INTRODUCTION

Trypanosoma brucei sp. is a parasitic protozoan responsible for sleeping sickness in man and Nagana in domestic animals. In recent years, the study of this organism at the molecular level has been facilitated by the development of both transient and stable transfection techniques. Stable transformation has been obtained with positively selectable marker genes encoding resistance to the antibiotics neomycin/geneticin (1), hygromycin (2) and in both major life-cycle stages (4). As yet, however, no negatively selectable marker has been shown to work in Trypanosoma brucei. As yet, however, no negatively selectable marker has been shown to work in Trypanosoma brucei. The experiments described below show that a viral TK gene can be used as a negatively selectable marker in African trypanosomes. However, the gene is readily inactivated by mutation. This has allowed us to estimate the minimal rate of mutation in this organism to be 10⁻⁹ per base pair per cell generation. The rate of mutation may have implications for the evolution of the variant surface glycoprotein (VSG) genes on which the trypanosome depends for survival.

MATERIALS AND METHODS

Constructs

Plasmid pSPIRαβ: The αβ tubulin intergenic region was obtained by PCR with primers: 5’-gcTCTAGAAGTGTGACAAAACTG and 5’-ggCgggtcGACTATTTTCTTTTGATG (lower case letters indicate bases added to produce useful restriction sites). The fragment was cloned into the XbaI and SmaI sites of pSP72 (Promega).

Plasmid pSPIRαβ*: pSPIRαβ was digested with SmaI and XbaI. The XbaI end was made blunt with Klenow enzyme, and the plasmid religated.

Plasmid pSN: The 1.2 kb partial PstI fragment of pRK20(+) containing the neo gene and the βα intergenic region of the tubulin array (6), was cloned into the PstI site of pSPIRαβ*.

ABSTRACT

We have tested the use of thymidine kinase as a negatively selectable system for Trypanosoma brucei. To this end we have targeted a construct containing a Herpes simplex virus thymidine kinase (TK) gene into the ribosomal DNA array of procyclic T.brucei. This resulted in TK activity 30–50-fold above background and in susceptibility to the nucleoside analogues ganciclovir, ethyl-deoxyuridine and 1-[2-deoxy,2-fluoro-8-α-arabinofuranosyl]-5-iodouracil, all of which have no effect on wild-type trypanosomes. TK trypanosomes, however, reverted to a ganciclovir resistant phenotype at a rate of 10⁻⁶ per cell-generation. A similar reversion rate was observed using the Varicella-zoster virus TK gene. Loss of TK activity was not due to detectable DNA rearrangements or a decrease in TK mRNA. Sequence analysis of the revertant genes demonstrated, however, the occurrence of point mutations and frameshifts. One revertant line had a mutation in the thymidine binding site leading to the substitution of a conserved arginine by a glycine. Other mutations included single base insertion, single base deletion and the introduction of a premature termination codon by point mutation.

The TK gene has been used to enrich for correctly targeted integrations of DNA into mammalian genomes and for elimination of certain cell types from a population. It is the promiscuity of the TK enzyme that allows it to be used as a negatively selectable marker. The enzyme will phosphorylate a wide variety of modified nucleosides and nucleoside analogues which remain unphosphorylated in cells lacking the viral TK. The phosphorylated analogues then act as competitive inhibitors of DNA polymerase or as DNA chain terminators. These analogues include the antiviral therapeutic agents acyclovir and ganciclovir, and less well-known compounds such as 1-[2-deoxy,2-fluoro-8-α-arabinofuranosyl]-5-iodouracil (FIAU). The HSV-1 TK gene has been demonstrated to work as a negative selection system in conjunction with ganciclovir in Leishmania major, a protozoan related to T.brucei (5).

The experiments described below show that a viral TK gene can be used as a negatively selectable marker in African trypanosomes. However, the gene is readily inactivated by mutation. This has allowed us to estimate the minimal rate of mutation in this organism to be 10⁻⁹ per base pair per cell generation. The rate of mutation may have implications for the evolution of the variant surface glycoprotein (VSG) genes on which the trypanosome depends for survival.
**Plasmid pβαTKO**: The 176 bp SpeI fragment from pSP1pβα (7) containing the α-tubulin trans-splice acceptor site was cloned into the XhoI site of pTKO (a kind gift from Dr Heim te Riele, Netherlands Cancer Institute), which contains the HSV-1 TK gene.

**Plasmid pSTKN**: The α-tubulin splice acceptor-TK fragment was isolated from pβαTKO by digestion with HindIII followed by end repair with Klenow enzyme, and a further digestion with EcoRI. The fragment was subcloned into the EcoRI and SmaI sites of pSN.

**Plasmid pβTKN**: A 4.3 kb EcoRI–BglII fragment bearing the Tbrucei ribosomal promoter from plasmid pR4 (8) was cloned into the EcoRI and BglII sites of pSTKN.

**Plasmid pVTK**: This construct is analogous to pβTKN but contains the VZV TK gene and the hygromycin resistance selectable marker. It was derived from pBluescript KS(+) and has the following sequences inserted in the polylinker (5′→3′): a 4.3 kb EcoRI–BglII fragment bearing the Tbrucei ribosomal promoter (as in pβTKN); the 402 bp intergenic region between actin genes 1 and 2 as splice acceptor site; the VZV TK gene; the 270 bp ββ intergenic region of the tubulin array as polyadenylation/splice acceptor site; the hygromycin resistance gene; the 330 bp αβ intergenic region of the tubulin array as polyadenylation site.

A detailed description of how this plasmid was constructed can be obtained from the authors upon request.

**Growth and transformation of trypanosomes**

All studies were carried out using strain 427-60 cultured procyclic forms of Tbrucei (9). The trypanosomes were cultured in a semi-defined medium as described (9). Constructs pβTKN (see Fig. 1 and below) and pVTK were linearised by digestion with ClaI. Trypanosomes (2.5 × 10⁸) were electroporated with 5 µg of linearised pβTKN or pVTK DNA as described (10). Transformants were selected by growth in medium supplemented with 20 µg/ml G418 (Gibco-BRL) for pβTKN and with 20 µg/ml hygromycin for pVTK and thereafter continuously maintained on selection. Transfection efficiency was measured and transformant lines were cloned, by limiting dilution, mixing in wild-type lines of pβTKN. The rate of reversion to a TK− phenotype was measured using the Luria–Delbrück fluctuation test essentially as described (11). Stock cultures of different transformants were inoculated into 150 wells in microtitre plates, and a bulk culture containing the same number of trypanosomes as had been seeded in the entire wells in microtitre plates, and a bulk culture containing the same number of trypanosomes as had been seeded in the entire microtitre plate was set up as a control. Cultures were incubated at 28°C for 3–6 days depending on the cell line, until the density reached 1–2 × 10⁶ trypanosomes per well.

**Thymidine kinase assay**

Thymidine kinase activity was measured in crude soluble protein preparations. Trypanosomes (50 ml) in late logarithmic phase culture were pelleted and washed in cold PSG (0.59 M Na₂HPO₄, 0.03 M NaH₂PO₄, 0.044 M NaCl, 1% w/v glucose). All procedures in lysate preparation after this stage were carried out at 4°C. The cell pellet was resuspended in 1 ml 50 mM sodium phosphate buffer (pH 7.5). Seventy-five µl 10% NP-40, 1.5 µl 100 mM Nε-p-tosyl-L-lysine chloromethylketone (TLCK) and 1.5 µl 1 M dithiothreitol were added and the suspension shaken several times until clear. The lysate was freeze–thawed three times in liquid nitrogen. It was then centrifuged for 15 min at top speed in a microfuge and the
supernatant was cleared in a Beckman SW 50.1 rotor at 30 000 g for 35 min. The protein content was assayed using the Bradford method (BioRad). Lysates were stored at −70°C until required.

For the TK assay the volume of lysate equivalent to 10 µg protein was mixed in a final volume of 25 µl with 200 mM Tris–HCl, pH 7.5, 10 mM NaF, 2 mM MgCl₂, 5 mM ATP and 40 µM [³²P]thymidine (6.7 Ci/mmol, NEN-Dupont). All assays were done in duplicate. The reaction was incubated for 30 min at 37°C. Two 11 µl aliquots were taken from each tube and spotted onto half-discs of Whatman DE81 filters. One half was dried immediately and the second washed three times in ethanol. The percentage of label remaining bound to the second filter is then a measure of the thymidine kinase activity in that sample. The length of time of incubation and the amount of protein used were shown to be within the linear range of the assay in control experiments, as was the ATP dependency of the measured activity (data not shown).

**DNA and RNA analyses**

DNA and RNA preparation, blotting and hybridisation were performed as described (10,13). Probes were prepared by random priming. Blots were washed at a stringency of 0.1 × SSC, 0.1% SDS at 65°C.

**Rescue of plasmid DNA from trypanosome lines**

Total genomic DNA from the transformed lines was digested with ClaI. The DNA was diluted to 200 ng/ml to enhance the chance of intramolecular ligation, and circularised with T4 DNA ligase (Boehringer Mannheim). The ligated DNA was introduced into *Escherichia coli* DH5α by electroporation as described (12), using a Gene Pulser with pulse controller (BioRad). Ampicillin resistant colonies were picked and plasmid DNA isolated by standard methods (13). Colinearity of rescued plasmids with input DNA was checked by restriction enzyme analysis. The rescued plasmids were retransfected into wild-type trypanosomes as above. After selection cloned lines from each transfection were assayed for TK enzyme activity and for expression of TK mRNA.

**Sequence analysis of plasmid DNA**

DNA sequencing was performed using the dideoxy chain termination method with the Sequenase™ enzyme as described by the manufacturer (US Biochemicals).

**PCR amplification from trypanosome genomic DNA and linear amplification sequencing**

Regions identified as containing putative mutations by plasmid sequencing were amplified and sequenced directly using the polymerase chain reaction (PCR). The amplification conditions were as follows: 2 µg genomic DNA was mixed with 1× PCR buffer containing 200 µM dNTPs, 3.0 mM MgCl₂, 5% dimethylsulphoxide and 160 ng of each primer in a total volume of 50 µl. This mix was incubated at 94°C for 5 min, then allowed to cool to 50°C for 2 min after which 0.5 U *Taq* polymerase was added to each tube. The tubes were incubated at 72°C for 1 min and then taken through 25 cycles of 30 s at 94°C, 30 s at 45°C, 1 min at 72°C and a final cycle of 1 min at 94°C, 1 min at 55°C and 5 min at 72°C. The product was precipitated with PEG-8000 for linear amplification sequencing (LAS).

For the LAS reaction the DNA pellet was resuspended in 10 µl H₂O and divided between the four dideoxynucleotide reactions. For this reaction the primer was end-labelled using T4 poly-nucleotide kinase and [γ-³²P]ATP. The sequencing reaction was performed as described (13). The cycling conditions were: 30 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C. The reactions were fractionated on 7% polyacrylamide sequencing gels and autoradiographed at −70°C.

**RESULTS**

**Stable integration and expression of TK gene**

The TK construct was designed with the neo gene downstream of the HSV-1 TK gene such that the trypanosomes could not switch off transcription of TK without losing G418 resistance (Fig. 1). The TK construct was linearised by restriction enzymes and transfected into wild-type trypanosomes. The pBTKN construct integrated at a frequency of 1.3 × 10⁻⁴ per cell as measured by outgrowth of dilution series. This was somewhat less efficient than the integration of our standard control construct pUCTbNeo3 targeted to the tubulin gene array but was as expected for a single-crossover integration (1,15). The pBTKN construct integrated at a frequency of 1.3 × 10⁻⁴ per cell as measured by outgrowth of dilution series. This was somewhat less efficient than the integration of our standard control construct pUCTbNeo3 targeted to the tubulin gene array but was as expected for a single-crossover integration (1,15). Four of the lines obtained (5.3, 5.7, 5.8 and 5.31) were analysed in detail. Correct targeting of the construct to a ribosomal array in each cell line was confirmed by Southern hybridisation (data not shown).

**Growth and reversion of parasites in the presence of toxic nucleoside analogues**

The growth of wild-type trypanosomes was unaffected by concentrations of ganciclovir ≤ 1 mM (data not shown). The TK⁺ transformants, in contrast, were inhibited and the degree of growth inhibition was dependent upon the concentration of ganciclovir. After an interval, however, trypanosomes began to grow out at a rate which matched the wild-type, suggesting reversion of the cells to a TK⁻ phenotype. This phenomenon was also observed with other nucleoside analogues (ethyl deoxyuridine and FIAU; data not shown), thus suggesting that the reversion mechanism is independent of the type of nucleoside analogue involved. When these resistant trypanosomes were seeded into fresh medium containing ganciclovir and G418 there was no lag phase and the rate of growth was identical to that of wild-type trypanosomes (Fig. 2B). Growing the resistant trypanosomes in the absence of ganciclovir selection for four passages did not result in a return to ganciclovir sensitivity suggesting that the reversion was a permanent mutation rather than a transient adaptation.

**Levels of TK mRNA and TK activity**

RNA blotting (Fig. 3) showed the expected 2.1 kb transcript in all transformants including those that had reverted to a TK⁻ phenotype. In addition to this, a second higher molecular weight transcript was observed which corresponds to an unspliced
precursor RNA. This RNA was also seen by Rudenko et al. (8) with other plasmid transformants containing this version of the ribosomal promoter. Interference with splicing could be due to secondary structure formation in the 5' part of the primary transcript, which is derived from the rDNA unit and therefore not normally spliced, blocking access of the spliceosome to the tubulin splice acceptor site.

Since TK mRNA was still being produced, the trypanosomes were assayed for TK enzyme activity. Figure 4A shows that extracts from trypanosomes transfected with the neo gene alone (pTN3) can phosphorylate thymidine at a low rate in agreement with a previous report (17). In the TK transfectants the activity is much higher whereas the revertants have lost all activity above the endogenous level. Extracts from the revertants did not inhibit TK activity suggesting that the reversion phenotype was due to an alteration in the TK itself and not a secondary mutation such as the activation of a phosphatase degrading TMP. This was confirmed by rescuing the inserted plasmid DNA from the genome of stably transfected trypanosome revertants that had been isolated independently, and reintroducing the plasmid into fresh wild-type trypanosomes. The results of this TK assay are illustrated in Figure 4B. The cells transfected with a TK gene from non-revertant trypanosomes (cell lines 5.31, 5.3 and 5.7) show a comparable level of TK activity to the original transfec-
tants, whilst those transfected with a revertant TK gene (cell lines 5.31r, 5.3r, 5.7r, 5.7r1, 5.7r2 and 5.7r3) show only wild-type background activity.

Sequence analysis of inactivated TK genes

The TK genes from four revertants were sequenced and the results are summarised in Table 1. Each gene was found to contain a mutation which should inactivate the gene product. Since we also identified mutations that arose during the rescue in E. coli, the trypanosomal origin of the mutations was confirmed by direct linear amplification sequencing from trypanosome genomic DNA or by sequencing the entire ORF and confirming that only one mutation was present (data not shown).

Table 1. Mutations found in the revertant-derived TK genes

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Position in gene</th>
<th>Effect on TK protein</th>
</tr>
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<tbody>
<tr>
<td>C deleted</td>
<td>212</td>
<td>Frameshift at codon 70</td>
</tr>
<tr>
<td>C→G</td>
<td>487</td>
<td>Arg → Gly in thymidine binding site</td>
</tr>
<tr>
<td>C inserted</td>
<td>532</td>
<td>Frameshift at codon 177</td>
</tr>
<tr>
<td>C→T</td>
<td>748</td>
<td>Creation of stop codon at codon 250</td>
</tr>
</tbody>
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The TK genes from the revertant lines were rescued and sequenced. When a putative inactivating mutation was identified this region of the TK gene was ampli-
fied and sequenced directly from trypanosomal genomic DNA to prove that the mutation was present in the revertant cells and not introduced during post-res-
cue manipulation in E. coli. The nucleotide positions are numbered from the ade-
nine of the translational start codon.
The most interesting mutation was that found in revertant 5.7r3. In this case a C→G transversion had occurred within the sequence encoding the thymidine binding site of the enzyme. This change resulted in the substitution of an arginine residue that is absolutely conserved in the TK genes of all members of the herpesvirus family studied so far, by a glycine. A similar mutation in which this arginine is replaced by glutamine has been shown to correlate with acyclovir resistance and loss of TK activity in Varicella Zoster virus (18,19). In our (limited) analysis no common mutation was found although all mutations were single base-pair changes.

Measurement of inactivation rate

No data are available on the natural mutation rate in trypanosomes or related organisms and it would therefore be of interest to determine the rate of inactivation by the Luria–Delbrück fluctuation test. The results are shown in Table 2. This analysis indicated that both transformant lines tested reverted to a TK– phenotype at approximately the same rate, giving a mean value of $8.9 \times 10^{-7}$ cell-generation$^{-1}$. The similarity of the reversion rates of two individual cell lines suggests that the inactivation mechanism is characteristic for the TK gene in trypanosome DNA and not influenced by incidental factors such as the particular ribosomal array targeted. The inactivation rate could, however, be due to some specific property of the HSV-1 TK gene. Since this gene is unusually GC rich (65%; ref. 20), it is possible that the trypanosomal DNA polymerase might have problems replicating it. To address this possibility, we tested the Varicella-Zoster virus TK gene (VZV TK) which has a GC content of 46% (21), similar to the 48% mole GC content reported for T. brucei (22), and is of similar length to the HSV-1 TK gene.

Inactivation of trypanosomes expressing a VZV TK gene

Trypanosomes were transformed with a construct designed similarly to pBTKN but containing the VZV TK gene and the hygromycin resistance selectable marker. This DNA was also targeted to the ribosomal array and transformant cell lines were characterised for correct integration (data not shown). Cells expressing VZV TK were found to be resistant to ganciclovir but sensitive to FIAU. This result was not unexpected since VZV is much less susceptible than HSV-1 to acyclovir, a compound closely related to ganciclovir (23). As had been observed for trypanosomes expressing the HSV-1 TK gene, the VZV TK transformants reverted back to a FIAU resistant phenotype and grew out at a rate which matched the wild-type, indicating mutation of the VZV TK gene (data not shown).

To see if the inactivation rate of the two different TK genes in T. brucei was the same, we performed a fluctuation test on both transformants in parallel. FIAU instead of ganciclovir was used to select TK– mutants as both VZV and HSV-1 TK expressing cell lines were equally sensitive to this nucleoside analogue. Both transformant lines reverted to FIAU resistance at a similar rate (Table 2). The reversion rate of HSV-1 TK cell line TKN5.3 was found to be slightly lower in this experiment than in the previous one, being $1.2 \times 10^{-7}$ cell-generation$^{-1}$ as opposed to $8.3 \times 10^{-7}$ cell-generation$^{-1}$. However, different nucleoside analogues were used for the selection of revertants in the two experiments (FIAU as opposed to ganciclovir), and this may influence the rate estimate to a minor extent.
### DISCUSSION

We have targeted the herpesvirus TK gene into the ribosomal array of *T. brucei*. The gene is initially expressed in a functional form which renders the trypanosome susceptible to antiviral nucleoside analogues. The activity of TK found in our transformants is consistently much higher than the activity of the putative trypanosomal TK homologue in our assay system. Since the enzyme assay is performed on crude soluble protein extracts, which do not contain detectable TK inhibitors, it is unlikely that the trypanosomal TK activity measured is significantly underestimated.

To our knowledge, our experiments provide the first measurement of the mutation rate in a kinetoplastid protozoan. We think that the rate measured here reflects the mutation rate of trypanosome genes in general. Our arguments for this are as follows. All transformant lines tested gave similar growth curves in toxic nucleoside analogues (data not shown), and the fluctuation test showed similar rates of inactivation in two different lines. It is unlikely, therefore, that inactivation is a consequence of the integration of the TK gene into a particular ribosomal array influencing adjacent genes. Although we cannot rule out that the position of the TK gene in the genome affects the mutation frequency, it seems unlikely that ribosomal arrays are more prone to point mutation than any other locus. The growth curves showing reversion to resistance were similar with different nucleoside analogues and different concentrations of any one nucleoside, and reversion occurred with every nucleoside tested. This indicates that the rate of mutation is dependent on the number of generations and not due to specific mutagenic effects of a particular nucleoside analogue. It is also unlikely that high level expression of TK results in an imbalance in dNTP pools at the replication fork because of increased synthesis of dTMP and dCMP. Evidence from other systems suggests that the dCTP:dTTP ratio rather than the absolute pyrimidine dNTP concentration is important and as long as both nucleotide levels increase proportionally there is no mutagenic effect (24).

The high GC content of the HSV-1 TK gene does not seem to contribute to the rate of mutation. None of the mutations identified occur within poly G runs and therefore polymerase slippage does not appear to cause the mutations. Moreover, the less GC rich VZV TK gene is inactivated at a similar rate. It is therefore probable that we are measuring the basal mutation rate in the trypanosome and not a gene-specific event.

The overall mutation rate in an organism is commonly expressed as the number of mutations per base pair per cell generation. This is estimated by dividing the rate of inactivation of a given gene by the length of that gene. In *E. coli*, inactivation of genes of the *lac* and *his* operons gives a mutation rate of 5 × 10⁻¹⁰ (25). Using the same approach, we estimate the mutation rate of *T. brucei* to be ~10⁻⁹ per base pair per cell generation. Drake (25) observed that a diverse set of microbes, which use DNA to encode their genes and which includes the eukaryotes *Saccharomyces cerevisiae* and *Neurospora crassa*, exhibit widely varying spontaneous mutation rates per base pair. However, when expressed as mutations per genome, the rates are remarkably similar at 0.003 mutations per genome per DNA replication. Drake concluded that such a common mutation rate must have been shaped in response to evolutionary forces of a very general nature. Based upon the inactivation of a TK gene, we calculate the mutation rate in *T. brucei* to be 12-fold higher, at 0.037 mutations per genome per DNA replication. Why such a high mutation rate has arisen during the evolution of *T. brucei* is unclear, but it may be related to the requirement for diversity among the VSG genes on which the trypanosome depends for survival. In this context it is of interest that Lu et al. (26) have reported evidence for a highly mutagenic DNA copying process in African trypanosomes. This process is limited to the duplication transposition of one exceptional VSG gene in bloodstream form trypanosomes and is thought to involve mutation rates in excess of 10⁻² per copying event. This is clearly unrelated to the basal mutation rate studied here.

We have nevertheless tested whether the TK gene can be used as a negative selection system for studies of the mechanism of VSG expression site switching. We have inserted the HSV-1 TK gene in the active VSG expression site in bloodstream form *T. brucei*, and have found that it is possible to select trypanosomes which have changed their VSG coat in the presence of nucleoside analogues (unpublished results). Thus, in spite of the high
inactivation frequency, it appears that the TK system will prove useful not only for the study of recombination and gene conversion but also in the analysis of the regulation of gene expression in African trypanosomes.

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