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Control of variant surface glycoprotein gene-expression sites in *Trypanosoma brucei*

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Trypanosoma brucei **has 20 similar telomeric-expression sites for variant surface glycoprotein genes. Expression sites appear to be controlled at the level of transcription initiation, resulting in only one site being active at any time. Switching between expression sites occurs at a low rate. To analyse the switching mechanism, we used trypanosomes with two expression sites tagged with two different drug-resistance genes and selected these on agarose plates containing both drugs. Double-resistant clones arose at a low frequency of 10^{-7} per cell, but these behaved as if they rapidly switched between the two tagged expression sites and lost double resistance in the absence of selection. Using *in situ* hybridization we found that only 10% of the double-resistant cells had two fluorescent spots corresponding to transcribed expression sites. Our results suggest that: (i) a double expressor is not a stable intermediate in expression site switching; (ii) expression sites are not independently switched on and off; and (iii) expression sites can be in a 'pre-active' silent state from which they can be readily activated.**

Keywords: allelic exclusion/antigenic variation/ expression site switching/mono-allelic expression/ telomeric silencing

Introduction

The African trypanosome *Trypanosoma brucei* multiplies freely in the blood of its mammalian host, using antigenic variation to escape total elimination by the host immune system (reviewed in Barry, 1997; Borst *et al.*, 1998; Cross *et al.*, 1998; Pays and Nolan, 1998). The trypanosome coat consists primarily of a single protein, the variant surface glycoprotein (VSG), and a trypanosome can change the antigenic determinants in its coat by changing the *VSG* gene transcribed. Transcription of the active *VSG* invariably occurs in a telomeric-expression site (ES). Trypanosomes can change the *VSG* expressed either by replacing the *VSG* in an active expression site, or by silencing the active expression site and activating another one. The number of expression sites in *T.brucei* is ~20, but normally only one of these is active at any one time.

Why *T.brucei* should need 20 expression sites, where one would be sufficient for expression of each of the ~10^3 *VSG*s, was unclear. Recent work suggests, however, that the expression site-associated genes (ESAGs) may provide the answer (Bitter *et al.*, 1998). Expression sites are generally long 40–60 kb polycistronic transcription units under the control of a single promoter, containing at least eight ESAGs in addition to a *VSG* (Johnson *et al.*, 1987; Kooter *et al.*, 1987; Pays *et al.*, 1989). Two of these ESAGs encode subunits for a heterodimeric transferrin receptor (Schell *et al.*, 1991; Chaudhri *et al.*, 1994; Ligtenberg *et al.*, 1994; Salmon *et al.*, 1994; Steverding *et al.*, 1994). Receptors encoded by different expression sites differ >100-fold in their ability to bind transferrins from different mammals (Salmon *et al.*, 1997; Bitter *et al.*, 1998). Multiple expression sites therefore appear to help *T.brucei* cope with the diversity in macromolecules that it acquires from its large range of mammalian hosts (reviewed in Borst *et al.*, 1997).

Having 20 similar but not identical expression sites at its disposal, the trypanosome needs a system to coordinate the expression of these sites. Only one out of 20 should be active at any time and silencing the other 19 should be reversible. In other words, the trypanosome requires mono-allelic expression of *VSG*s, but the exclusion of the other 19 expression site alleles should not be irreversible. The problem is analogous to the selection of a single *var* gene out of 50 in *Plasmodium* (Qijun *et al.*, 1998; Scherf *et al.*, 1998), a selection which is also reversible. Usually, however, the choice for mono-allelic expression is irreversible (see Chess, 1998; Ohlsson *et al.*, 1998).

Early experiments on the control of *VSG* expression in trypanosomes seemed compatible with the idea that the telomeric-expression sites are regulated by telomeric silencing (Horn and Cross, 1995; Rudenko *et al.*, 1995; Borst *et al.*, 1997), as proposed by Gottschling *et al.* (1990). In this *T.brucei* strain, switching of expression sites is due to infrequent stochastic events, (~10^{-7}–10^{-6} per cell division) which do not appear to involve DNA rearrangements near the promoter (Zomerdijk *et al.*, 1990, 1991; Horn and Cross, 1997a; Rudenko *et al.*, 1998). The control of expression sites does not depend on the exact promoter sequence, as an expression site in which the promoter is replaced by a ribosomal RNA promoter can still switch in a normal fashion (Rudenko *et al.*, 1995). Moreover, expression sites are switched on and off as a complete unit, since a promoter placed in the middle is suppressed when the expression site promoter is switched off (Horn and Cross, 1997b).

An important prediction of the telomeric silencing model is that each expression site is controlled by stochastic events and independently of all other sites. This

implies that double expressors with two active expression sites are obligatory intermediates in switching. Double expressors with a mixed coat were indeed found in *Trypanosoma equiperdum*, a close relative of *T.brucei* (Baltz *et al.*, 1986). Moreover, Cornelissen *et al.* (1985) reported indirect evidence for a *T.brucei* clone with two simultaneously active *VSG*-expression sites. More recent experiments, however, have not substantiated the existence of double expressors. The introduction of selectable marker genes into expression sites made it possible to select for trypanosomes that had activated a new expression site, and these had invariably switched off or lost the old site (Horn and Cross, 1997a; Cross *et al.*, 1998; Rudenko *et al.*, 1998). This result suggested a selection against double expressors. As trypanosomes with a mixed VSG coat appear to be perfectly normal (Muñoz-Jordan *et al.*, 1996), this selection against double expressors raised the possibility that expression sites are not controlled as independent entities, but that there is cross-talk between expression sites.

To analyse how switching occurs, we have generated trypanosomes with markers in two different expression sites (Rudenko *et al.*, 1998) and selected for (high) expression of both markers. Our results show that double expressors are not a stable intermediate in switching, and we provide evidence for a new unstable state (a putative switching intermediate) in which *T.brucei* can rapidly switch back and forth between two expression sites.

Results

Selecting for trypanosomes with two active VSG-expression sites

To allow stringent selection for trypanosomes with two simultaneously active *VSG*-expression sites, we generated a trypanosome line in which two expression sites were tagged with different selectable marker genes as indicated in Figure 1A (top). Two versions of this line were used for selection experiments: HN.221, in which the 221-expression site is active, and HN.VO2, in which the VO2-expression site is the active one.

To determine the level of drug resistance in trypanosomes expressing both marker genes in an expression site, we used trypanosome clone 3174 (McCulloch *et al.*, 1997) in which the two markers are both integrated into the active 221-expression site, as indicated in the bottom part of Figure 1A. Clone 3174 can grow in up to 50 μg/ml hygromycin plus 10 μg/ml G418 without a significant effect on growth rate (Figure 1B, bottom). These drug concentrations (or even 10 μg/ml hygromycin plus 5 μg/ml G418) are sufficient to kill cells expressing only one of the marker genes, as shown in the top part of Figure 1B.

Double-resistant clones derived from HN.221 trypanosomes (DR.221)

When large numbers of HN.221 trypanosomes were grown on agarose plates containing 10 μg/ml hygromycin plus 5 μg/ml G418, double-resistant clones (DR.221) arose at a frequency of ~10^{-7} per trypanosome (ranging from 7×10^{-8} to 2×10^{-7}). This is approximately half the frequency at which *in situ* switches from the 221- to the VO2-expression site are obtained (Rudenko *et al.*, 1998). To exclude the possibility that double-resistant cells had

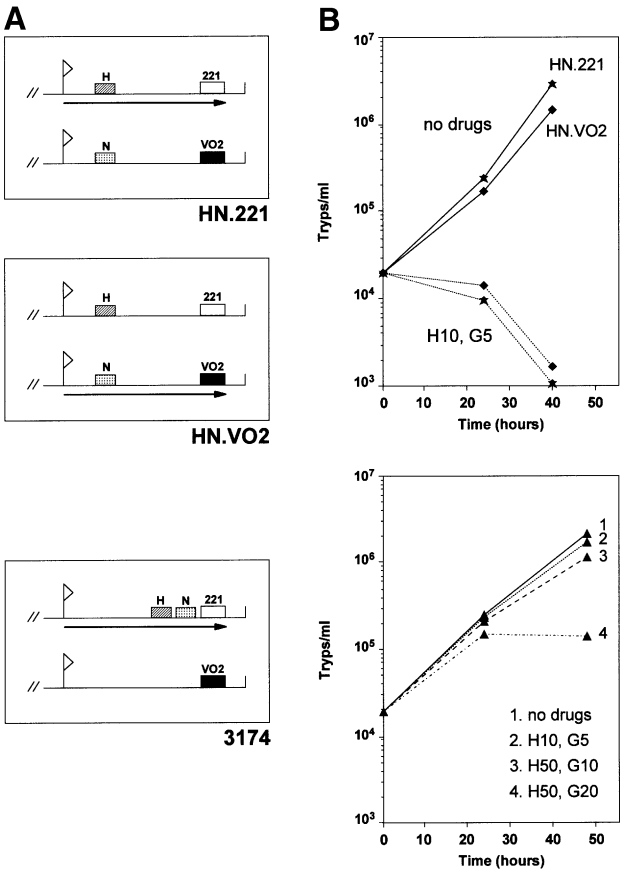

Fig. 1. (**A**) Schematic representation of the trypanosome clones HN.221 (top) and HN.VO2 (middle), and 3174 (bottom). Each box represents a trypanosome. In each case the two expression sites studied here are represented as a simplified version: the flag represents the promoter, the white rectangle is the 221 *VSG*, the black rectangle is the VO2 *VSG*, the striped rectangle is the hygromycin phosphotransferase gene (H) and the dotted rectangle the neomycin phosphotransferase gene (N). The arrow represents the direction of transcription. (**B**) Growth curves of the clones represented in (A). HN.221 and HN.VO2 are shown in the top panel, represented by a star and a square, respectively; 3174 is shown in the bottom panel, represented by a triangle. The filled lines correspond to growth in the absence of drug selection. The other lines correspond to different drug concentrations, in μg/ml, of hygromycin (H) and G418 (G): H10 G5 (dotted), H50 G10 (dashed) and H50 G20 (dash and dot).

recombined the marker genes into a single expression site, we analysed the DNA from these cells by pulsed-field gel electrophoresis, under conditions which separate the two chromosomes containing the 221- and VO2-expression sites. We also checked whether the promoter region had been rearranged. No DNA rearrangements were detected in the 10 independent DR.221 clones analysed (data not shown). Also, the cell cycle pattern of the DR clones was unaltered when compared with a control cell line (see below).

To determine whether both tagged expression sites were being transcribed over their entire length including the *VSG*s, we used RT–PCR. In the double-resistant cells approximately equal amounts of product were obtained with primers for each VSG mRNA, whereas the initial single expressor clones HN.221 or HN.VO2 yielded no detectable VSG mRNA from the silent expression sites (data not shown). To test whether the products of both expression sites were present in the same trypanosome,

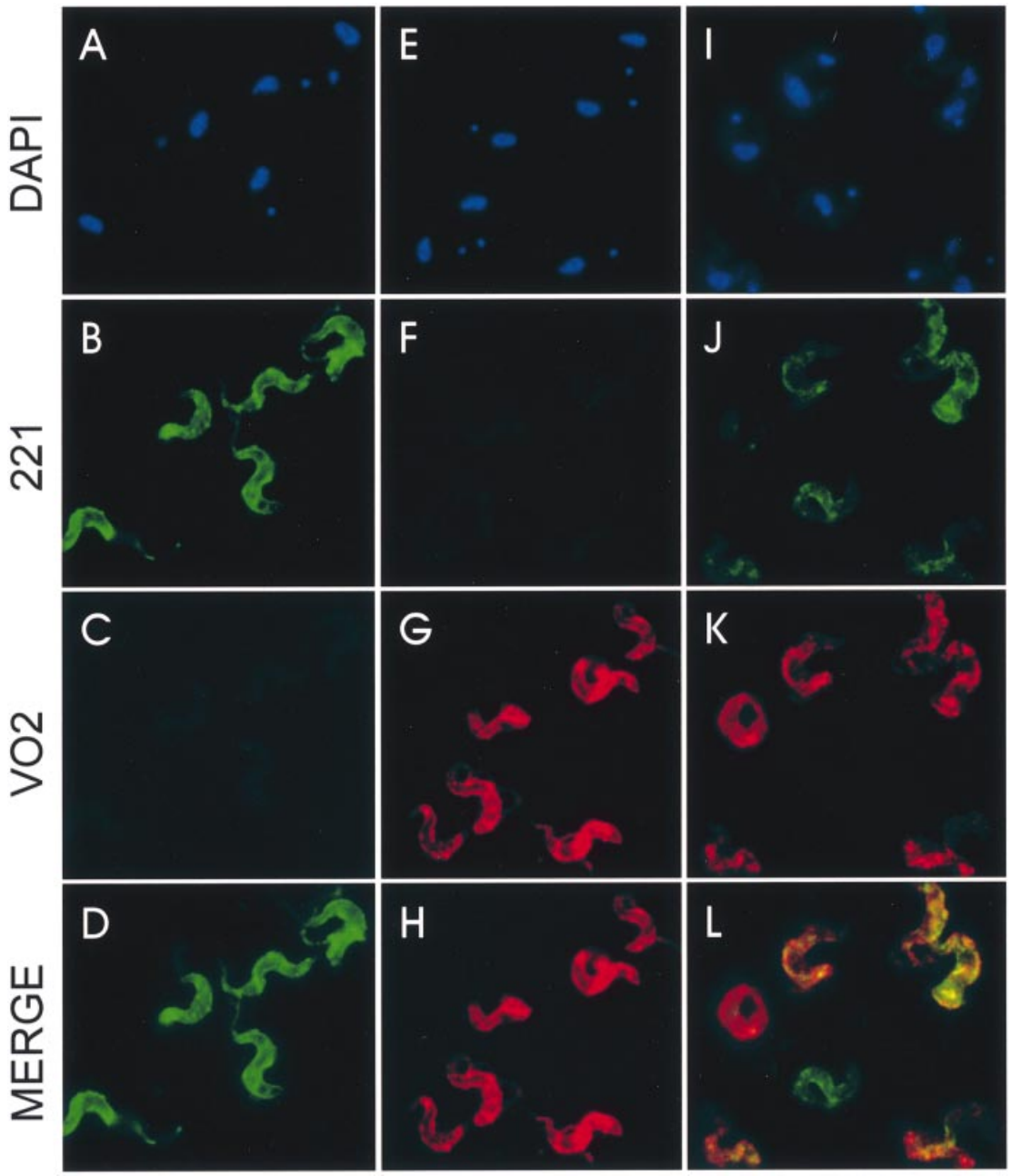

Fig. 2. Immunofluorescence images of trypanosomes stained with antibodies against the 221 and the VO2 VSG coats. The anti-221 antibody, labelled with digoxin and detected with Alexa 488-conjugated antibodies, is shown in green and the anti-VO2 antibody, labelled with biotin and detected with streptavidin-Alexa 594, is shown in red (see Materials and methods for details). HN.221 trypanosomes are shown in (**A**–**D**), HN.VO2 trypanosomes in (**E**–**H**) and DR.221 trypanosomes in (**I**–**L**).

we used fluorescence *in situ* hybridization (FISH) with *VSG* probes and an immunofluorescence assay (IFA) with antibodies against the VO2 or 221 VSG coats.

IFA with antibodies against the 221 or VO2 VSGs detected a mixed VSG coat in ~68% of the DR.221 cells (Figure 2I–L and Table I), whereas HN.221 or HN.VO2 were 100% single positive for 221 or VO2, respectively (Figure 2A–H and Table I). In FISH analysis, using 221 and VO2 *VSG* probes, 26% of the cells stained positive for both 221 and VO2 mRNA (Table I). Both in IFA and FISH analysis the cells that stained positive for a single VSG were approximately half 221 and half VO2 (Table I).

The fact that we found DR.221 cells with mixed VSG coats and transcripts from two expression sites does not necessarily mean that both sites were active at the same time. The half-lives of VSG protein and RNA are, respectively, 30–40 h (reviewed in Cross *et al.*, 1998) and 4.5 h (Ehlers *et al.*, 1987). Hence, our results are readily explainable by rapid switching back and forth between the VO2- and 221-expression sites. In fact, as only one-quarter of the DR.221 cells contained detectable amounts of both mRNAs (Table I), rapid switching between expression sites is necessary to account for our data.

To test whether any DR.221 cells transcribed both expression sites at the same time, we looked for expression site-derived nuclear transcripts by RNA–FISH. We have shown previously that nuclear RNA derived from the active expression site can be visualized as a single intense

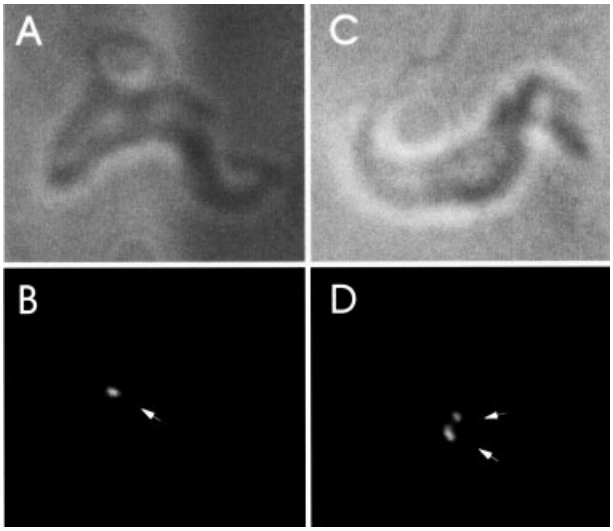

Fig. 3. *In situ* hybridization, with probe Mix 4, of DNase-treated trypanosomes (procedure described in Materials and methods). (**A** and **B**) An image of a HN.221 trypanosome, showing a single spot derived from probe Mix 4 (B, arrow). (A) Nomarski picture of the cell. (**C** and **D**) An example of a DR.221 trypanosome showing a double spot derived from probe Mix 4 (D, arrows). (C) Nomarski picture of the cell.

Table I. Single-cell analysis of double-resistant clones

	HN.221	DR.221	HN.VO2	DR.VO2
VSG coat				
221	100	14 ± 4	0	18 ± 2
VO2	0	17 ± 5	100	29 ± 5
MIX	0	68 ± 3	0	62 ± 4
VSG RNA				
221	100	31 ± 2	0	34 ± 4
VO2	0	43 ± 3	100	42 ± 5
MIX	0	26 ± 2	0	24 ± 2
ES nRNA				
double signal	<1	10 ± 2	<1	9 ± 1

The results are all given in percentages, and correspond to the average ± SD of at least three measurements. A minimum of 150 cells was analysed per measurement.

fluorescent spot in >80% of the trypanosomes analysed (Chaves *et al.*, 1998; Figure 3B). To obtain sufficient signal, we used a mixture of DNA probes (Mix 4) which cover most of the expression site, but not the *VSG* itself. When DR.221 cells were analysed with the Mix 4 probe, we unexpectedly found that 10% of the cells had two fluorescent nuclear signals instead of one (Figure 3D; Table I). Such a clear double signal has not been seen before with trypanosomes that have only a single active expression site. Nevertheless, it was necessary to exclude the possibility that the 10% of cells with a double spot were abnormal cells stalled in the G_2 phase of the cell cycle, with two copies of a single active expression site. We therefore sorted the trypanosome culture by flow cytometry on the basis of DNA content (Figure 4), and analysed the resulting G_1 and $S+G_2$ phase fractions separately for double nuclear spots. As a control cell line we used the 3174 clone (Figure 1), rather than the parent strain HN.221 or HN.VO2, to rule out possible differences

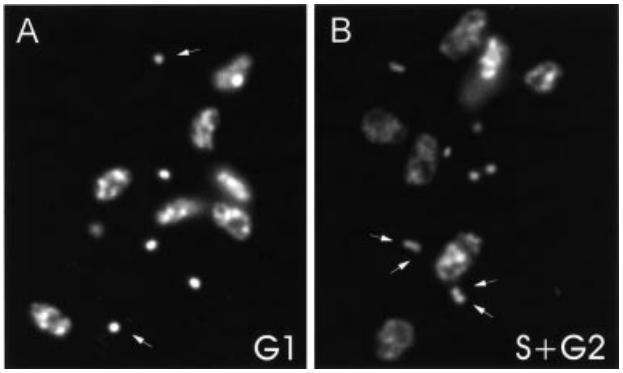

Fig. 4. DAPI staining of DR.221 trypanosomes which were sorted on the basis of their DNA content in two populations: **(A)** G_1 and (**B**) $S+G_2$. The arrows indicate the kinetoplast DNA, which enters S phase before the nuclear DNA. Cells in G_1 phase should only have one kinetoplast, whereas most cells in $S+G_2$ phases should have a dividing kinetoplast or two.

Table II. Cells with two spots for expression site nuclear transcripts in G_1 and $S+G_2$ cells

Cell line	G_1 (%)	$S+G_2$ (%)
3174	1.5[a]	0.8[a]
DR.221	14	9
DR.VO2	13	9

[a]This value is considered to be background due to ambiguous signals.

in the cell cycle pattern due to the simultaneous presence of hygromycin- and neomycin-resistance gene products in the same cell (the cell cycle pattern of DR cells was unaltered compared with the control). The results of this analysis are presented in Table II. Clearly, cells in G_1 are as likely to have two nuclear spots of expression site-derived RNA as cells in S or G_2. We do not see cells with two spots in the control cell line, or cells with four spots in the DR clones, indicating that post-replication cells are either not transcribing the expression site or have the two spots close enough to be seen as one. We conclude that the two spots identify a sub-fraction of trypanosomes in which two expression sites are transcribed at the same time. The analysis is not sensitive enough to verify whether both sites are simultaneously transcribed over their full length. When we used probes corresponding to half of the expression site, the fraction of cells in which an active expression site could be detected dropped below 80% and the analysis became unreliable. However, the fact that the two spots are of equal intensity, and also equal in intensity to single spots, makes it likely that most of the double spots represent two expression sites transcribed over their entire length in one cell.

Analysis of the distance between the two signals derived from expression site nuclear transcripts

We have shown previously that the two alleles of the tubulin gene clusters appear to be randomly distributed in the diploid trypanosome nucleus (Chaves *et al.*, 1998). In comparison, the two nuclear spots corresponding to two active expression sites appeared to be closer together. This impression was substantiated by the data presented in

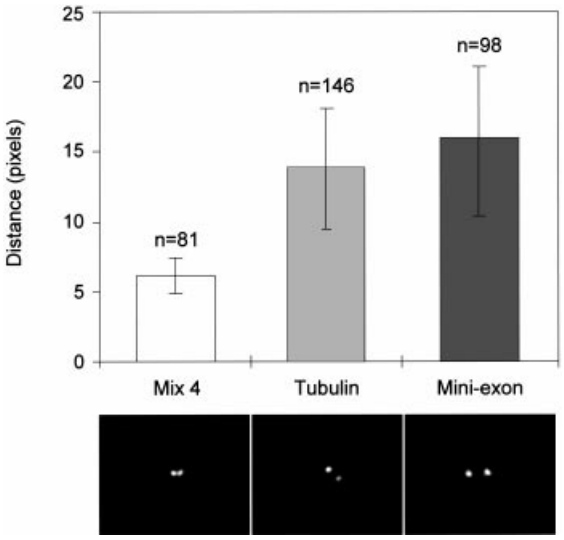

Fig. 5. Measurement of the distance (in pixels) between two fluorescent signals in the nucleus of *T.brucei*. Distance between the two signals derived from each of the probes used (Mix 4, tubulin and mini-exon) was measured using IPLab software (the distance between the centre of two spots was measured). The graph shows the mean of the measurements ± SD. The number of cells analysed is indicated above each histogram. The distance between the two Mix 4 spots is always smaller than the distance between the two tubulin or the two spliced leader spots (the difference is significant at $P < 0.001$). The pictures below show an example of the fluorescent signals analysed (*in situ* hybridization described in Materials and methods).

Figure 5. Both the average distance between the expression site spots and the standard deviation of distances are less than half of the corresponding values of the spots detected with tubulin gene probes or probes for the mini-exon arrays, which are also present in two large arrays (de Lange *et al.*, 1983; Nelson *et al.*, 1983). The evidence available indicates that these spots identify nascent RNA and not sites where RNA is processed (Chaves *et al.*, 1998). We therefore tentatively conclude that the active *VSG*-expression sites are not randomly distributed in the nucleus, in contrast to expression sites in general which are distributed throughout the nucleus (Chaves *et al.*, 1998).

Stability of double expressors

The single-cell analysis of VSG coat, mRNA and expression site nuclear transcripts shows that DR.221 clones are heterogeneous. Only 25% of the cells have detectable mixed VSG mRNA and only 60–70% have mixed coats (we can score cells as having a mixed coat or mixed mRNA when the ratio of the two VSGs is in the range of 1:10 or higher). Nevertheless, growth of these cells is only slightly slower in the presence of hygromycin plus G418 than in its absence, as shown in Figure 6A. Hence, ~90% of the cells must be able to maintain sufficient levels of both marker enzymes to cope with the drug challenge. As only 10% of the cells are transcribing both tagged expression sites simultaneously, the cells must be switching back and forth between the 221- and VO2-expression sites in order to maintain resistance.

To determine how stable this putative rapid-switching state in the DR.221 cells is, double-resistant cultures were grown in the absence of drug selection for increasing time periods, after which hygromycin and G418 were added back to the culture and growth was measured (see stability test, Materials and methods). Figure 6A shows the growth curves of DR.221 trypanosomes after the culture had been 0, 12, 22 or 31 days off selection and was put back on drugs. In the absence of selection, the double-resistant phenotype was gradually lost. From the lag in growth we deduce that after 12 days ~12% of the cells are still capable of surviving in the presence of drugs, and this number is reduced to ~0.06% after 22 days. No double-resistant cells remained after 31 days off selection.

The same cultures that were used for the growth analysis were harvested for single-cell analysis. In the absence of drug selection, the percentage of cells with a mixed VSG coat decreases (Figure 6B and C). The percentage of cells with mixed mRNA and two expression site-derived nuclear transcripts (double nRNA) also decreases, but faster. Such cells are undetectable after 4 days and 1 day off selection, respectively (Figure 6C). Eventually the cultures resolve into ~50% 221 and 50% VO2 cells (Figure 6B). To determine whether the 221 and VO2 cells derived after discontinuation of drug selection were altered in their switching proficiency, they were tested for their ability to switch to the other expression site and to yield double-resistant clones. The results obtained were the same as for HN.221 and HN.VO2 trypanosomes (which had never been double selected). The frequency of switching to neomycin resistance was ~10^{-6} and that of switching to hygromycin resistance was 10^{-5}, similar to what was previously reported (Rudenko *et al.*, 1998), and the frequency of obtaining double-resistant clones was 10^{-7}. We conclude that the rapidly switching, double-resistant state is not the result of a mutation altering switching rates, but an epigenetic state that can be erased over time. We think that this state is a naturally occurring intermediate in expression-site switching, and is stable enough to allow growth in the presence of hygromycin and G418.

Double-resistant clones derived from HN.VO2 trypanosomes (DR.VO2)

Double-resistant clones were also obtained starting from trypanosomes transcribing the VO2-expression site. Using 40 μg/ml hygromycin plus 3 μg/ml G418, a drug regimen that differed somewhat from that used to select the DR.221 cells (10 μg/ml hygromycin plus 5 μg/ml G418), we reproducibly obtained double-resistant DR.VO2 clones at a frequency of 10^{-7}. These were similar to the DR.221 clones in all properties tested, as shown by the results in Table I.

When HN.VO2 cells were selected for resistance to 10 μg/ml hygromycin plus 5 μg/ml G418, we predominantly obtained double-resistant clones that differed from the DR.221 clones (Borst *et al.*, 1998). These sDR.VO2 ('s' for stable) clones arose at relatively high frequency (10^{-5} per trypanosome), and were VSG VO2 positive and VSG 221 negative. They were found to transcribe the VO2-expression site completely, but not to contain any VSG 221 mRNA detectable by RT–PCR and only up to 0.1% of the maximal level of hygromycin-resistance gene

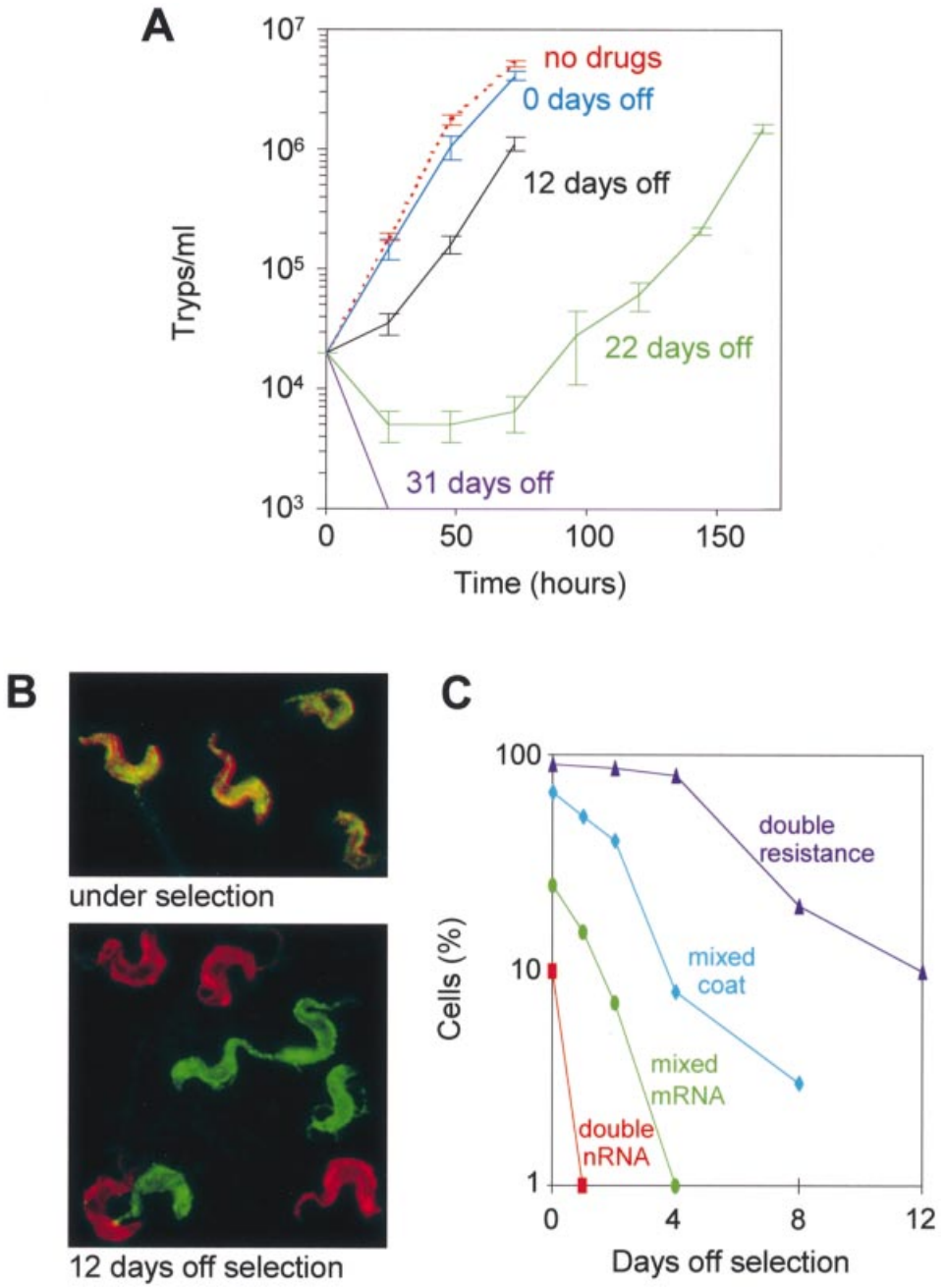

Fig. 6. (**A**) Stability of drug resistance of double-resistant trypanosomes in the absence of drug selection. Growth curves of DR.221 trypanosomes in the absence (dotted line) or presence (filled lines) of drug selection. Each curve represents a culture that has been grown in the absence of selection for different time periods (indicated on the graph by number of days off). At time 0, 10 μg/ml hygromycin and 5 μg/ml G418 were added back to the cultures and growth was measured (stability test described in Materials and methods). (**B**) Immunofluorescence of DR.221 trypanosomes stained with antibodies against the 221 (green) and the VO2 (red) VSGs (see Materials and methods). A cell with a double VSG coat appears as orange or yellow (merge of green and red). The top panel shows a picture of cells from a culture grown in the presence of drug selection. The bottom panel shows a picture of cells from a culture grown in the absence of selection for 12 days (cells with a double VSG coat are no longer visible). (**C**) Graphic representation of the decay of the double-resistance phenotype (DR), mixed VSG coat, mixed VSG mRNA, and double ES nuclear transcripts (nRNA). The curve for the decay of double resistance was based on the growth curves of cultures put back on selection following 2, 4, 8 and 12 days off selection [the last one presented in (A)].

mRNA. This level of mRNA is apparently sufficient to confer resistance to the drug concentrations used. Remarkably, the sDR.VO2 completely retained their hygromycin resistance after 50 generations of growth in the absence of selection, and this stable low activation state of the 221-expression site was only erased by complete activation of the 221 site (selection for high hygromycin resistance) followed by a switch back to G418 resistance.

A clone resembling the sDR.VO2 cells was only obtained once in 11 selection experiments with HN.221, and such clones presumably arise at a frequency far below 10^{-7} per trypanosome. The results for the sDR clones will be presented in detail elsewhere.

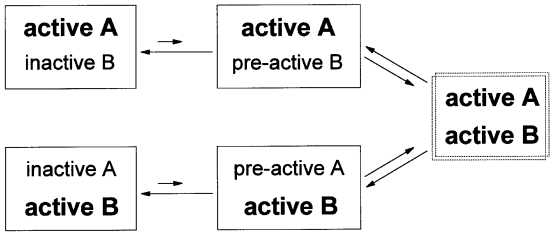

Fig. 7. Model for VSG-expression site switching. The boxes represent trypanosomes. A and B represent two expression sites. The active expression site is shown in bold.

Discussion

Trypanosoma brucei has ~20 telomeric *VSG*-expression sites available, and has developed mechanisms to silence 19 out of 20 and it is able to do this reversibly. With low frequency, transcription of the active site can be shut off and transcription of a silent site activated. To study the mechanisms of silencing and switching, we have tagged two expression sites with selectable drug-resistance genes and selected trypanosomes *in vitro* for resistance to both drugs. The double-resistant clones obtained at a low frequency have peculiar properties which are not compatible with simultaneous, stable high-level transcription of two expression sites. Rather, our data suggest the model summarized in Figure 7, in which two expression sites switch back and forth between an active and a 'pre-active' state, with a brief transient state in which both sites are simultaneously active.

On the one hand, our results cannot be explained by partial activation of one expression site and complete activation of the other one. To obtain double-resistant cells with a mixed coat, both expression sites must be completely transcribed down to the *VSG* (see Figure 1A, top). On the other hand, the heterogeneity of the double-resistant clones is incompatible with continued stable transcription of two expression sites. Only two-thirds of the cells have a mixed coat, only one-quarter stain positive with probes for both VSG mRNAs, and only one-tenth have a double spot in the nucleus corresponding to two active expression sites. Although the precision of these analyses is limited, it is highly unlikely that we would underestimate the fraction of cells with two spots by more than a factor of two. Single nuclear spots are observed in >80% of control cells (Chaves *et al.*, 1998) and there is no indication that double spots are less accurately scored than single spots, even though superimposition of spots could theoretically result in missing 6% double spot cells (see Materials and methods for calculation). We doubt whether that many are missed in practice, as even double spots that are partially overlapping can be recognized under the microscope.

From the data in Figure 6 it is clear that the rapid-switching state is not stable. The ability to grow in both drugs is lost with a $t_{1/2}$ of ~4–5 days, corresponding to ~12–15 cell doublings. We infer that this loss of resistance also occurs in the presence of drugs, as DR cells multiply 10–20% slower with drugs than without drugs. The slower growth is not due to drug toxicity *per se* or the presence of high levels of drug-resistance proteins, since the 3174 cells, which have both resistance genes in a single active VSG-expression site, are not affected by the drug levels used (Figure 1). Hence, it seems likely that the slower growth of the DR cells is due to insufficient levels of one of the proteins conferring drug resistance in a fraction of the trypanosomes. This fraction may have failed to switch in time to retain sufficient levels of one of the drug-resistance proteins, or may have gone back to a stage in which one of the expression sites is inactive, rather than pre-active (see Figure 7).

The rate at which the DR cells switch is probably high. The $t_{1/2}$ of VSG mRNA is only 4.5 h (Ehlers *et al.*, 1987), but nevertheless we see a mixed VSG mRNA population in up to 25% of the cells, even though only 10% of the cells have two active expression sites. Using a rough calculation, presented in Materials and methods, we infer from these data that cells switch every six generations. This must be the minimal switching rate, as the marker enzyme encoded by the switched off expression site is diluted out 30-fold after six generations, even if the mRNA for this enzyme were stable.

The rapid-switching state that we have identified here is rather remarkable. The overall rate of switching between two expression sites in naive cells is ~10^{-7} to 10^{-6} per cell doubling (Rudenko *et al.*, 1998), whereas in our rapidly switching cells this rate has increased to $<10^{-1}$. This is not due to a mutation, but to an epigenetic state that is completely erased when the cells are grown without drugs. The nature of this state is unknown, but we think that it represents a natural intermediate in switching, made visible by our selection procedure. This selection process may actually trap this intermediate, in view of the unusual kinetics of the decay of the rapid switching state. The two-spot nuclei disappear within two doublings after transfer of the cells to drug-free medium, and this is not what one would expect if the growth rate in the presence of drug were only 10–20% less than in its absence (see Materials and methods for calculation). Moreover, the loss of the DR state shows a lag phase (Figure 6), raising the possibility that the presence of drugs not only selects for the rapid switching state, but also stabilizes it. We speculate that this might be because the rate of switching between the pre-active and active states increases when protein synthesis is partially inhibited by hygromycin or G418.

We have shown previously that inactive expression sites are not clustered in trypanosome nuclei (Chaves *et al.*, 1998), but the resolution of this analysis was insufficient to test whether active and inactive expression sites occupy different domains within the relatively small (2 μm in diameter) trypanosome nucleus. We now find that two active expression sites are much closer together than the two loci of the tubulin gene clusters or the mini-exon clusters. This suggests that active sites occupy a specific nuclear domain comparable in size to the nucleolus (~0.5 μm). Whether the 'pre-active' expression site remains in this domain cannot be tested, as we cannot detect the short marker genes by DNA–DNA hybridization under the conditions required for visualization of RNA.

Our present results and conclusions contrast with the conclusion of a previous paper from this laboratory by Cornelissen *et al.* (1985). This paper described a clone of *T.brucei* variant 221a (221ar2 cl.1) which had activated a new expression site, containing the 1.1 *VSG*, but appeared to stably maintain transcription of the 221-expression site. As it is now possible to separate the two chromosomes

that contain the 221 and 1.1 *VSG*s by pulsed-field gel electrophoresis, we repeated the analysis and found that the switch from 221a to 221ar2 cl.1 involved a telomere exchange between the 221- and 1.1 *VSG*-containing chromosomes (unpublished data). Telomere exchanges are now known to be frequent in *T.brucei* (Rudenko *et al.*, 1996), but the first example (Pays *et al.*, 1985) was only described a few months after the paper of Cornelissen *et al.* (1985) went to press. Our conclusion now is that clone 221ar2 cl.1 does not stably transcribe two expression sites simultaneously.

Although the rapid switching state identified here has not been seen before, the model in Figure 7 can account for several unusual results reported in the literature. Baltz *et al.* (1986), working with *T.equiperdum*, found large numbers of cells with mixed coats in a switched culture. The mixed coat phenotype was relatively stable; after 30 days in culture 16% of cells still had a mixed coat, whereas in our experiments cells with a mixed coat disappear within 12 days. Nevertheless, we think that Baltz *et al.* (1986) may have seen the same rapidly switching intermediate described here, and that this intermediate is more stable in the *T.equiperdum* experiments than in ours.

Horn and Cross (1997a) analysed several switched *T.brucei* clones and found that a third of the clones had a tendency to switch back to the original expression site, resulting in mixed coats. Although this is reminiscent of our results, in their case the fraction of cells with a mixed coat increased rather than decreased in the absence of selection. The authors interpreted their results as showing that 'the machinery maintaining all but one expression site in a repressed state can be compromised in a heritable fashion' (Horn and Cross, 1997a).

An apparent coupling between the activation of an expression site and the inactivation of the previously active site has been observed in several types of experiments. Davies *et al.* (1997) noted that alteration of the region just upstream of the *VSG* in an active expression site led to an immediate switch to another expression site. This raised the possibility that this upstream region contains a 'locus-stabilizing element' required for continued high-level transcription. Subsequent results from the Cross laboratory have confirmed that tampering with an expression site results in a switch, suggesting that expression sites may compete for activity (see Cross *et al.*, 1998; Navarro *et al.*, 1999). Conversely, we have shown that switches may be accompanied by destruction of the previously active site (Cross *et al.*, 1998; Rudenko *et al.*, 1998), as if some switches can only be accomplished if the old site, that does not easily switch off, is deleted by a rare, random event.

Although activation of an additional entire expression site is a rare event, a trypanosome can transcribe the upstream part of multiple expression sites at a low level (Rudenko *et al.*, 1994). The nature of the selection against double expressors is not yet clear. It is unlikely that the gene products of two different expression sites would be incompatible. Muñoz-Jordan *et al.* (1996) have shown that trypanosomes with mosaic coats grow well and there is no reason to think that mixed ESAGs would be a problem either. If this were the case, we would have been unable to maintain double-resistant trypanosomes over many generations. We therefore conclude that allelic exclusion is due to competition for conditions required for high-level transcription, be it *trans*-acting factors or a privileged domain in the nucleus. As it is possible to find trypanosomes that transiently transcribe two expression sites at high rate, the privileged domain must be able to accommodate two expression sites, at least temporarily.

For a time, telomeric silencing seemed a reasonable model to explain the control of expression of multiple telomeric *VSG*-expression sites by trypanosomes (Gottschling *et al.*, 1990). The fact that trypanosomes cannot maintain two active expression sites over longer time periods now argues against this model, and it is possible that the telomeric location is not essential for expression site control. The *var* gene family of *Plasmodium falciparum* consists of 50 members spread over several chromosomes, and the expression of these genes is controlled at the transcriptional level and does not necessarily involve telomeric-expression sites (Qijun *et al.*, 1998; Scherf *et al.*, 1998). Control of the *var* genes of *Plasmodium* and the *VSG*-expression sites of *T.brucei* may, therefore, have mechanistic elements in common.

Materials and methods

Trypanosome clones and growth conditions

The trypanosomes used belong to strain 427 of *T.brucei brucei* (Cross, 1975) and were cultured *in vitro* in HMI-9 medium (Hirumi and Hirumi, 1989). *Trypanosoma brucei* variant used: 221a (MiTat 1.2a), which expresses the 221 *VSG* from the 221-expression site (Cross, 1975). Transformants of variant 221a used: 3174, which contains the drug-resistance gene for neomycin and hygromycin between *ESAG1* and the 221 *VSG* (McCulloch *et al.*, 1997), HN.221 and HN.VO2 (Figure 1A; Rudenko *et al.*, 1998).

Single-cell analysis (IFA and FISH)

Cell fixation, immunohistochemistry, probe labelling and probe hybridization were performed essentially as described (Chaves *et al.*, 1998). For immunocytochemical detection goat anti-mouse-Alexa 488 and streptavidin-Alexa 594 (Molecular Probes) were used instead of the corresponding FITC and Texas Red conjugates, respectively. The IFA was carried out with polyclonal antisera raised against the 221 and VO2 VSGs as primary antibodies. The anti-221 antibody was labelled with digoxin (and detected with Alexa 488 conjugated antibodies) and the anti-VO2 antibody was labelled with biotin (and detected with streptavidin–Alexa 594 conjugates). Rabbit anti-221 polyclonal antiserum was directed against a maltose binding protein–VSG 221 N-terminus fusion protein. The N-terminal part of the 221 VSG was used (amino acids 33–223). Rabbit anti-VO2 polyclonal antiserum was directed against the complete VO2 VSG purified according to Paturiaux-Hanocq *et al.* (1997).

The 221 *VSG* probe is a 590 bp *Pst*I fragment of the 5′ end of the gene cloned in pGEM3. The VO2 *VSG* probe is a 600 bp *Eco*RI–*Hin*dIII fragment from the 5′ end of the gene cloned in pGEM3. The expression site probe Mix 4 used in the *in situ* hybridization shown in Figure 3G and I contained the clones pTg221.8, pTg221.4, pTg221.14 and pTg221.11 (described in Chaves *et al.*, 1998). The tubulin probe is a 2.9 kb *Eco*RI–*Hin*dIII fragment of one αβ repeat unit, cloned in pGEM4. The spliced leader probe is the T7-SL plasmid, which contains the mini-exon gene (Cross *et al.*, 1991). Plasmid sequences alone were used as a negative control and did not detectably hybridize to fixed trypanosome nuclei (data not shown). The VO2 *VSG* probe was labelled with biotin-16-dUTP and the other probes were labelled with digoxigenin-11-dUTP.

Cell sorting by flow cytometry

Trypanosomes were fixed as described in Chaves *et al.* (1998). After fixation, cells were washed twice in PBS (0.15 M NaCl, 10 mM sodium phosphate pH 7.2) and resuspended to a final concentration of 5×10^6/ml. 4′,6-diamidino-2-phenylindole (DAPI) was added to a final concentration of 100 ng/ml. Cells were sorted directly on microscope slides (50 000 cells per drop) based on their DAPI fluorescence. Sorting was

done with a high speed sorter (Moflo, Cytomotion, USA) equipped with a UV laser (360 nm). After sorting, one of the slides was re-stained with anti-fading solution {9 parts glycerol, 1 part 0.2 M Tris–HCl pH 7.5, 2% 1,4-diazobicyclo-[2,2,2]-octane (DABCO), 0.02% NaN_3} containing 75 ng/ml DAPI, and the G_1 and $S+G_2$ fractions analysed under the fluorescence microscope (the analysis was based on the presence of one or two kinetoplasts in the cells of each fraction, as G_1 cells should have a single kinetoplast).

Microscopy

A Zeiss Axiovert 100 TV microscope equipped with a 100× objective (numerical aperture 1.3), single band-pass filters for red, green and blue fluorescence and a double band-pass filter for simultaneous detection of red and green fluorescence was used for visual analysis. For photography, digital images were acquired with a Photometrics CCD camera series 200 with a KAF 1400 chip. Exposure times were adjusted to optimize detection of nuclear signals.

Growth curves and stability analysis

Growth curves were done in a 2 ml volume, starting at a density of trypanosomes of 2×10^4/ml. Samples were analysed every 24 h until the culture was grown to full density. For the stability analysis, the cultures were released from drug selection and grown for the indicated number of days without drugs. At time 0, drugs were added back to the cultures and growth was measured.

Calculations

Using FISH we find that the DR clones have 25% of cells with a mixed VSG mRNA and only 10% of cells with a double signal for expression-site nuclear RNA (corresponding to two active expression sites). By subtracting these two numbers, we determined that ~15% of cells which have a mixed VSG mRNA, but are not simultaneously transcribing both expression sites. We think that our detection limit for mRNA is ~10% of the total VSG mRNA. Suppose that we start with a cell which has both sites on, hence 100% of both VSG mRNAs. If one of the sites is switched off, then after one doubling (~9 h, which is approximately two VSG mRNA half-lives; Ehlers *et al.*, 1987), the daughter cells will have 100% of the mRNA from one VSG and 12.5% of the other (which is no longer produced). After a second division, cells will be scored as single positive for the VSG mRNA. Hence, switching should occur approximately every six doublings [100% divided by 15% (which is the percentage of single spotted cells with detectable amounts of both VSG mRNAs)].

We have shown previously that the probability of accidental overlap of a fluorescent signal with the nucleolus of trypanosomes is 20% (Chaves *et al.*, 1998). In the analysis of the signals derived from the two tubulin or mini-exon spots, we have an accidental overlap of 10%. This should be approximately the same in the case of the two ES nRNA spots, if the two signals were randomly distributed. However, the two signals seem to be restricted to a subnuclear compartment which has maximally half the diameter of the nucleus. In this case, we could expect an accidental overlap (total or partial) of ~40%, meaning that we are missing 5 or 6% of cells with a double spot. We find this unlikely as partially overlapping spots can be readily distinguished.

From the difference in growth in the presence and absence of drug selection, we deduce that the DR cells are losing the double resistance at a rate of 10–20% per cell doubling. In that case, we would expect the number of cells with a double spot for ES nRNA to be ~5% after three divisions (1 day). It seems, therefore, that these cells are lost faster than expected (see Figure 6C). The same occurs for the cells with a mixed VSG coat and a mixed VSG mRNA, but the difference is less pronounced.

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