accounted for in calculating the switch frequencies. Furthermore, we find no evidence that the *RAD51*$^{-/-}$ cells are significantly impaired in their ability to grow in vitro after recovery from mice. It is also unlikely that the *RAD51* mutation affects a *VSG*-switching mechanism that is undetectable in our assay, as all switching events thus far characterized fit with the scheme predicted in Figure 4 (see also McCulloch et al. 1997). Therefore, we interpret these results as meaning that the *RAD51* mutation affects both pathways of switching equally, and the low level of residual events is due to RAD51-independent mechanisms. We suggest that the variability in the level of reduction in switching is due to a founder effect in the assay we have used. RAD51-independent switchers arise at random during the growth of the *RAD51*$^{-/-}$ cells prior to selection of switchers, and in some instances, these can accumulate to such sufficiently large numbers, that it appears antigenic variation is little affected by the *RAD51* mutation.

The fact that we can detect switched variants that have deleted the *VSG* and associated marker genes from the active expression site suggests that gene conversion-like recombination events can occur in the absence of RAD51. How these reactions have occurred has not been examined in detail, but a number of recombination pathways that could potentially generate these types of events have been described in yeast and other organisms. RAD51 is normally considered to catalyze homologous recombination reactions that proceed from a double-strand DNA break and use a Holliday junction intermediate (so called double-strand break repair recombination). Other recombination reactions exist that do not conform to this pathway, and some have been shown not to involve RAD51 (Shinohara and Ogawa 1995; Baumann and West 1998). Single-strand annealing is a recombination reaction that has been shown to occur between directly repeated DNA sequences, and may be catalyzed by a conserved eukaryotic recombination enzyme called RAD52 (Mortensen et al. 1996). Work in *Drosophila* has detailed reactions associated with P-element transposition that also appear not to involve Holliday intermediates and can occur within, and between, chromosomes (Nassif et al. 1996). The genetic requirements of this so-called synthesis-dependent strand-annealing model have not yet been determined, but it has been noted that a number of the features of this reaction are salient for the requirements of trypanosome *VSG* switching (Borst et al. 1996). In yeast, DNA breaks have been shown to be repaired by DNA replication in the absence of RAD51, resulting in nonreciprocal transfer of sequence information from homologous sites (Malkova et al. 1996) up to and including the telomere; such events could readily provide new *VSG*s being expressed with concomitant deletion of sequence within the expression site as we see here. To establish whether or not any of the above types of recombination reaction are occurring in the *RAD51*$^{-/-}$ switchers, further experiments will be required. Interestingly, in one switch that we have analyzed by pulsed-field gel electrophoresis, we can see that the newly expressed *VSG* has made a new copy, but the *VSG221* expression-site chromosome appears to have changed size (R. McCulloch, unpubl.), suggesting that these can involve nonstandard means of gene conversion.

How do we account for our observation that the *RAD51* mutation affects in situ transcriptional switching, which has not been considered previously to require genetic exchange? The simplest explanation is that RAD51 is directly involved, and switching occurs by undetected recombination reactions between expression sites. This seems unlikely. Although DNA rearrangements can be detected around the expression site promoter during in situ switching (for review, see Pays et al. 1994), and alteration of expression-site sequences by recombination can apparently precipitate an in situ switch (Davies et al. 1997), a number of studies have detailed transcriptional switches that show no structural rearrangements either at the promoter or in upstream sequence elements (Horn and Cross 1997; Rudenko et al. 1998). A second explanation might be that the transcriptional switching reaction does not require RAD51 for recombination, but instead for transient association be-

tween the expression sites. Although there is no direct evidence that this is the case, most work has focused on the proposed mechanisms that control the activity of the expression sites, and has not considered whether association between the active site and an inactive site is needed to effect a switch (Horn and Cross 1995; Rudenko et al. 1995). Whether this would require some form of RAD51-dependent homology sensing is conjecture, however, and is argued against by the fact that the expression-site promoter can be changed to a nonhomologous ribosomal promoter without affecting switching (Rudenko et al. 1995). On the other hand, a number of reports have shown that pairing of chromosomes in homologous regions, or pairing of a chromosome with an injected plasmid (Ashe et al. 1997), can cause up- or down-regulation of transcription in a number of organisms (for review, see Hensikoff 1997; Grant 1999). A final explanation would be that it is not the absence of RAD51 itself that causes an impairment in in situ switching, but the effect this has on a protein(s) that RAD51 interacts with. In higher eukaryotes, RAD51 interacts with a number of cellular factors that are not directly involved in recombination, for example, the tumor suppressers p53 and BRCA1 and 2 (Sturzbecher et al. 1996; Scully et al. 1997; Sharan et al. 1997). It is possible that RAD51 provides a means by which the cell can integrate DNA repair and recombination with a number of other processes, such as cell cycle progression and transcriptional control (Baumann and West 1998). In other words, disruption of RAD51 may have an indirect effect that disrupts the normal way that trypanosomes co-ordinate in situ transcriptional switching, and VSG recombination, with other aspects of cell metabolism. Further studies of the genetics of trypanosome antigenic variation will be required to address these complex issues.

Materials and methods

Trypanosome strains and transformation

T. brucei 221a trypanosomes (MITat1.2a) of strain 427 (Cross 1975) were used. Bloodstream cells were grown in vitro at 37°C in HMI-9 medium (Hirumi and Hirumi 1989), whereas procyclic cells were grown at 27°C in SDM-79 (Brun and Schonenberger 1979). Trypanosomes were transformed by electroporating 5×10^7 cells with ~5 µg of plasmid DNA that had been restriction digested, phenol/chloroform treated, and ethanol precipitated. Electroporation was performed in 0.5 ml of Zimmerman Post-Fusion medium (132 mM NaCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.5 mM Mg acetate, 0.09 mM Ca acetate at pH7.0), supplemented with 1%glucose for the bloodstream cells, with a Biorad Gene Pulser II set at 1.4 kV and 25 µF capacitance. Cells were allowed to recover for 18 hr before transformants were selected. Bloodstream RAD51 transformants were selected on semisolid agarose plates, whereas procyclic transformants were selected in 5–10 ml of liquid medium supplemented with ~10^6 untransformed cells.

Cloning and sequencing the T. brucei RAD51 gene

To obtain a probe to clone the T. brucei RAD51 gene, PCR was performed on T. brucei genomic DNA with oligonucleotides

directed against two conserved regions of the RAD51 protein family identified by Shinohara et al. (1993): 5′RAD51, 5′-CC-<u>GAATTC</u>TT(C/T)GGIGA(A/G)TT(T/C)(A/C)GIACIGG(G/A/T/C)AA-3′ (EcoRI underlined); 3′RAD51, 5′-CC<u>GGATCC</u>IGTI-CC(T/C)TCIGT(A/G)TC(G/A/T/C)AT-3′ (BamHI underlined). PCR was carried out with ~30 pmoles of each primer with an initial 5 min denaturation at 94°C followed by 30 cycles of 94°C for 1 min, 42°C for 1 min, and 72°C for 1 min A 120-bp product was generated and cloned into pBluescript (Stratagene) which, on sequencing, was predicted to encode the expected region of T. brucei RAD51 (Fig. 1). The probe was then used to screen two T. brucei mini-libraries generated in pBluescript with 2–3 kb of PstI-digested, and 5–6 kb of BamHI-digested, genomic DNA. This allowed us to isolate a 2.5-kb PstI fragment (Fig. 2) that contained the complete RAD51 ORF, as well as a larger 5.5-kb BamHI fragment. The sequence of the PstI fragment was determined on both strands by ABI-PRISM automated sequencing with custom-designed primers. The sequence predicts a polypeptide with 60 amino acids more at its carboxyl terminus than suggested by a database entry from Haag (accession no. Y13144). We believe ours to be correct, as the start codon we have used is the first that is present ~180-bp downstream of a large polypyrimidine tract (Matthews et al. 1990; Schurch et al. 1990) that is likely to dictate the trans-splicing that creates the 5′ end of RAD51 mRNA (corroborated by RT–PCR; data not shown).

Generation and analysis of T. brucei RAD51 knockouts

For mapping of the transformants, genomic DNA was restriction digested and separated on 1.0% agarose gels in 1× TBE (90 mM Tris-borate, 2 mM EDTA). Intact chromosomes were separated by pulsed-field electrophoresis in 90 mM Tris-borate, 0.2 mM EDTA on 1.2% agarose gels with a Bio-Rad CHEF-DRIII system. Conditions for electrophoresis were from S. Melville (University of Cambridge, UK), 144 hr at 15°C and 2.5 V/cm with 1400–700 sec switch times. Each gel lane contained ~2.5×10^7 cells embedded in 0.5% low melting point agarose. All DNA was blotted onto Hybond N (Amersham) following the manufacturer's instructions. Hybridization of probes (labeled with [α-^{32}P]dATP by random priming) was as described in Sambrook et al. (1989), and all blots were washed to a stringency of 0.2× SSC, 0.1% SDS at 65°C. All PCR for generating and analyzing transformants (and VSG switchers) used an initial 5 min denaturation at 95°C followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1–2 min.

The constructs used to generate T. brucei RAD51 knockout mutants contain 300 bp of 5′ and 3′ RAD51 sequences (that act as targeting flanks) separated by one of two antibiotic resistance cassettes. The RAD51 sequences were amplified by PCR from genomic DNA with Pfu DNA polymerase, and cloned into pBluescript. 5′ flank primers: 5′-CC<u>GGATCC</u>ATGCAGCAG-CAAGTTGGTGA-3′ (BamHI), and 5′-CC<u>GAATTC</u>CTCACG-GCTGCCTGTCGTGA-3′ (EcoRI). 3′ flank primers, 5′-CC<u>GG-TACC</u>GCACGGCAGATGCATCTTGG-3′ (KpnI), and 5′-CC-<u>AAGCTT</u>GTCCCTAACGTCTCCCACA-3′ (HindIII). The antibiotic resistance cassettes contained either the 400-bp bleomycin phosphotransferase ORF or the 600-bp puromycin N-acetyltransferase ORF (both gifts of J. Mottram, University of Glasgow) flanked by 100 bp of 5′ and 300 bp of 3′ processing signals from the PARP locus (gifts from S. Graham, University of Glasgow). The cassettes were cloned between the RAD51 targeting sequences to generate ΔRAD51::BLE and ΔRAD51::PUR constructs, respectively. The plasmids were digested with BamHI and XhoI prior to being transformed into T. brucei, and transformants were selected with 2.0 µg/ml bleomycin or 1.0 mg/ml puromycin. Note that the constructs con-

tain no promoter elements to drive the antibiotic resistance cassette expression (and therefore rely on endogenous *RAD51* transcription), and the *RAD51* trans-splicing and polyadenylation signals are not altered by the knockout strategy because only internal *RAD51* coding sequence is removed.

Correct integration of the constructs into *RAD51* was determined in two ways. First, PCR was performed on the transformants' genomic DNA with primers outside of the *RAD51* sequence included in the constructs (Fig. 2), ko5′, 5′-GATGAT-GCCGCAGTGGATGCGG-3′, and ko3′, 5′-GCACTTCTCC-GCTTTCGGAGTG-3′. Because of the high GC content of the resistance cassettes, this PCR was performed with 5%DMSO. Second, to determine that *RAD51* wild-type was removed from the genome in the puromycin and bleomycin double-resistant clones, genomic DNA from the transformants was digested with either *Pst*I or *Eco*RV and probed with *RAD51* wild-type ORF amplified by PCR with the *Bam*HI and *Hin*dIII construct primers (above). In both digests a 2.5-kb fragment corresponding to intact *RAD51* was detected in both the wild-type and heterozygous cells, but was absent in the homozygous mutants. The only other hybridising bands corresponded to the Δ*RAD51::BLE* and Δ*RAD51::PUR* alleles (data not shown).

Total RNA from the *RAD51* transformants was prepared with TRIzol, and cDNA was synthesized with random hexamers and Superscript reverse transcriptase (all reagents from Life Technologies). To assess whether or not intact *RAD51* mRNA was present in the knockouts, PCR was performed on the cDNAs with primers rad51d6 (5′-TGGTGACGCTGCCGGTGGGC-3′) and rad51u3 (5′-TTTCCAAGATGCATCTGCCG-3′). The primers used to perform a control PCR on cDNA from RNA polymerase I mRNA have been described before (Rudenko et al. 1996).

Re-expressing RAD51 in the homozygous knockouts

The construct *tub::RAD51–BSR* contains 240 bp of β-α tubulin and 330 bp of α-β tubulin intergenic sequence (derived from the construct hygrotub; Blundell et al. 1996) at its 5′ and 3′ flanks, respectively; these allow integration into the tubulin locus, replacing an α tubulin gene. Between the flanks, a 400-bp Blasticidin S deaminase ORF (*BSR*; a gift from M. Cross, Netherlands Cancer Institute) was cloned and, downstream, a 2.27-kb *Bgl*II–*Pst*I *T. brucei* genomic DNA fragment containing the complete *RAD51* ORF and ~1.0-kb of 5′ sequence. On correct integration, the endogenous *RAD51* signals should dictate the 5′ splicing of *RAD51* RNA and 3′ polyadenylation of *BSR* RNA, whereas the β tubulin gene signals 3′ of *RAD51* will dictate its polyadenylation. We did not test whether this ectopic expression strategy resulted in the same steady-state levels of *RAD51* mRNA as endogenous *RAD51*. *tub::RAD51–BSR* was digested with *Xba*I and *Xho*I prior to electroporation, and transformants were selected on semisolid agarose plates containing 2 µg/ml Blasticidin (Invitrogen), or in 10 ml of HMI-9 medium containing 5 or 10 µg/ml. Integration of the constructs was examined by Southern analysis of *Eco*RV or *Bam*HI-digested genomic DNA, and RT–PCR to check for intact *RAD51* mRNA was performed as described in the previous section (data not shown).

Measuring the growth of trypanosome RAD51 mutants

To measure the growth rate of the *RAD51* transformants in vivo, 2×10^5 trypanosomes growing at mid-log in vitro were injected into an ICR mouse (which, in some experiments, had been immunocompromised 1–24 hr previously with 300 mg/kg of cyclophosphamide). At intervals thereafter, small blood samples were removed from the mouse tail into heparinized

capillary tubes, and 1 µl of the blood was diluted into 99 µl of 0.85%ammonium chloride. Erythrocytes were allowed to lyse for 5 min at room temperature before the concentration of trypanosomes in the blood was measured with a bright-line hemacytometer (Sigma). For each growth rate measurement, three mice were independently infected.

In vitro growth rates were measured by diluting mid-log bloodstream trypanosomes to a concentration of 1×10^5/ml in 2.5 ml of HMI-9 medium in 6-well culture dishes. Cell concentrations were measured at time intervals thereafter with a hemacytometer. Methyl methanesulphonate (Sigma) was diluted in HMI-9 from a 99%stock solution, and 3-aminobenzamide (Sigma) was dissolved in 100%DMSO and then diluted in HMI-9.

Generation and analysis of trypanosome variants that have switched their VSG coat

The approach adopted to isolate VSG-switched variants is essentially that described in McCulloch et al. (1997), but with some modifications. To generate mice with immunity to the VSG221 coat, a number of ICR mice were intraperitoneally injected with 2×10^5 to 4×10^5 3174 *RAD51* wild-type trypanosomes (McCulloch et al. 1997; Fig. 5) growing on hygromycin (5.0 µg/ml) and G418 (2.5 µg/ml); 3–4 days later, the infections were cured by injection of cymelarsan (Rhone Merieux; 5 mg/kg). The immunized mice were used between 7 and 35 days post-curing. To select for switch variants, the 3174 *RAD51* transformants were passaged from medium containing hygromycin and G418 into nonselective medium at a density of 0.5×10^4 to 1.0×10^4 cells/ml and grown until they reached densities of 1×10^6 to 3×10^6/ml. Measured numbers were then intraperintoneally injected into immunized mice in 300 ml of HMI-9. Twenty-four hours later, the mice were bled by cardiac puncture and trypanosomes isolated and cloned as described (McCulloch et al. 1997). In calculating the VSG switching frequencies from the numbers of clones that grew out, we have assumed (as before, McCulloch et al. 1997) that the 3174 *RAD51* wild-type and 3174 *RAD51*$^{+/-}$ cell, populations double every 8 hr; for the 3174 *RAD51*$^{-/-}$ cells, we have assumed that the doubling time is increased by 15%, which is slightly greater than was measured in vivo. The drug sensitivities of the switched variant clones, as well as the presence or absence of the marker genes within the marked *VSG221* expression site, were assayed as described previously (McCulloch et al. 1997).

Note added in proof

The EMBL/GenBank accession no. for the sequence reported is AF174136.

References

Aline, R.F. and K.D. Stuart. 1989. *Trypanosoma brucei*-con-

served sequence organization 3′ to telomeric variant surface glycoprotein genes. *Exp. Parasitol.* **68:** 57–66.

Ashe, H.L., J. Monks, M. Wijgerde, P. Fraser, and N.J. Proudfoot. 1997. Intergenic transcription and transinduction of the human B-globin locus. *Genes & Dev.* **11:** 2494–2509.

Barry, J.D. 1997. The relative significance of mechanisms of antigenic variation in African trypanosomes. *Parasitol. Today* **13:** 212–218.

Basile, G., M. Aker, and R.K. Mortimer. 1992. Nucleotide sequence and transcriptional regulation of the yeast recombination repair gene *RAD51. Mol. Cell Biol.* **12:** 3235–3246.

Baumann, P. and S.C. West. 1997. The human RAD51 protein: Polarity of strand transfer and stimulation by hRP-A. *EMBO J.* **16:** 5198–5206.

———. 1998. Role of the human RAD51 protein in homologous recombination and double-stranded-break repair. *Trends Biochem. Sci.* **23:** 247–251.

Baumann, P., F.E. Benson, and S.C. West. 1996. Human RAD51 protein promotes ATP-dependent homologous pairing and strand transfer reactions in vitro. *Cell* **87:** 757–766.

Benson, F.E., A. Stasiak, and S.C. West. 1994. Purification and characterization of the human Rad51 protein, an analogue of *E.coli* RecA. *EMBO J.* **13:** 5764–5771.

Bernards, A., L.H.T. van der Ploeg, A.C.C. Frasch, P. Borst, J.C. Boothroyd, S. Coleman, and G.A.M. Cross. 1981. Activation of trypanosome surface antigen genes involves a duplication-transposition leading to an altered 3′ end. *Cell* **27:** 497–505.

Bishop, D.K., D. Park, L. Xu, and N. Kleckner. 1992. DMC1: A meiosis-specific yeast homolog of *E.coli* RecA required for recombination, synaptonemal complex formation and cell cycle progression. *Cell* **69:** 439–456.

Blundell, P.A., G. Rudenko, and P. Borst. 1996. Targeting of exogenous DNA into *Trypanosoma brucei* requires a high degree of homology between donor and target DNA. *Mol. Biochem. Parasitol.* **76:** 215–229.

Borst, P. and D.R. Greaves. 1987. Programmed gene rearrangements altering gene expression. *Science* **235:** 658–667.

Borst, P., G. Rudenko, M.C. Taylor, P.A. Blundell, F. van Leeuwen, W. Bitter, M.A. Cross, and R. McCulloch. 1996. Antigenic variation in trypanosomes. *Arch. Med. Res.* **27:** 379–388.

Borst, P., W. Bitter, P.A. Blundell, I. Chaves, M.A. Cross, H. Gerrits, F. van Leeuwen, R. McCulloch, M.C. Taylor, and G. Rudenko. 1998. Control of VSG gene expression sites in *Trypanosoma brucei. Mol. Biochem. Parasitol.* **91:** 67–76.

Brun, R. and M. Schoenenberger. 1979. Cultivation and in vitro cloning of procyclic culture forms of *Trypanosoma brucei* in a semi-defined medium. *Acta Trop.* **36:** 289–292.

Campbell, D.A., M.P. van Bree, and J.C. Boothroyd. 1984. The 5′ limit of transposition and upstream barren region of a trypanosome VSG gene: Tandem 76 bp repeats flanking (TAA)90. *Nucleic Acids Res.* **12:** 2759–2774.

Capaldo, F.N., G. Ramsey, and S.D. Barbour. 1974. Analysis of the growth of recombination-deficient strains of *Escherichia coli* K12. *J. Bacteriol.* **118:** 242–249.

Chaves, I., J. Zomerdijk, A. Dirks-Mulder, R.W. Dirks, A.K. Raap, and P. Borst. 1998. Subnuclear localization of the active variant surface glycoprotein expression site in *Trypanosoma brucei. Proc. Natl. Acad. Sci.* **95:** 12328–12333.

Cornelissen, A.W.C.A., P.A.M. Michels, P. Borst, W. Spanjer, J.A.M. Versluis-Broers, C. van der Meer, F. Farzaneh, and S. Shall. 1985. Effect of 3-aminobenzanide on antigenic variation of *Trypanosoma brucei. Biochem. Pharmacol.* **34:** 4151–4156.

Cross, G.A.M. 1975. Identification, purification and properties of clone-specific glycoprotein antigens constituting the surface coat of *Trypanosoma brucei. Parasitology* **71:** 393–417.

———. 1996. Antigenic variation in trypanosomes: Secrets surface slowly. *BioEssays* **18:** 283–291.

Cross, G.A.M., L.E. Wirtz, and M. Navarro. 1998. Regulation of VSG gene expression site transcription and switching in *Trypanosoma brucei. Mol. Biochem. Parasitol.* **91:** 77–91.

Cross, M, M.C. Taylor, and P. Borst. 1998. Frequent loss of the active site during variant surface glycoprotein expression site switching in vitro in *Trypanosoma brucei. Mol. Cell Biol.* **18:** 198–205.

Cully, D.F., H.S. Ip, and G.A.M. Cross. 1985. Coordinate transcription of variant surface glycoprotein genes and an expression site associated gene family in *Trypanosoma brucei. Cell* **42:** 173–182.

Davies, K.P., V.B. Carruthers, and G.A.M. Cross. 1997. Manipulation of the *VSG* co-transposed region increases expression site switching in *Trypanosoma brucei. Mol. Biochem. Parasitol.* **86:** 163–177.

Deitsch, K.W., E.R. Moxon, and T.E. Wellems. 1997. Shared themes of antigenic variation and virulence in bacterial, protozoal and fungal infections. *Microbiol. & Mol. Biol. Rev.* **61:** 281–293.

de Lange, T., J.M. Kooter, P.A. Michels, and P. Borst. 1983. Telomere conversion in trypanosomes. *Nucleic Acids Res.* **11:** 8149–8165.

Dunderdale, H.J. and S.C. West. 1994. Recombination genes and proteins. *Curr. Biol.* **4:** 221–228.

Edelmann, W. and S.C. Kucherlapati. 1996. Role of recombination proteins in mammalian cell survival. *Proc. Natl. Acad. Sci.* **93:** 6225–6227.

Eggleston, A.K. and S.C. West. 1996. Exchanging partners: Recombination in *E.coli. Trends Genet.* **12:** 20–26.

Eid, J. and B. Sollner-Webb. 1991. Stable integrative transformation of *Trypanosoma brucei* that occurs exclusively by homologous recombination. *Proc. Natl. Acad. Sci.* **88:** 2118–2121.

Fingerhut, R., J. Kiefer, and F. Otto. 1984. Cell cycle parameters in radiation sensitive strains of *Saccharomyces cerevisiae. Mol. Gen. Genet.* **193:** 192–194.

Game, J.C. 1983. Radiation-sensitive mutants and repair in yeast. In: *Yeast genetics: Fundamental and applied aspects*, (ed. J. Spencer, D. Spencer, and A. Smith), pp. 109–137. Springer-Verlag, New York, NY.

Grant, S.R. 1999. Dissecting the mechanisms of posttranscriptional gene silencing: Divide and conquer. *Cell* **96:** 303–306.

Grivell, L.A. 1996. Transposition: Mobile introns get into line. *Curr. Biol.* **6:** 48–51.

Haber, J.E. 1995. In vivo biochemistry: Physical monitoring of recombination by site-specific endonucleases. *BioEssays* **17:** 609–620.

———. 1998. Mating-type gene switching in *Saccharomyces cerevisiae. Annu. Rev. Genet.* **32:** 561–599.

Hensikoff, S. 1997. Nuclear organization and gene expression: Homologous pairing and long-range interactions. *Curr. Opin. Cell Biol.* **9:** 388–395.

Hirumi, H. and K. Hirumi. 1989. Continuous cultivation of *Trypanosoma brucei* bloodstream forms in a medium containing a low concentration of serum protein without feeder cell layers. *J. Parasitol.* **75:** 985–989.

Hoeijmakers, J.H.J., A.C.C. Frasch, A. Bernards, P. Borst, and G.A.M. Cross. 1980. Novel expression-linked copies of the genes for variant surface antigens in trypanosomes. *Nature* **284:** 78–80.

Horn, D. and G.A.M. Cross. 1995. A developmentally regulated position effect at a telomeric locus in *Trypanosoma brucei. Cell* **83:** 555–561.

———. 1997. Analysis of *Trypanosoma brucei*. VSG expression site switching in vitro. *Mol. Biochem. Parasitol.* **84:** 189–201.

Jeggo, P.A. 1998. PARP - another guardian angel?. *Curr. Biol.* **8:** R49–R51.

Johnson, P.J., J.M. Kooter, and P. Borst. 1987. Inactivation of transcription by UV irradiation of *Trypanosoma brucei* provides evidence of a muligene transcription unit, including a VSG gene. *Cell* **51:** 273–281.

Kooter, J.M., H.J. van der Spek, R. Wagter, C.E. d'Oliveira, F. van der Hoeven, P.J. Johnson, and P. Borst. 1987. The anatomy and transcription of a telomeric expression site for variant-specific surface antigens in *T. brucei. Cell* **51:** 261–272.

Lamont, G.S. and G.A.M. Cross. 1987. Effect of 3-aminobenzanide on the frequency of antigenic switching of *Trypanosoma brucei. in vitro. Biochem. Pharmacol.* **36:** 1750–1751.

Laurent, M., E. Pays, E. Magnus, N. van Meirvenne, G. Matthyssens, R.O. Williams, and M. Steinert. 1983. DNA rearrangemements linked to the expression of a predominant sufrace antigen gene of trypanosomes. *Nature* **302:** 263–266.

Lee, M.G.-S. and L.H.T. Van der Ploeg. 1990. Homologous recombination and stable transfection in parasitic protozoan *Trypanosoma brucei. Science* **250:** 1583–1587.

Lim, D.S. and P. Hasty. 1996. A mutation in mouse *RAD51* results in an early embryonic lethal that is suppressed by a mutation in p53. *Mol. Cell. Biol.* **16:** 7133–7143.

Lindhal, T., M.S. Satoh, G.G. Poirier, and A. Klugland. 1995. Post-translational modification of poly (ADP-ribose) polymerase induced by DNA strand breaks. *Trends Biochem. Sci.* **20:** 405–411.

Liu, A.Y.C., L.H.T. Van der Ploeg, F.A.M. Rijsewijk, and P. Borst. 1983. The transposition unit of variant surface glycoprotein gene 118 of *Trypanosoma brucei*. Presence of repeated elements at its border and absence of promoter-associated sequences. *J. Mol. Biol.* **167:** 57–75.

Malkova, A., E.L. Ivanov, and J.E. Haber. 1996. Double strand break repair in the absence of Rad51 in yeast: A possible role for break-induced DNA replication. *Proc. Natl. Acad. Sci.* **93:** 7131–7136.

Matthews, K.R., C. Tschudi, and E. Ullu. 1994. A common pyrimidine-rich motif governs trans-splicing and polyadenylation of tubulin polycistronic pre-mRNA in trypanosomes. *Genes* & *Dev.* **8:** 491–501.

McCulloch, R., G. Rudenko, and P. Borst. 1997. Gene conversions mediating antigenic variation in *Trypanosoma brucei* can occur in Variant Surface Glycoprotein expression sites lacking 70 bp repeat sequences. *Mol. Cell. Biol.* **17:** 833–843.

Mhlanga, J. 1994. "Antigenic variation in *Trypanosome brucei*, a relationship with poly ADP-ribose polymerase." PhD thesis, University of Sussex, UK.

Michels, P.A.M., L.H.T. van der Ploeg, A.Y.C. Liu, and P. Borst. 1984. The activation and reactivation of an expression-linked copy for a variant surface glycoprotein in *Trypanosoma brucei. EMBO J.* **3:** 1345–1351.

Mortensen, U.H., C. Bendixen, I. Sunjeravic, and R. Rothstein. 1996. DNA strand annealing is promoted by the yeast protein Rad52. *Proc. Natl. Acad. Sci.* **93:** 10729–10734.

Myler, P.J., R.G. Nelson, N. Agabian, and K.D. Stuart. 1984. Two mechanisms of expression of a predominant variant antigen gene of *Trypanosoma brucei. Nature* **309:** 282–284.

Nassif, N., J. Penney, S. Pal, W. Engels, and G.B. Gloor. 1994. Efficient copying of nonhomologous sequences from ectopic sites via P element-induced gap repair. *Mol. Cell. Biol.* **14:** 1613–1625.

Navarro, M. and G.A.M. Cross. 1996. DNA rearrangements associated with multiple consecutive directed antigenic

switches in *Trypanosoma brucei. Mol. Cell. Biol.* **16:** 3615–3625.

Ogawa, T., A. Shinohara, A. Nabetani, T. Ikeya, K. Yu, E.H. Egelman, and H. Ogawa. 1993a. RecA-like recombination proteins in eukaryotes: Functions and structures of *RAD51* genes. *Cold Spring Harb. Symp. Quant. Biol.* **58:** 567–576.

Ogawa, T., K. Yu, A. Shinohara, and E.H. Egelman. 1993b. Similarity of the yeast RAD51 filament to the bacterial RecA filament. *Science* **259:** 1896–1899.

Pays, E. and D.P. Nolan. 1998. Expression and functions of surface proteins in *Typanosoma brucei. Mol. Biochem. Parasitol.* **91:** 3–36.

Pays, E., van N. Meirvenne, D. Le Ray, and M. Steinert. 1981. Gene duplication and transposition linked to antigenic variation in *T. brucei. Proc. Natl. Acad. Sci.* **78:** 2673–2677.

Pays, E., S. van Assel, M. Laurent, M. Darville, T. Vervoort, N. van Meirvenne, and M. Steinert. 1983a. Gene conversion as a mechanism for antigenic variation in trypanosomes. *Cell* **34:** 371–381.

Pays, E., M.F. Delauw, S. van Assel, M. Laurent, T. Vervoort, N. van Meirvenne, and M. Steinert. 1983b. Modifications of a *Trypanosoma b. brucei* antigen gene repertoire by different DNA recombinational mechanisms. *Cell* **35:** 721–731.

Pays, E., M. Guyaux, D. Aerts, N. van Meirvenne, and M. Steinert. 1985. Telomere reciprocal recombination as a possible mechanism for antigenic variation in trypanosomes. *Nature* **316:** 562–564.

Pays, E., P. Tebabi, A. Pays, H. Coquelet, P. Revelard, D. Salmon, and M. Steinert. 1989. The genes and transcripts of an antigen gene expression site from *T. brucei. Cell* **57:** 835–845.

Pays, E., L. Vanhamme, and M. Berberof. 1994. Genetic controls for the expression of surface antigens in African trypanosomes. *Annu. Rev. Microbiol.* **48:** 25–52.

Pittman, D.L., J. Cobb, K.J. Schimenti, L.A. Wilson, D.M. Cooper, E. Brignull, M.A. Handel, and J.C. Schimenti. 1998. Meiotic prophase arrest with failure of chromosome synapsis in mice deficient for Dmc1, a germline-specific RecA homolog. *Mol. Cell* **1:** 697–705.

Rudenko, G., P.A. Blundell, A. Dirks-Mulder, R. Kieft, and P. Borst. 1995. A ribosomal DNA promoter replacing the promoter of a telomeric variant surface glycoprotein gene expression site can be efficiently switched on and off in *Trypanosoma brucei. Cell* **83:** 547–553.

Rudenko, G., R. McCulloch, A. Dirks-Mulder, and P. Borst. 1996. Telomere exchange can be an important mechanism of variant surface glycoprotein gene switching in *Trypanosoma brucei. Mol. Biochem. Parasitol.* **80:** 65–75.

Rudenko, G., I. Chaves, A. Dirks-Mulder, and P. Borst. 1998. Selection for activation of a new variant surface glycoprotein expression site in *Trypanosoma brucei* can result in deletion of the old one. *Mol. Biochem. Parasitol.* **95:** 97–109.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: A laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sancer, A. 1994. Mechanisms of DNA excision repair. *Science* **266:** 1954–1956.

Scholler, J.K., P.J. Myler, and K.D. Stuart. 1989. A novel telomeric gene conversion in *T. brucei. Mol. Biochem. Parasitol.* **35:** 11–19.

Schurch, N., A. Hehl, E. Vasella, R. Braun, and I. Roditi. 1994. Accurate poyadenylation of procyclic mRNAs in *T. brucei*. is determined by pyrimidine-rich elements in the intergenic regions. *Mol. Cell. Biol.* **14:** 3668–3675.

Scully, R., J.J. Chen, A. Plug, Y.H., Y.H. Xiao, D. Weaver, J. Feunteun, T. Ashley, and D.M. Livingston. 1997. Associa-

tion of BRCA1 with RAD51 in mitotic and meiotic cells. *Cell* **88:** 265–275.

Sharan, S.K., M. Morimatsu, A. Albrecht, D.-S. Lim, E. Regel, C. Dinh, A. Sands, G. Eichele, P. Hasty, and A. Bradley. 1997. Embryonic lethality and radiation hypersensitivity mediated by Rad51 in mice lacking BRCA2. *Nature* **386:** 804–810.

Shinohara, A. and T. Ogawa. 1995. Homologous recombination and the role of double strand breaks. *Trends Biochem. Sci.* **20:** 387–391.

Shinohara, A., H. Ogawa, and T. Ogawa. 1992. Rad51 protein involved in repair and recombination in *S. cerevisiae* is a RecA-like protein. *Cell* **69:** 457–470.

Shinohara, A., H. Ogawa, Y. Matsuda, N. Ushio, K. Ikep, and T. Ogawa. 1993. Cloning of human, mouse and fission yeast recombination genes homologous to *RAD51* and *recA. Nat. Genet.* **4:** 239–243.

Sturzbecher, H.-M., B. Donzelmann, W. Henning, U. Knippschild, and S. Buchop. 1996. p53 is linked directly to homologous recombination processes via Rad51/RecA protein interaction. *EMBO J.* **15:** 1992–2002.

Story, R.M., D.K. Bishop, N. Kleckner, and T.A. Steitz. 1993. Structural relationship of bacterial recA proteins to recombination proteins from bacteriophage T4 and yeast. *Science* **259:** 1892–1896.

Sung, P. 1994. Catalysis of ATP-dependent homologous DNA pairing and strand exchange by yeast RAD51 protein. *Science* **265:** 1241–1243.

———. 1997. Yeast Rad55 and Rad57 proteins form a heterodimer that functions with replication protein A to promote DNA strand exchange by Rad51 recombinase. *Genes & Dev.* **11:** 1111–1121.

Sung, P. and D.L. Robberson. 1995. DNA strand exchange mediated by a RAD51-ssDNA nucleoprotein filament with polarity opposite to that of RecA. *Cell* **82:** 453–461.

Ten Asbroek, A.L.M.A., M. Ouellette, and P. Borst. 1990. Targeted insertion of the neomycin phosphotransferase gene into the tubulin gene cluster of *Trypanosoma brucei. Nature* **348:** 174–175.

Tsuzuki, T., Y. Fujii, K. Sakumi, Y. Tominaga, K. Nakao, M. Sekigughi, A. Matsushiro, Y. Yoshimura, and T. Morita. 1996. Targeted disruption of the *Rad51* gene leads to lethality in embryonic mice. *Proc. Natl. Acad. Sci.* **93:** 6236–6240.

Van der Ploeg, L.H.T., D. Valerio, T. de Lange, A. Bernards, P. Borst, and F. Grosveld. 1982. An analysis of cosmid clones from *Trypanosoma brucei* shows that the genes for variant surface gclycoproteins are clustered in the genome. *Nucleic Acids Res.* **10:** 5905–5923.

Van Leeuwen, F., R. Kieft, M. Cross, and P. Borst. 1998. Biosynthesis and function of the modified DNA base B-D-glucosyl-hydroxymethyluracil in *Trypanosoma brucei. Mol. Cell. Biol.* **18:** 5643–5651.

Xong, H.V., L. Vanhamme, M. Chamekh, C.E. Chimfwembe, J. van den Abbeele, A. Pays, N. van Meirvenne, R. Hamers, P. de Baetselier, and E. Pays. 1998. A VSG expression site-associated gene confers resistance to human serum in *Trypanosoma rhodesiense. Cell* **95:** 839–846.

Yoshida, K., G. Kondoh, Y. Matsuda, T. Habu, Y. Nishimune, and T. Morita. 1998. The mouse *RecA*-like gene *Dmc1* is required for homologous chromosome synapsis during meiosis. *Mol. Cell* **1:** 707–718.