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Position-dependent and promoter-specific regulation of gene expression in *Trypanosoma brucei*

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Trypanosoma brucei **evades the mammalian immune response by a process of antigenic variation. This involves mutually exclusive and alternating expression of telomere-proximal variant surface glycoprotein genes (*vsg*s), which is controlled at the level of transcription. To examine transcription repression in *T.brucei* we inserted reporter genes, under the control of either *rRNA* or *vsg* expression site (ES) promoters, into various chromosomal loci. Position-dependent repression of both promoters was observed in the mammalian stage of the life cycle (bloodstream forms). Repression of promoters inserted into a silent ES was more pronounced closer to the telomere and was bi-directional. Transcription from both ES and *rRNA* promoters was also efficiently repressed at a non-telomeric *vsg* locus in bloodstream-form trypanosomes. In cultured tsetse fly midgut-stage (procyclic) trypanosomes, in which *vsg* is not normally expressed, all inserted *rRNA* promoters were derepressed but ES promoters remained silent. Our results suggest that *vsg* promoters and ectopic *rRNA* promoters in bloodstream-form *T.brucei* are restrained by position effects related to their proximity to *vsg*s or other features of the ES. Sequences present in *rRNA* promoters but absent from *vsg* ES promoters appear to be responsible for *rRNA* promoter-specific derepression in procyclic cells.**

Keywords: silencing/telomere/*T.brucei*/variant surface glycoprotein

Introduction

Trypanosoma brucei is a protozoan parasite transmitted between mammalian hosts by the tsetse fly (*Glossina* spp.). In the mammal the so-called bloodstream forms are covered by a dense coat of a single type of variant surface glycoprotein (Vsg), which represents ~10% of total cell protein (Cross, 1975). Long-term survival in the mammalian host is dependent upon antigenic variation, which involves periodically replacing the Vsg with an antigenically distinct species on a minority of cells (reviewed by Vanhamme and Pays, 1995; Cross, 1996).

Trypanosoma brucei contains ~1000 Vsg genes (*vsg*s) and pseudo-*vsg*s (Van der Ploeg *et al.*, 1982). The majority of silent *vsg*s are probably clustered at non-telomeric positions while transcriptionally competent *vsg*s are located adjacent to telomeres. The *vsg* active in the bloodstream forms is found in a polycistronic expression site (ES). The bloodstream-form *vsg* ES promoter (Zomerdijk *et al.*, 1990) is usually 30–50 kbp upstream of the *vsg* and only one ES is active at a time, while multiple others are somehow silenced.

When bloodstream-form *T.brucei* are ingested by the tsetse fly during a blood meal they differentiate into procyclic-form cells, which proliferate in the insect midgut. Vsg synthesis ceases during differentiation and established procyclic forms do not express Vsg and cannot infect mammals. Procyclic forms express an invariant surface glycoprotein coat composed of procyclins, also known as procyclic acidic repetitive proteins (Parps) (reviewed by Hehl and Roditi, 1994). Infectivity is reacquired after trypanosomes migrate to the tsetse fly salivary glands and differentiate into metacyclic forms, which express a distinct subset of telomeric *vsg*s. Metacyclic *vsg*s are transcribed in monocistronic transcription units where the promoter lies ~2 kbp upstream of the *vsg* and within ~5 kbp of the telomeric repeats (Alarcon *et al.*, 1994; Graham and Barry, 1995). Metacyclic forms switch to transcription of bloodstream-form ESs shortly after their introduction into the mammalian host (Hajduk and Vickerman, 1981; Esser and Schoenbechler, 1985).

vsg expression is controlled at the level of transcription in the two life cycle stages (bloodstream and procyclic) that are readily accessible for laboratory study. One of the mechanisms for antigenic variation in metacyclic or bloodstream forms is to switch transcription between telomeric loci (see for example Zomerdijk *et al.*, 1990; Rudenko *et al.*, 1995; Graham and Barry, 1995; reviewed by Vanhamme and Pays, 1995; Cross, 1996). ES switching can occur in the absence of detectable genomic rearrangements (Navarro and Cross, 1996; Horn and Cross, 1997). In bloodstream forms repression of transcription from all metacyclic *vsg* loci and all but one of the bloodstream-form ESs is critical for mutually exclusive *vsg* expression and parasite survival in the mammalian host. Similarly, all but a single metacyclic *vsg* locus must be repressed in metacyclic forms.

Promoters for *vsg*s and *parp* resemble those for *rRNA*, based on similarities in their transcription behavior in the presence of α-amanitin and Sarcosyl (see Chung *et al.*, 1992). All four promoters display a similar level of activity in transient transfection assays (see Zomerdijk *et al.*, 1991; Alarcon *et al.*, 1994; Graham and Barry, 1995; Vanhamme and Pays, 1995), but when mapping elements important for promoter activity in these assays the *rRNA* (Zomerdijk *et al.*, 1991; Janz and Clayton, 1994) and *parp* (Sherman *et al.*, 1991; Brown *et al.*, 1992) promoters were both found to have an 'upstream control element' (UCE), whereas the *vsg* ES (Vanhamme *et al.*, 1995; Pham *et al.*,

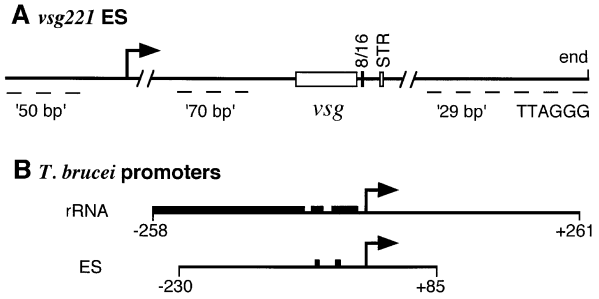

Fig. 1. Physical maps of the *vsg221* expression site and *T.brucei* promoters. (**A**) Salient non-coding motifs in the *vsg221* ES. The arrow represents the *vsg* ES promoter. Repeats that occur in tandem arrays are labeled below the map. Other sequences of interest are labeled above the map; 8/16, octamer/hexadecamer conserved sequences; STR, sub-telomeric repeat. (**B**) The promoters used in this study are shown. The arrows indicate the transcription initiation points, which are designated as position +1. Black boxes upstream of the initiation point indicate regions encompassing elements that are critical for promoter activity in transient transfection assays.

1996) and metacyclic *vsg* (Alarcon *et al.*, 1994; Vanhamme *et al.*, 1995) promoters can be truncated to within ~70 bp upstream of the initiation point without any loss of activity. Similarly truncated *rRNA* and *parp* promoters, in which the UCE has been removed, direct only 3–5% of full promoter activity in transient transfection assays (Sherman *et al.*, 1991; Zomerdijk *et al.*, 1991; Brown *et al.*, 1992; Janz and Clayton, 1994).

When transcription is assayed using chromatin templates rather than 'naked' templates (as in transiently transfected cells), *cis*-acting elements may add an extra dimension to the regulatory process by generating chromatin domains that are refractory to transcription. Sequence-specific transcription factors, although present, may be unable to counteract chromatin-mediated repression (reviewed by Felsenfeld, 1996). A number of reports indicate that *T.brucei* promoters are unregulated following transient transfection (see for example Zomerdijk *et al.*, 1991). In chromatin, transcription from endogenous ES promoters is repressed at, or soon after, initiation. This appears to be the case for all but one ES in bloodstream forms (Zomerdijk *et al.*, 1990) and at all ESs in procyclic forms (Rudenko *et al.*, 1994). In addition, when a *rRNA* promoter containing a UCE replaces an endogenous ES promoter (Rudenko *et al.*, 1995) or is inserted into an ES (Horn and Cross, 1995) this promoter can be efficiently repressed in bloodstream forms. These results indicated that regions of the chromosome mediate transcription repression within a *vsg* ES in a promoter-independent manner in bloodstream-form cells.

In the current study, to further our understanding of how *T.brucei* maintains the mutually exclusive but alternating transcription of *vsg*s and repression of all *vsg*s in procyclic cells, we have measured position-dependent and promoter-dependent repression at multiple loci in bloodstream and procyclic trypanosomes.

Results

Figure 1A shows some salient features of the *vsg221* ES, highlighting putative regulatory elements. '50 bp' repeats (Zomerdijk *et al.*, 1990) are found upstream of the endogenous ES promoter and '70 bp' repeats (see Campbell *et al.*, 1984) are found upstream of the *vsg*. Conserved octamer and hexadecamer motifs (Majumder *et al.*, 1981) are found immediately downstream of all *vsg*s and sub-telomeric repeats or GC-rich repeats (Aline and Stuart, 1989; Weiden *et al.*, 1991; D.Horn and G.A.M.Cross, unpublished data) are found further downstream. *Trypanosoma brucei* chromosome ends have subterminal '29 bp' repeats (Weiden *et al.*, 1991) and appear to end in tandem arrays of TTAGGG (Blackburn and Challoner, 1984; Van der Ploeg *et al.*, 1984).

Both the *T.brucei rRNA* and ES promoters display a similar level of activity in transient transfection assays (Zomerdijk *et al.*, 1991) and the *rRNA* (Zomerdijk *et al.*, 1991; Janz and Clayton, 1994) and ES promoter (Vanhamme *et al.*, 1995; Pham *et al.*, 1996) fragments used in the current study contain all the elements required for full activity in transient assays (Figure 1B). All of the experiments below were repeated at least twice, with similar results, but the data shown were derived from the same gel and filter in order to facilitate signal quantification and accurate comparisons.

Transcription from promoters inserted into the non-transcribed rRNA spacer

Stable transformation of *T.brucei* appears to occur exclusively by homologous recombination (Blundell *et al.*, 1996), so we can efficiently target specific loci. Promoter sequences were flanked by selectable markers (Figure 2A), allowing us to assign activity to endogenous transcription at the target locus (indicated by transcription of the upstream selectable marker *ble*) or, if this was undetectable, to the inserted promoter (indicated by transcription of the downstream selectable marker *neo*). Each selectable marker gene was flanked by well-characterized *T.brucei* splice acceptor and polyadenylation signals (Hug *et al.*, 1993; Horn and Cross, 1995).

Before testing repression at various loci in bloodstream forms we wanted to test the activity of the *rRNA* and ES promoters at loci that we expected to reflect a transcriptionally competent chromatin structure. For this experiment we chose the non-transcribed *rRNA* gene spacer (White *et al.*, 1986). We expected an inserted *rRNA* promoter to be active at this locus as it is within a few kilobases of an endogenous *rRNA* promoter (see Figure 2A). Because the non-transcribed ribosomal spacer constitutes a multiple copy target and because promoters may behave differently at different loci, we generated multiple clones. We obtained three independent clones with *rRNA* promoters integrated into at least two different spacer loci on different chromosomes and four independent clones with ES promoters integrated at four different spacer loci. The size of the four chromosomes targeted in these experiments were consistent with the previously reported location of *rRNA* gene arrays as determined by pulsed field gel electrophoresis (Gottesdiener *et al.*, 1990; data not shown).

ble mRNA was not detected by Northern analysis in any of these clones, confirming that the targeted loci were not transcribed (Figure 2B). We could, therefore, attribute *neo* transcription to initiation at the integrated promoters. Cells with a *ble*–promoter–*neo* cassette integrated in an active ES acted as a positive control for transcription through the entire cassette. The *rRNA* (R) promoter

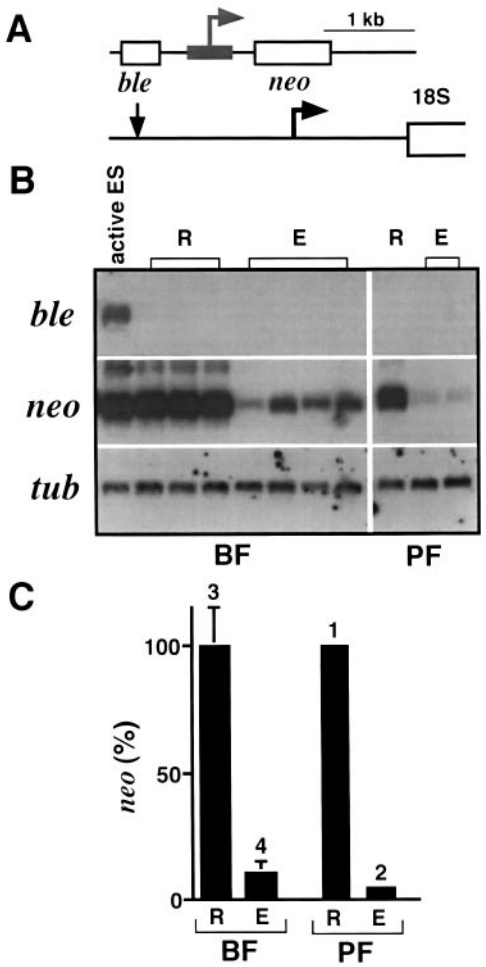

Fig. 2. Transcription from promoters inserted into the non-transcribed *rRNA* gene spacer. (**A**) Physical map of the *ble*–promoter–*neo* cassette and the integration site (vertical arrow) upstream of the endogenous *rRNA* promoter. The exogenous promoter and the endogenous *rRNA* promoter are indicated by horizontal arrows. (**B**) A Northern blot was hybridized with the probes indicated to the left of each panel. Results are shown for three clones with the *rRNA* promoter cassette (R) and four with the ES promoter cassette (E) in bloodstream forms (BF) and one and two procyclic form (PF) derivatives respectively. The tubulin (*tub*) probe served as a loading control. Cells with a cassette integrated in an active ES are shown as a positive control for transcription through the entire cassette (active ES). (**C**) *neo* mRNA signals were quantified by phosphorimager analysis. These values were corrected for loading variations based on *tub* mRNA signals and then related to a reference value of 100% for *neo* mRNA in bloodstream or procyclic clones where *neo* was driven by an *rRNA* promoter. The numbers above each bar represent the number of independent clones in each data set and error bars indicate standard deviation. Symbols are as in (B).

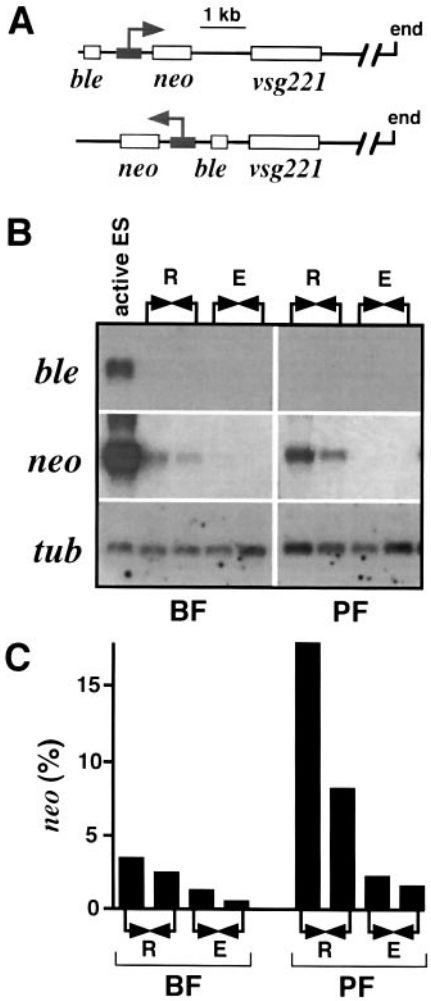

Fig. 3. Transcription repression and promoter orientation. (**A**) Physical map of the *ble*–promoter–*neo* cassettes integrated, in either orientation, upstream of *vsg221* in the 'silent' *vsg221* ES. The exogenous promoters are indicated (arrows). (**B**) A Northern blot was hybridized with the probes indicated to the left of each panel. Arrowheads indicate the direction of transcription in each clone. BF, bloodstream forms; PF, procyclic forms; R, cassette with *rRNA* promoter; E, cassette with ES promoter. Cells with a cassette integrated in an active ES are shown as a positive control (active ES). (**C**) *neo* mRNA signals were quantified by phosphorimager analysis. Symbols are as in (B).

was highly active in all three bloodstream-form clones, generating a similar quantity of *neo* mRNA at different spacer loci (standard deviation 15%, $n = 3$; Figure 2C) as was produced in an active ES (Figure 2B). The ES (E) promoter was ~10-fold less active than the *rRNA* promoter at four different spacer loci. Following differentiation to procyclic forms, the *rRNA* promoter remained highly active, while the ES promoter was less active than in bloodstream forms, generating a 20-fold lower signal than the *rRNA* promoter. *rRNA* promoters inserted at *rRNA* gene spacers did not appear to be subject to repression, so we chose the *neo* values derived from these bloodstream and procyclic clones as a universal reference value in all of the experiments presented below.

Telomere-proximal repression is bi-directional

We previously reported that *rRNA* and ES promoters were repressed when inserted at a telomere-proximal locus with transcription directed towards the telomere (Horn and Cross, 1995). We wanted to determine if promoter orientation, relative to the telomere, had any significant effect on repression, so we constructed clones in which transcription from either promoter was directed away from the telomere (see Figure 3A). Northern blotting indicated the absence of a detectable *ble* signal in each clone with cassettes integrated into a repressed ES, showing that no significant transcription was derived from the endogenous ES promoter or from elsewhere beyond the integrated cassette (Figure 3B). Both promoters were repressed in either orientation in bloodstream forms, as demonstrated by *neo*

mRNA levels, indicating that repression was bi-directional for both the *rRNA* (R) and ES (E) promoters. When these clones were differentiated to procyclic forms the ES promoter remained strongly repressed while the *rRNA* promoter was partially derepressed (Figure 3C). The activities were expressed as percentages of a fully active *rRNA* promoter in the cell lines illustrated in Figure 2. The extent of repression is greater when either promoter is directed away from the telomere. This is probably because the promoter is closer to the telomere in these clones (see Figure 3A and below).

Repression is more pronounced closer to the telomere

In *Saccharomyces cerevisiae* (Gottschling *et al.*, 1990) and *Schizosaccharomyces pombe* (Nimmo *et al.*, 1994) telomeres repress transcription of genes located nearby. Such repression is dependent upon the promoter and distance from the telomere. Telomere position effects in yeast originate at the telomere and spread 3–5 kbp towards the centromere, so silencing of inserted promoters is inversely related to their distance from the telomere (Renauld *et al.*, 1993). In *T.brucei* endogenous *vsg* ES promoters are located 30–50 kbp from the telomeric TTAGGG repeats while metacyclic *vsg* promoters are located within 5 kbp. All but one of these promoters are repressed in bloodstream and metacyclic forms respectively (Zomerdijk *et al.*, 1990; Graham and Barry, 1995). To determine if telomere length or proximity influences promoter-independent transcription repression in bloodstream-form *T.brucei* we constructed isogenic bloodstream-form clones with reporter cassettes inserted at different positions within the silent *vsg221* ES. We generated two independent clones with the *rRNA* promoter inserted at four different positions (Figure 4A) and tested two different positions for the ES promoter. We were unable to obtain clones with an ES promoter integrated downstream of *vsg221* in the repressed ES, possibly reflecting the low activity of this promoter at this position (see Figure 4).

Northern analysis indicated that no significant transcription from the endogenous ES promoter reached the *ble* markers in either life cycle stage in any of the targeted positions (Figure 4B). Telomere position effects in yeast are highly sensitive to changes in telomere length, with longer telomeres increasing the frequency of transcription repression (Kyrion *et al.*, 1993). *Trypanosoma brucei* telomeres increase and decrease in length during mitotic growth (Bernards *et al.*, 1983; Pays *et al.*, 1983). To test whether proximity to the physical end of the chromosome or the length of TTAGGG repeats might influence repression within a *T.brucei vsg* ES, we determined the predominant length of the *vsg221*-associated telomeres in all 10 clones that had cassettes inserted in the repressed *vsg221* ES. Transcription repression as a function of telomere length or distance from the telomere was analyzed by determining the level of *neo* mRNA expression in each clone (Figure 4B and C). Although telomere length ranged from 4 to 20 kbp (data not shown) in some pairs of clones with *rRNA* (R) promoters at identical locations, each pair generated similar levels of *neo* signal. This indicated that, unlike yeast, telomere length variation in *T.brucei* had no significant effect on position effect repression. The position

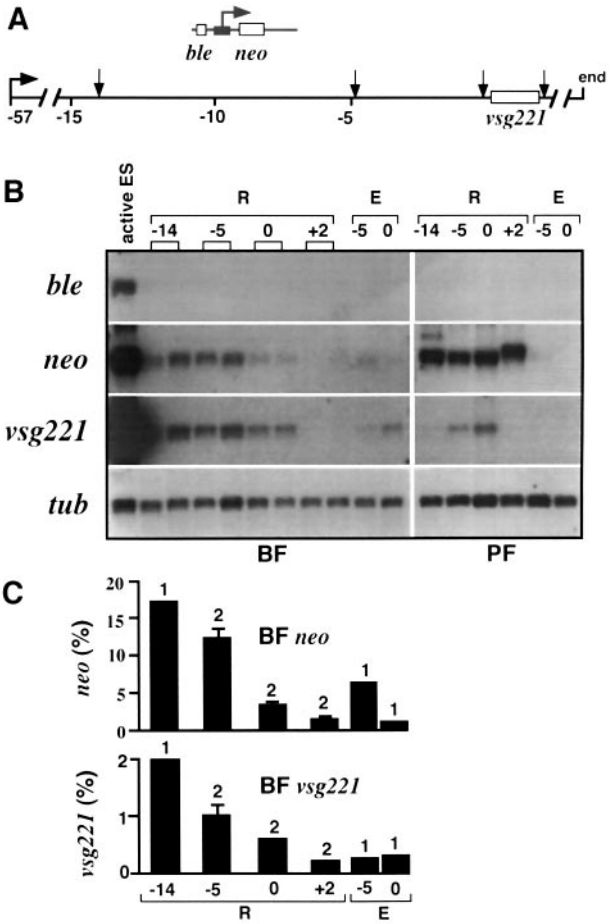

Fig. 4. Transcription repression and telomere proximity. (**A**) Physical map of the *ble*–promoter–*neo* cassette and locations of four integration sites (vertical arrows) in the 'silent' *vsg221* ES. The exogenous promoter and the endogenous ES promoter are indicated by horizontal arrows. (**B**) A Northern blot was hybridized with the probes indicated to the left of each panel. The position of integration (relative to the *vsg221* start codon) in each clone, in kbp, is indicated. For the inserted *rRNA* promoter (R) results are shown for two independent bloodstream-form (BF) clones and one procyclic form (PF) derivative at each integration site. Only one clone was derived for each of the two insertion sites of the ES promoter (E). Cells with a cassette integrated in an active ES are shown as a positive control (active ES). (**C**) Bloodstream-form *neo* and *vsg221* signals were quantified by phosphorimager analysis. *neo* signals were normalized against clones with the same cassettes integrated at each position into an active ES (see text). The numbers above each bar represent the number of independent clones in each data set and error bars indicate standard deviation. Symbols are as in (B).

of integration did have a significant effect, however. The *neo* mRNA levels in bloodstream forms indicated that repression at both promoters was more pronounced closer to the telomere (Figure 4B and C), similar to the situation in yeast. Differentiation of these bloodstream forms to procyclic forms showed that the *rRNA* (R) promoter was partially derepressed at all four loci whereas the ES (E) promoter remained strongly repressed at the two loci tested (Figure 4B, extreme right lanes). Similar to the *rRNA* promoter, the activity of a *parp* promoter was unaffected by its distance from the telomere when introduced into procyclic forms on artificial linear chromosomes (Patnaik *et al.*, 1996).

The Northern blot indicated that *neo* transcripts derived

from a gene integrated downstream of *vsg221* were longer than *neo* transcripts generated at other loci. Mapping splice acceptor and polyadenylation sites revealed that cassettes inserted at this locus generated *neo* mRNA polyadenylated within downstream pGem3 vector-derived sequence rather than within the aldolase-derived 3′-UTR sequence (data not shown). Because insertion at different loci can influence post-transcriptional processing of the reporter transcript and, therefore, steady-state mRNA levels, we carried out control experiments in which the same cassettes were inserted at the same loci in the active *vsg221* ES, where it may be assumed that they will be transcribed equally from the endogenous ES promoter and differences in steady-state mRNA will reflect post-transcriptional differences. Steady-state *neo* mRNA levels from all four loci in an active ES differed by up to 2.3-fold (data not shown), showing that any variations in post-transcriptional processing at different integration sites had little effect on *neo* mRNA levels, which therefore truly reflect differences in transcription in our experiments. We corrected for these small differences in computing the results shown in Figure 4C.

Because *vsg221* transcription is normally driven by a promoter located ~50 kbp upstream of the gene, *vsg* transcription could be regulated at the level of initiation or elongation. In bloodstream forms ES transcription is controlled at or near initiation (Zomerdijk *et al.*, 1990). In procyclic forms transcription initiated at endogenous ES promoters (Rudenko *et al.*, 1994) and at a *parp* promoter inserted into an ES (Lee, 1995) was repressed at the level of transcription elongation. Rehybridizing the Northern blot with a *vsg221* probe allowed us to gain some insight into the mode of regulation operating on promoters inserted into the silent *vsg221* ES, in bloodstream and procyclic forms. If only transcription initiation were repressed, we would expect to see similar ratios of *neo* signal to *vsg221* signal as the distance between the two markers increased. If transcription elongation were repressed we would expect to see increasing ratios of *neo* signal to *vsg221* signal as the distance between the two genes increased. The results (Figure 4B and C) suggest that transcription from the *rRNA* (R) promoter is repressed predominantly at the level of initiation in bloodstream forms. However, the ratios of *neo* signal to *vsg221* signal at all four loci was between 6 and 12, suggesting that attenuation occurs within or just upstream of the *vsg221* gene.

Although *vsg221* signals were detectable in procyclic forms (Figure 4B), *vsg* mRNA is unstable in this life cycle stage (Ehlers *et al.*, 1987), so the levels of *vsg* mRNA in bloodstream and procyclic forms cannot be directly compared. However, the results suggest that transcription from the *rRNA* promoter is being repressed at the level of elongation in procyclic forms. Elongation from the ES (E) promoter appears to be repressed in bloodstream forms (Figure 4B and C), although the data were very limited. This promoter was so strongly repressed in procyclic forms that we did not detect any significant *neo* or *vsg221* signal by Northern analysis (Figure 4B). Values below 0.2% are not significant as this is within the range of variation obtained for different background values.

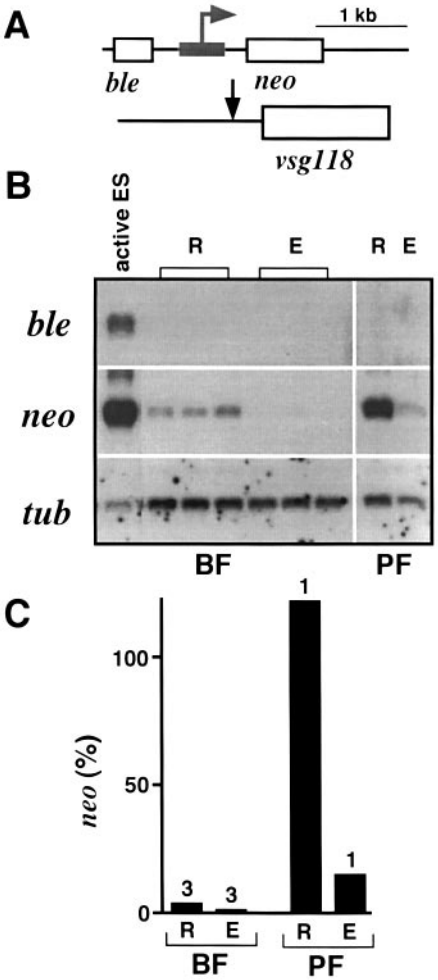

Fig. 5. Transcription repression at the non-telomeric *vsg118* locus. (**A**) Physical map of the *ble*–promoter–*neo* cassette and the integration site (vertical arrow). (**B**) A Northern blot was hybridized with the probes indicated to the left of each panel. BF, bloodstream forms; PF, procyclic forms; R, cassette with *rRNA* promoter; E, cassette with ES promoter. Cells with a cassette integrated in an active ES are shown as a positive control (active ES). (**C**) *neo* signals were quantified by phosphorimager analysis. The numbers above each bar represent the number of independent clones in each data set. Standard deviation was negligible in these samples. Symbols are as in (B).

Transcription is strongly repressed at a telomere-distal vsg locus

ES and *rRNA* promoters can be repressed at least 50 kbp from telomeres in bloodstream-form *T.brucei* (Rudenko *et al.*, 1995), but it is unclear if this repression is mediated by the telomere (see Figure 1A). Most silent *vsg*s are not located adjacent to telomeres. To test how promoters behave at a non-telomeric *vsg* locus, we generated three independent clones with an *rRNA* (R) promoter and three with an ES (E) promoter placed directly upstream of the non-telomeric copy of *vsg118* (Figure 5A).

Northern blotting (Figure 5B) with a *ble* probe indicated no detectable endogenous transcription at the *vsg118* locus and strong repression (more pronounced for the ES promoter) of both promoters in bloodstream forms. Differentiation to procyclic forms resulted in complete derepression of the *rRNA* promoter and some derepression of the ES promoter (Figure 5B and C).

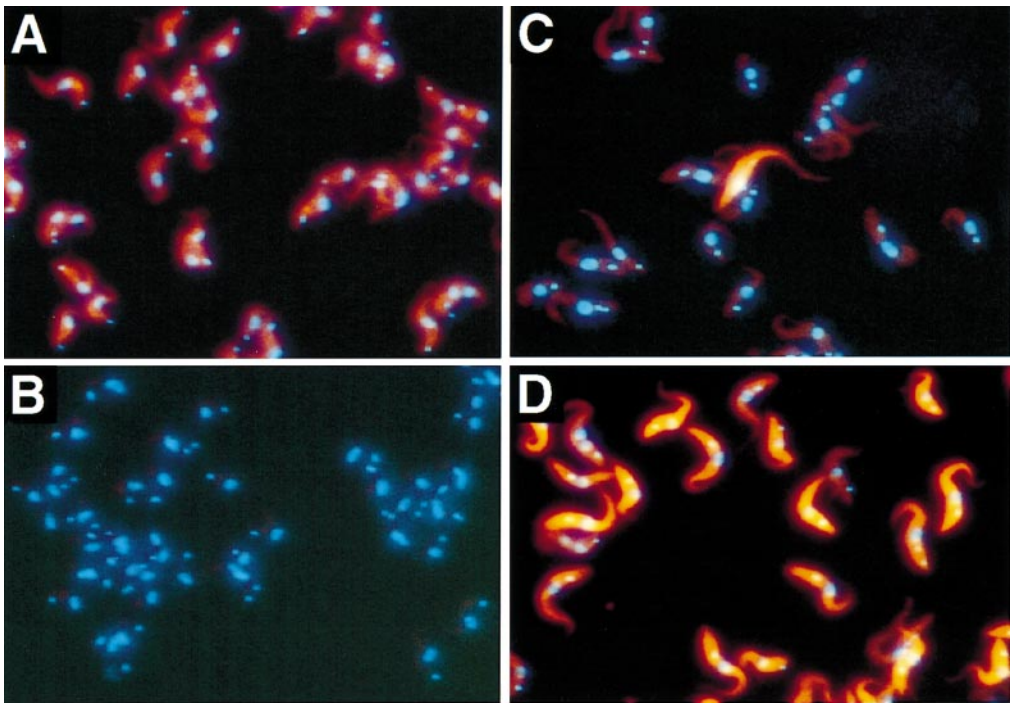

Fig. 6. Neo expression in the *vsg221* ES in individual cells. Rabbit anti-Neo primary antibody and rhodamine-conjugated goat anti-rabbit secondary antibody were used to detect Neo (red). DAPI (Sigma) was used to stain nuclear and mitochondrial DNA (blue). In bloodstream forms Neo expression was driven by the endogenous active ES promoter (**A**) or an ES promoter integrated at a repressed ES (**B**). In procyclic forms Neo expression was driven by an ES promoter within an ES (**C**) or an *rRNA* promoter within an ES (**D**).

Individual cell analysis

Northern analysis only allows us to determine the average amount of mRNA in a cell population. To identify possible differences between the activity of promoters in individual cells we carried out drug selection assays and immunofluorescence analysis with antibodies against Vsg221, Vsg118, Neo and Ble.

Bloodstream-form clones with *ble*–promoter–*neo* cassettes inserted into an active ES displayed EC_{50} values of 175 μg/ml phleomycin (selection for *ble* expression) (Horn and Cross, 1995). Growth in phleomycin, therefore, provided a sensitive phenotype assay for *ble* expression driven by endogenous transcription in individual cells. Testing bloodstream-form clones with cassettes inserted at the ribosomal spacer and at the non-telomeric *vsg118* locus, for *ble* expression (resistance to 1 μg/ml) yielded no resistant cells from populations of 10^7, except when using one of the clones with a cassette integrated at a ribosomal spacer. All clones with cassettes integrated within the repressed *vsg221* ES (with promoters directed towards the telomere) yielded resistant clones at a low frequency, representing a switch to the *vsg221* ES (Horn and Cross, 1997). Our results indicated that endogenous transcription was greatly reduced, relative to an active ES, in the vast majority of cells, at most ribosomal spacer loci, at the non-telomeric *vsg118* locus and within a repressed ES.

Immunofluorescence analysis indicated that all (>99% detection level) bloodstream forms expressed homogeneously high levels of Neo and Vsg221 when cassettes were inserted into an active ES (Figure 6A). In contrast, bloodstream forms with promoters inserted into the repressed *vsg221* ES efficiently silenced this locus. No (<0.1% detection level) cells expressed detectable Vsg221 and all (>99%) cells expressed homogeneously low levels of Neo (Figure 6B). Results obtained with procyclic forms were independent of whether the *vsg221* ES was previously active or inactive. The results, instead, were dependent upon the promoter that had been inserted. The majority of procyclic form cells with an ES promoter inserted in an ES, including cells in which transcription was directed away from the telomere, expressed low levels of Neo. However, a sub-population (<0.5%) of these cells expressed easily detectable Neo (Figure 6C). Such a sub-population was not detected in procyclic forms with an ES promoter at the non-telomeric *vsg118* locus. In contrast, procyclic forms with an inserted *rRNA* promoter displayed high but variable levels of Neo (Figure 6D). Of the clones in which Neo was easily detectable, bloodstream-form clones with promoters inserted at the *rRNA* spacer and procyclic-form clones with inserted *rRNA* promoters displayed variation of Neo expression between individual cells. Such heterogeneity was also observed for endogenous *rRNA* and *neo* mRNA transcribed at a *rRNA* locus, consistent with temporal activation of these genes during the cell cycle (Rudenko *et al.*, 1991). Vsg expressed in procyclic forms is efficiently shed into the medium (Bangs *et al.*, 1997), so Vsg can only be detected on a few cells in Vsg-expressing procyclic cultures (data not shown).

Discussion

In order to extend our understanding of how *vsg*s are regulated in bloodstream and procyclic *T.brucei* we have carried out a detailed comparison of *rRNA* and *vsg* ES promoter activities at multiple loci in both life cycle stages. Position-dependent and promoter-specific effects were demonstrated to quantitatively alter gene expression

Table I. Relative percentage of *neo* transcripts generated at different chromosomal loci

Locus	*rRNA* promoter		*vsg* ES promoter	
	BF	PF	BF	PF
Ribosomal spacer	[100]	[100]	10	4
Telomeric (*vsg221*, 0 kbp)	3	18	1	2
Chromosome internal *(vsg118*)	4	115	1	15

[100] indicates values assigned to these 'control' samples.
BF, bloodstream-form cells; PF, procyclic-form cells.

in *T.brucei* as measured by the amount of stable mRNA in cell populations (see Table I) and by drug-resistant phenotype and reporter protein levels in individual cells.

Position-dependent repression and vsg regulation

In other organisms position effects are mitotically heritable but reversible. Changes in state can occur in an epigenetic and stochastic manner, generating isogenic but phenotypically distinct populations of cells (Aparicio and Gottschling, 1994; Csink and Henikoff, 1996; Dernburg *et al.*, 1996). *vsg* ES switching appears to occur in an epigenetic and stochastic manner and changes in transcription status are usually co-ordinated at different loci to maintain mutually exclusive *vsg* expression (see Horn and Cross, 1997). As *vsg* ESs are invariably telomeric, this location is probably important for regulation.

Evidence from other organisms suggests that region-dependent or position-dependent gene repression reflects the packaging of such regions into a silent chromatin structure. Histone modification is thought to alter chromatin structure and compact chromatin or heterochromatin is thought to prevent the transcription machinery from effectively accessing its target sites (reviewed by Lohe and Hilliker, 1995; Loo and Rine, 1995; Felsenfeld, 1996; Wade *et al.*, 1997). These structures may be organized as functional domains (reviewed by Gerasimova and Corces, 1996) or topologically isolated loops (Cook, 1994) which may, in turn, be sequestered to compartments within the nucleus (reviewed by Strouboulis and Wolffe, 1996). Covalent DNA modification may also lead to gene repression (reviewed by Laird and Jaenisch, 1996). These mechanisms are not mutually exclusive, but in some cases it is unclear whether a particular phenomenon represents a cause or a consequence of transcription repression.

Telomere biology has been studied in some detail in yeast (reviewed by Zakian, 1996). Yeast telomeres repress transcription of genes located nearby and both the composition and orientation of DNA sequence located between the telomere and marker influence silencing (Gottschling *et al.*, 1990). In bloodstream-form *T.brucei* transcription repression was bi-directional at a telomere-proximal locus. Position-dependent repression in *T.brucei* was influenced by the distance between the promoter and the telomere. By analogy to similar results in yeast (Renauld *et al.*, 1993), these data are consistent with a model in which repressed domains in *T.brucei* are propagated along the DNA. In *T.brucei*, however, repression appears to be sustained over longer distances and does not appear to reflect the exponentially decreasing frequency of repression, which is thought to be related to the cooperative assembly of silent chromatin subunits in yeast. The repressive effect in *T.brucei* may originate at the telomere or at another conserved sequence associated with the *vsg* or the ES. Strong position effect repression is also seen at the endogenous *vsg* ES promoter locus, ~50 kb from the telomere (Rudenko *et al.*, 1995). Based on our results, which show that repression is progressively diminished further from the telomere, it seems that repression of endogenous *vsg* ES promoters is dependent upon an additional or separate mechanism. A conserved ES promoter-associated element(s) (see Figure 1A) may mediate transcription repression or, via a chromatin loop, the telomeric domain may associate with the other end of the ES. *vsg* ESs may constitute regulatory domains, flanked on one side by the telomere and upstream by 50 bp repeats, which may act as an insulator or boundary (see Gerasimova and Corces, 1996), preventing ES status from affecting other regions of the same chromosome.

Our observation of position-dependent repression at a non-telomeric *vsg* suggests that a *vsg*-associated sequence may initiate repression. Sub-telomeric repeats, 29 bp motifs or TTAGGG repeats, are not found at this locus (Liu *et al.*, 1983) but conserved octamer and hexadecamer sequences are found 3′ of all *vsg*s (Majumder *et al.*, 1981). Experiments are underway to test the ability of this sequences to influence local transcription. Another ES-associated sequence, possibly the telomere, may be necessary to act as an enhancer and overcome repression. This could explain why active *vsg*s are invariably found at telomeres and is compatible with the fact that telomere tract length variation has no significant effect on position effect repression in *T.brucei*. The proximity (Bayne *et al.*, 1994) or the length (Sprung *et al.*, 1996) of the same telomeric repeat sequence (TTAGGG) in human cells mediates no significant position effect repression, whereas in yeast telomere-mediated transcription repression is highly sensitive to telomere tract length variation (Kyrion *et al.*, 1993). It is unclear whether the novel *T.brucei* telomeric DNA modification, which is found in bloodstream forms but not in procyclic forms (Gommers-Ampt *et al.*, 1993), is involved in ES regulation. In these studies we observe transcription repression at a chromosome-internal *vsg* locus where modified DNA is undetectable (see Table I).

Differentiation of *T.brucei* from bloodstream to procyclic forms demonstrates developmental regulation of position effects at multiple loci. It appears, from our results, that multiple loci, possibly all those containing *vsg*s, become available for transcription by RNA polymerase I in procyclic trypanosomes. This indicates a global change in chromatin structure and is consistent with the relative instability of procyclic-form chromatin (reviewed by Hecker *et al.*, 1995), suggesting that a specific repressive machinery exists in bloodstream forms and that some chromatin components are developmentally regulated.

Repression of the vsg ES promoter in procyclic forms

The *vsg* ES promoter can drive high levels of expression following transient transfection in both bloodstream and procyclic-form cells (Jefferies *et al.*, 1991; Zomerdijk *et al.*, 1991). Levels of activity are similar to those from

the *rRNA* or *parp* promoters (Zomerdijk *et al.*, 1991). The *vsg* ES promoter is specifically repressed in chromatin however. Repression in bloodstream forms (Jefferies *et al.*, 1993; Horn and Cross, 1995; Rudenko *et al.*, 1995), where a single *vsg* is expressed, and in procyclic forms (Jefferies *et al.*, 1993; Zomerdijk *et al.*, 1993; Rudenko *et al.*, 1994; Urményi and Van der Ploeg, 1995; Pham *et al.*, 1996; Qi *et al.*, 1996), where all *vsg*s are normally silent, is affected by both the genomic context and the DNA sequence of the promoter. We have extended these studies by showing that the *vsg* ES promoter is repressed by a bi-birectional promoter-non-specific position effect at multiple loci in bloodstream-form cells (Table I). Although the position effect is relieved in procyclic forms, the *vsg* ES promoter remains repressed in the majority of cells.

A hybrid *vsg* ES promoter with an upstream control element (UCE), derived from a *parp* promoter, placed upstream of the core region was 3-fold more active than a wild-type promoter in the context of an expression site in procyclic-form cells. At another locus the hybrid promoter appeared to be 6- to 10-fold more active (Qi *et al.*, 1996). The UCE present in the *rRNA* promoter may allow that promoter to be more active in procyclic-form cells in the context of a more relaxed chromatin structure (see above). In bloodstream-form cells the position effect represses transcription of promoters containing or lacking a UCE (Horn and Cross, 1995; Rudenko *et al.*, 1995; the present work).

Previous studies on the relative activity of different endogenous *vsg* ES promoters in procyclic-form cells have yielded contrasting interpretations. It was suggested that the *vsg* ES promoter active in bloodstream forms remained constitutively active in procyclic forms (Pays *et al.*, 1990). Transcription attenuation subsequently prevents transcription of the entire ES. A later paper established that many, or all, expression site promoters were simultaneously active at a low level in procyclic form cells (Rudenko *et al.*, 1994). Neither of these studies distinguished between individual cells however. Our analysis of individual cells shows that an ectopic *vsg* ES promoter is active at a high level in ~0.5% of procyclic-form cells. This result shows that even a promoter lacking a UCE can be highly active in chromatin in procyclic forms, suggesting that a single, but variable, telomeric locus may be activated in each procyclic-form cell and that the regulatory mechanism, which differs between active and inactive expression sites in bloodstream forms, may still be present in procyclic forms. Alternatively, these cells may represent an advanced developmental stage-specific phenotype and the ectopic promoter may reflect a phenotype normally observed at metacyclic *vsg* loci.

Conclusions

The strong repression we observed on promoters inserted immediately upstream of the telomeric *vsg* could provide the mechanism to silence the metacyclic *vsg* promoters, found ~2 kbp upstream of telomeric *vsg*s in bloodstream trypanosomes. Silencing of bloodstream-form *vsg* ES promoters, found 30–50 kbp from telomeres and *vsg*s may depend upon, or be supplemented by, additional mechanisms. We also observed ES promoter derepression in a sub-population (<0.5%) of procyclic-form cultures, but only when the promoter was inserted within an expression site. Since we only marked one of the myriad telomeres, this phenomenon may represent mutually exclusive transcription and rapid switching between telomeres at this stage of the life cycle or, alternatively, a further developmental transition in these cells. Further studies will be required to determine how these phenomena reflect the mechanisms regulating *vsg* expression and to clarify the interplay between different factors and promoters.

Materials and methods

Plasmid constructs

The target for the non-transcribed *rRNA* gene spacer has been described previously (Wirtz and Clayton, 1995). The *vsg221* ES, –14 kbp target was derived from pTg221.8 (Kooter and Borst, 1984) as a *Cla*I–*Eco*RI fragment and cloned into pBS SK+II, also digested with *Cla*I and *Eco*RI. A region at the 5′-end was removed by *Sac*I digestion and religation. The *vsg221* ES, –5 kbp target was derived from pBΨANA5′ (V.B.Carruthers and G.A.M.Cross, unpublished data) by removing a *neo* cassette and another *vsg221* ES sequence. The *vsg221* ES, +2 kbp target was cloned by constructing a genomic library of *Eco*RI and *Nar*I-digested fragments from 221a cells cloned into pBS SK+II digested with *Eco*RI and *Cla*I. The library was screened with a probe derived from the 5′-end of *vsg221* and the resulting positive clone was determined to be unrearranged by comparing its size with the same fragment in 221a genomic DNA (data not shown). Most of the *vsg221* gene was subsequently removed by digestion with *Bst*XI and religation. The *vsg118* target was generated using primers with homology to a region 5′ of *vsg118* (5′-primer CCCTCGAGAGAAGCGCCAATACAAC) and the 5′-end of *vsg118* (3′-primer GTTCTAGAATCTTCGTTATTTTGAGCT) (see Liu *et al.*, 1983) as well as convenient restriction enzyme sites (underlined). PCR was carried out using 100 ng genomic DNA, derived from 221a cells, per 50 μl sample with the Repli-pack Reagent set (Boehringer Mannheim). Five cycles were carried out at 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, followed by 15 cycles at 94°C for 30 s, 65°C for 30 s and 72°C for 1 min. Products were subsequently cloned using a TA Cloning Kit (Invitrogen). Two *Pst*I sites were removed from the vector by *Xba*I digestion and religation followed by *Sty*I digestion and religation.

The *ble–rRNA* promoter–*neo* (bRn) cassette (see Horn and Cross, 1995) was cloned: into the *Eco*RV site in the non-transcribed *rRNA* gene spacer target to generate pbRn1; into the *Pfl*MI site in the *vsg221* ES, −14 kbp target to generate pbRn3; into the *Pst*I site in the *vsg221* ES, −5 kbp target to generate pbRn4; into the *Eco*RI site of the *vsg221* target (see Horn and Cross, 1995) in inverse orientation to generate pbRn5<; into the *Xba*I site in the *vsg221* ES, +2 kbp target to generate pbRn6; into the *Pst*I site in the *vsg118* target to generate pbRn7. The *ble*–ES promoter–*neo* (bEn) cassette was derived from pbEn (see Horn and Cross, 1995) and was cloned: into similar sites in the non-transcribed *rRNA* gene spacer target to generate pbEn1; into the *vsg221* ES, −5 kbp target to generate pbEn4; into the *vsg221* target in inverse orientation to generate pbEn5<; into the *vsg221* ES, +2 kbp target to generate pbEn6; into the *vsg118* target to generate pbEn7.

Cells

Maintenance of *T.brucei* MiTat 427 bloodstream-form clones 118a and 221a and differentiation to procyclic forms have been described previously (Horn and Cross, 1995). Procyclic form cultures were established for at least 1 week prior to analysis. Bloodstream-form *T.brucei* were transformed as previously described (Carruthers *et al.*, 1993) except that cells were grown *in vitro* prior to electroporation and Cytomix (van den Hoff *et al.*, 1992) replaced ZPFMG. Clonal cell lines were derived from drug-resistant cultures by dilution (drug selection was removed at this point). Cells were diluted to a concentration of 0.3 cells/ml and were distributed in 1 ml aliquots in 24-well plates. Cloning efficiency was typically between 50 and 100%.

DNA, RNA and protein analysis

Southern, Northern and Western blotting; phosphorimager analysis and immunofluorescence analysis were carried out as previously described (Horn and Cross, 1995). Pulsed field gel electrophoresis, for separation of *T.brucei* chromosomes, was carried out as previously described (Horn and Cross, 1997). In all cases in which the map of the target locus was

known each clone was determined, by Southern blotting, to have a single cassette integrated at the expected locus in the desired orientation. In the case of the *rRNA* spacer clones we determined the chromosomal location of each cassette by pulsed field gel electrophoresis followed by Southern blotting.

Probes used for Southern and Northern analysis were the entire coding regions from *ble* and *neo*, 5′-end probes from the *vsg*s (see Horn and Cross, 1997) and the *tub* probe was a 760 bp *Eco*RI–*Hin*dIII coding region fragment from pBT100 (P.Hevezi and G.A.M.Cross, unpublished results). All Filters were washed in 0.2× SSC and 0.2% SDS at 65°C.

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