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In situ analysis of a variant surface glycoprotein expression-site promoter region in *Trypanosoma brucei*

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Abstract

In *Trypanosoma brucei*, the active variant surface glycoprotein genes (*vsg*) are located at telomeric expression sites (ES), whose expression is highly regulated during the life cycle. In the procyclic form, all ESs are repressed. In the bloodstream form, where antigenic variation occurs, only one of approximately 20 ESs is active at a given time. We have investigated chromatin structure and DNA sequence around the ES promoter to identify *cis*-acting regulatory regions. A marker gene, inserted 1 kb downstream of the ES promoter, was used as a specific probe to map the position of nuclease hypersensitive sites. A prominent hypersensitive site was detected within the core promoter. This site was present in both active and inactive ES promoters, suggesting that a protein complex is bound to the promoter irrespective of its transcriptional state. However, none of the regions showed differential nuclease sensitivity between active and inactive transcriptional states. A systematic deletion analysis of the sequences surrounding the active ES promoter in situ confirmed the absence of *cis*-regulatory elements. We find that only 70 bp within the ES promoter are necessary to support ES regulation. Analysis of the reporter activities in an inactive bloodstream-form ES revealed the existence of an intermediate promoter activity in some clones, but we never observed full activation of more than one ES. The *vsg* mRNA from this intermediate ES was expressed less efficiently.

Keywords: *Trypanosoma brucei*; Antigenic variation; Variant surface glycoprotein expression site; Expression site promoter; Nuclease hypersensitive sites

1. Introduction

Antigenic variation in African trypanosomes provides an interesting model system to study the regulation of gene expression in a 'primitive' eukaryote. The expressed variant surface glyco-

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protein gene (*vsg*) is located at the end of a polycistronic telomeric expression site (ES) [1,2], whose expression is remarkably regulated during the trypanosome life cycle (reviewed in Refs [3–5]). In the procyclic form, an insect stage of the parasite, all ESs are repressed. In the bloodstream form, where antigenic variation occurs, only one of many ESs is transcribed at a given time, resulting in the expression on the surface of a dense coat of a single Vsg [6]. Periodic *vsg* switching allows the parasite to escape the host immune response. This variation in Vsg expression can be achieved by replacing the resident *vsg* in an active ES. Alternatively, a switch can also occur by activation of another ES and simultaneous inactivation of the previously active one [7]. The ES promoter is located 40–60 kb upstream of the telomeric *vsg* [8,9] with a 90% sequence identity among ESs [10–12]. Hybridization analysis suggests the presence of approximately 20 ESs [13,14] and about 30 ES promoters [12,15]. ES transcription is driven by a polymerase having several characteristics of RNA polymerase I [8,16–18]. No sequence alterations within the promoter region have been detected that correlate with an in situ switch [10–12]. Although occasional DNA rearrangements have been described in the region upstream of the promoter, they are not necessary for ES activation or inactivation [12,15].

The position of nuclease hypersensitive sites has often been correlated with the presence of *cis*-acting regulatory DNA elements in other organisms (for review see [19]). The existence of sites showing preferential digestion with nuclease around the ES promoter might provide indications of *cis*-acting regulatory sequences. Earlier studies have shown an increased nuclease sensitivity of the chromatin in an expressed *vsg* gene using pancreatic DNase-I treatment [2]. The active *vsg* was preferentially digested by single-strand-specific endonucleases, though there was no difference in the nucleosome pattern of active or inactive telomeric *vsgs* when treated with micrococcal nuclease [20]. Previous work [21,22] has identified DNA elements named box 1 (-60) and 2 (-35), and the start site, as the only sequences necessary for ES-promoter activity in transient assays. In this study, the possible existence of other *cis*-acting regulatory sequences that may only work in situ, within the chromosomal context, is investigated. A marker gene that was previously inserted downstream of the ES promoter [15] was used as a specific probe to analyze nuclease hypersensitive sites, overcoming the problems associated with the high sequence similarity among ESs [23–26]. To monitor ES promoter regulation, a luciferase gene was inserted downstream of the promoter. This study will show an extensive in situ deletion analysis of the ES promoter region. After obtaining variants that have switched off the tagged ES, some clones were found that retained previously undescribed intermediate ES-promoter activities in the bloodstream form.

2. Materials and methods

2.1. Trypanosomes and switching experiments

Trypanosoma brucei clone 221a (Molteno Institute Trypanozoon antigenic type 1.2 (MITat 1.2) clone 221a) was derived from strain 427 [6]. The *neo* cassette was inserted into an inactive expression site (121 ES), on a 2.2-Mb chromosome [27–29], which contains tandem promoters located 50 kb upstream of the *vsg*121 [30]. The tandem promoter was deleted when the 121 ES was activated [15]. Trypanosomes were routinely cultured in HMI-9 at 37°C [31]. All of the cell populations used in this study were cloned by limiting dilution in vitro. The homogeneity of trypanosome populations (based on counts of approximately 500 parasites) was verified by indirect immunofluorescence using specific antibodies against Vsg121 and Vsg221, together with DAPI as a nuclear stain. Switching experiments were carried out as previously described [32], except that female Swiss white mice were immunized with 50 μg of purified Vsg121 in Freund's adjuvant (Sigma), injected twice in 3 weeks before intraperitoneal inoculation of 10^6 Vsg121-expressing trypanosomes. After 24 h, when the parasitemia was not detected, the mouse blood was isolated and cloned in 96-well microtiter plates in three serial dilutions of the blood, in medium

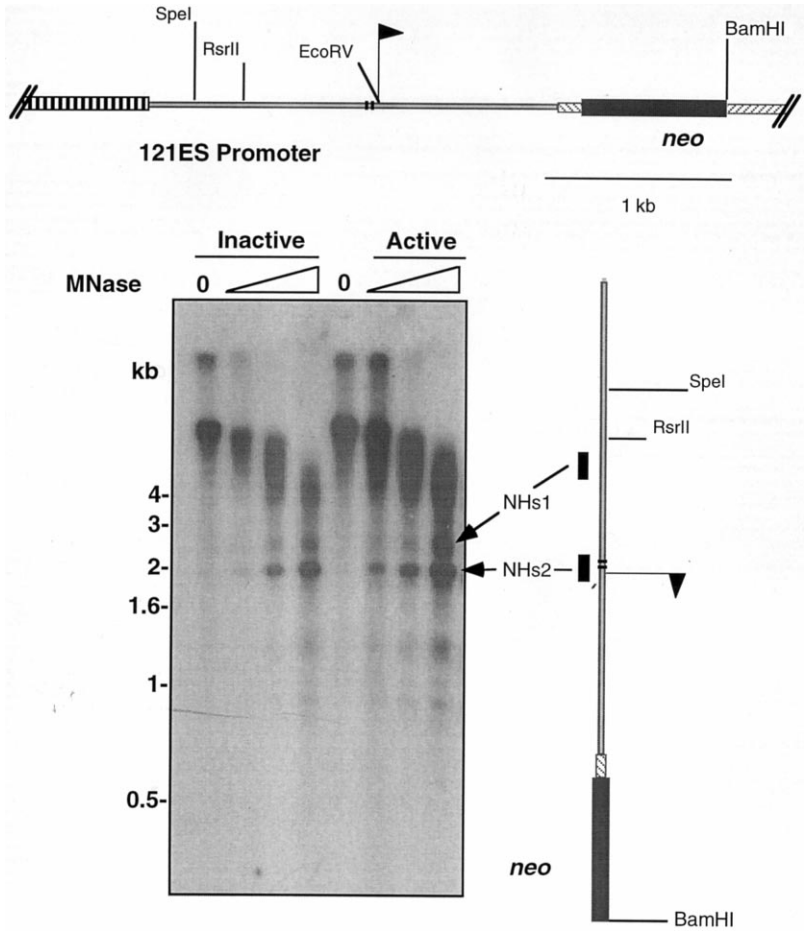

Fig. 1. Nuclease hypersensitive sites in active and inactive ES core promoters. After progressive treatment of the chromatin with micrococcal nuclease, DNA was isolated, digested with *Bam*HI and separated on an agarose gel. The neo marker gene, inserted 1 kb downstream of the 121 ES promoter, was used as probe. The locations of nuclease hypersensitive sites (NHs) in the ES promoter region are indicated by arrows.

without drug. Antigenic variation may occur by gene conversion of different *vsg* genes into a still active ES. Therefore, only clones showing sensitivity to high levels (50 μg ml^{-1}) of phleomycin and no expression of Vsg121 were considered to have inactivation of the target 121 ES.

2.2. Plasmid constructions and transformation conditions

The 121 promoter ES-targeting sequences were derived from a 3.2-kb *Spe*I–*Bam*HI genomic clone described previously [15]. The *Sma*I–*Pst*I fragment from pLew46 containing the luciferase gene flanked by *T. brucei* actin splice acceptor and polyadenylation sequences [33] was ligated into the *Stu*I–*Pst*I fragment containing Bluescript (Stratagene) and the *Spe*I–*Stu*I 1.9 kb fragment of the 121 ES promoter region. This construct was used to generate the first six deletions described in Fig. 3 by double digestion with the indicated restriction enzymes followed by filling or trimming of 5′ and 3′ overhanging with Klenow or T4 DNA polymerase, respectively. The *Sac*I fragments containing the deletions and the luciferase gene (with the actin splice acceptor) were ligated to a *Sac*I fragment from pLew20 (closely related to pDH430 [34] in which T7 pro-

moter is deleted) containing pGEM4, the *Streptoalloteichus hindustanus* bleomycin resistance gene (*ble*) flanked by *T. brucei* actin splice acceptor and polyadenylation sequences and a truncated version of the aldolase polyadenylation sequence that does not confer developmental regulation [35]. Deletions #7, 8, 9 were generated by polymerase chain reaction (PCR). Deletion #7 was obtained by PCR using the oligonucleotide (5′GGAATGTTGGTGATTTGTTCG3′) (ESP-875) priming upstream of the ES promoter and (5′GGGCCCCTCGAGCACCGTGAGTCTGATATC3′) (ESP + 8). Deletion #8 was obtained by PCR using the oligonucleotide ESP–875 priming upstream of the ES promoter and (5′GGGCCCCTCGAGTATATTATGAGTCTGATATCTGAGAT3′) (ESP + 3). Deletion #9 was obtained by PCR using the oligonucleotide (5′GCGATCCCCGGGCCGGCGCCCCTATTACCTCACCAT3′) (ESP–65) priming upstream of the ES promoter and the oligonucleotide ESP + 8. All the PCR products obtained using 3.2-kb *Spe*I–*Bam*HI ES promoter genomic clone as a template were digested with *Hpa*I and *Xho*I (for #9 was *Sma*I and *Xho*I) and ligated to the ES promoter luciferase-bleomycin cassette, described above and digested with the same restriction enzymes. All constructs were sequenced at The Rockefeller University Shared Technology Resource Center, from which synthetic oligonucleotides were also obtained. Plasmid DNA for transfection was prepared using anion exchange columns (Qiagen). To remove vector sequence from the targeting cassettes, the plasmid constructs were digested with *Spe*I and *Ase*I prior to electroporation. Trypanosomes were grown in HMI-9 at 37°C and transformed in Cytomix [36] with 10 μg of linearized plasmid DNA in each cuvette. After electroporation surviving cells were counted and distributed in a 24-well plate (1–4×10^4 cells per well). The next day, one volume of 2 μg ml^{-1} Phleomycin (Cayla, Toulouse, France)-containing medium was added to the wells. Clonal cell lines were derived directly from drug-resistant positive wells (approximately 10% of the wells). Cultures were subsequently grown in the absence of drug selection unless otherwise indicated.

2.3. Cell permeabilization and micrococcal nuclease treatment

Trypanosomes were washed and resuspended to a cell density of 2×10^8 ml^{-1} in permeabilization buffer (100 mM KCl; 10 mM Tris pH 8; 25 mM EDTA; 1 mM DTT). Digitonin (Calbiochem) was added to 40 μM and incubated for 3 min at room temperature. After this treatment, cells were washed in cold isotonic buffer (100 mM KCl; 10 mM Tris pH 8; 10 mM $CaCl_2$; 5% glycerol; 1 mM DTT; 1 mM PMSF). After resuspension in isotonic buffer, increasing amounts of micrococcal nuclease were added and incubated for 20 min at room temperature. DNA was isolated and digested with *Bam*HI before loading on agarose gels.

2.4. RNA analysis and probes

RNA was isolated using RNAstat60 (Tel-Test). Northern analysis was performed using 4 μg RNA/lane in formaldehyde agarose gel [37], which were transferred to Hybond membrane and cross-linked to the membrane in a UV Stratalinker 1800 (Stratagene). All the restriction fragments used as a probes were described previously [15], except for the luciferase probe that was obtained by digestion of the plasmid described above with *Hin*DIII and *Bam*HI: all DNA probes were purified from a gel. Probes were generated using the Prime-It II labeling kit (Stratagene) with Prep-A-Gene (Bio-Rad) gel purified DNA. All hybridization and washing of filters was at 65°C in a hybridization oven (Hybaid). Post-hybridization washes were for 30 min at 65°C in $0.2 \times$ SSC and 0.1% SDS. RNA size was estimated according to the migration of an RNA ladder (Gibco-BRL). After hybridization, the probes were stripped as recommended by the manufacture (Amersham). Hybridization signals were quantified using a Phosphorimager and Imagequant software (Molecular Dynamics).

3. Results

3.1. Chromatin alterations within the ES promoter region

Many studies have correlated the position of nuclease-hypersensitive sites in chromatin with the presence of regulatory elements close to promoters. This study was concerned with investigating putative chromatin alterations around the ES promoter that could be associated with *cis*-acting regulatory sequences. The presence of more than 20 ES promoter regions with almost identical sequences has complicated this analysis, since hybridization studies are not able to discriminate between the unique active ES and all the inactive ESs. DNA transformation techniques in the bloodstream form *T. brucei* [38], allowed us to target a specific region of an ES in the bloodstream form with drug-resistance genes [15,39–41]. Previously, we have described the insertion of the neomycin phosphotransferase gene (*neo*) cassette into the 121 ES [15]. Immune selection in rats allowed us to isolate another variant in which the 121 ES was inactive without detectable DNA rearrangements. This *neo* gene, inserted downstream of the ES promoter in these two clones, was used as a specific probe to map the position of hypersensitive sites by an undirected end-labeling technique [19].

To perform a chromatin sensitivity assay, we used whole cells permeabilized with Digitonin in the presence of a protease inhibitor in an isotonic buffer (Section 2). The permeabilization of the cell membrane allows the diffusion of nucleases and should approximate the in vivo situation of chromatin in the nucleus. Permeabilized cells were treated with increasing concentrations of micrococcal nuclease. The DNA from these cells was isolated and digested with *Bam*HI which produced a 50 kb fragment containing the *neo* gene at the 3′ end. After size separation in an agarose gel, the DNA was transferred to a membrane and hybridized with *neo* coding region as a probe (Fig. 1). A major hypersensitive site was detected that mapped at around 2 kb, within the core promoter located 1930 bp from the *Bam*HI site. Interestingly, this hypersensitive site was present in both active and inactive ES promoters. This site was also detected using DNase I, but the result is less clear due to the presence of high level of internal nucleases in trypanosome cells. Another less intense hypersensitive site was detected approximately 2.6 kb from the *Bam*HI restriction site (Fig. 1). However, neither of the regions showed any differential nuclease sensitivity between active and inactive transcriptional states.

Despite the absence of differences between both transcriptional states on the ES promoter region, we decided to investigate the effect of deleting sequences around the promoter on ES regulation. The DNA sequences upstream and downstream of the ES share a nucleotide identity of 90% among different ESs [8,14,42]. The presence of these highly conserved sequences around the ES promoter suggested that this region may play a role in either the control of transcription, switching between ES, or developmental down-regulation in the procyclic form.

3.2. Reporter activities in different ES transcriptional states

Firstly, we were interested in monitoring the activity of the ES promoter throughout the three transcriptional states using a single clonal cell line. Luciferase was used as a reporter, because of its high sensitivity. Thus, the neo cassette in the active 121 ES was replaced with a cassette containing the luciferase reporter gene flanked by *T. brucei* actin splice-acceptor sequences, to provide equivalent post-transcriptional processing in both developmental stages [33], and a bleomycin gene as selectable marker. From these clones, we obtained antigenic switches in which the 121 ES was inactivated. Switches were obtained by immune selection against Vsg121 in vivo, as described in Section 2. The activity of a luciferase reporter gene inserted 1 kb downstream of the ES promoter increased by three orders of magnitude in the active versus inactive 121 ES in bloodstream form (Fig. 2). Procyclic forms were assayed for luciferase activity at least 2 weeks after differentiation. The luciferase activities were about a hundred-fold lower than an active ES in bloodstream forms, regardless of whether the procyclic lines

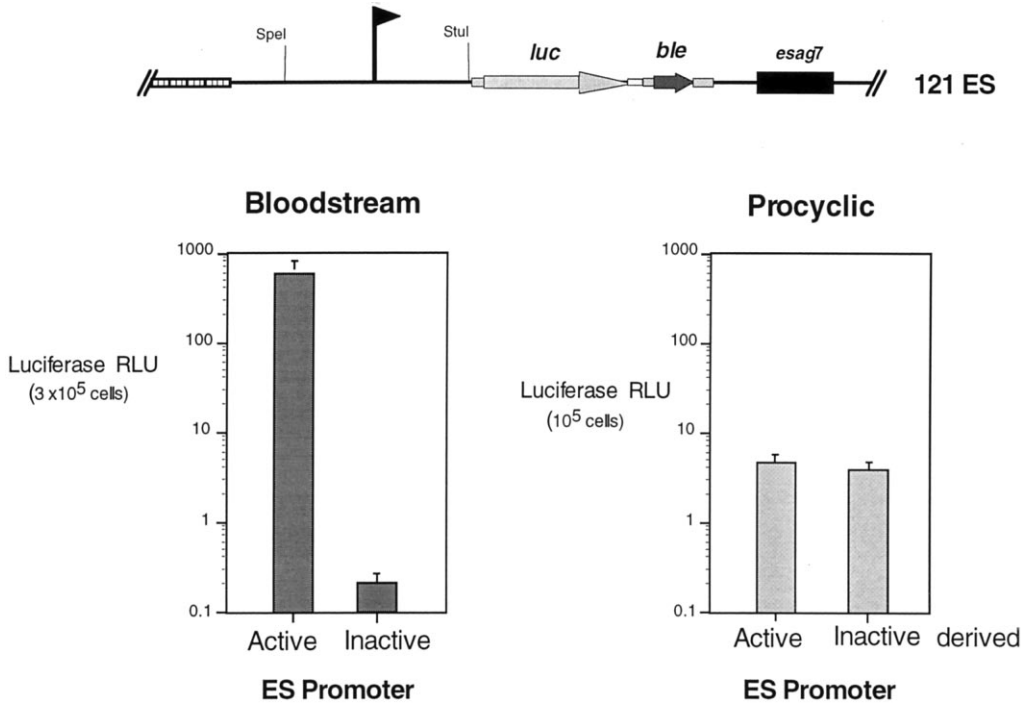

Fig. 2. Luciferase activity in different transcriptional states. Values are means of five luciferase assays on each of two independent transformants in which the *luc–ble* cassette was inserted into the active 121 ES. These clones were immune selected in vivo against Vsg121, and values represent the mean of the inactive state. The clones in both transcriptional states were differentiated to procyclic forms and the mean obtained from three independent differentiation experiments is shown in the right panel. The relative light units (RLU) in an inactive ES in bloodstream form were 0.2–0.5 RLU, which is 50- or 100-fold higher than background levels from a wild type cell lysate. The enzymatic activity of a commercial luciferase protein (Boehringer) showed to be two or three fold more active in a procyclic lysate than in a bloodstream form (Claudia Ochatt unpublished results), thus, we used three times less procyclic cells to reduce at least one of the parameters that differ between these two developmental stages.

were derived from bloodstream forms in which the 121 ES was active or inactive.

3.3. Deletions of the ES promoter region in situ

Although the flanking sequences are not necessary for the activity of the ES promoter in transient assays [14,43], they might play a regulatory role in the native chromosomal context. The 1.96-kb *Spe*I–*Stu*I DNA fragment, containing 1 kb upstream and 962 bp downstream of the 121 ES promoter region, was cloned upstream of a luciferase reporter gene (Fig. 3). The plasmid containing this sequence was used to generate deletions by digesting with restriction enzymes (Fig. 3). A second set of deletions, flanking the core promoter box 1 and 2 sequences identified by transient assays [21], was generated by PCR (Section 2).

As a first step to analyze the effects of these deletions upon the transcriptional activity of the ES promoter, a series of transient transfections were carried out in bloodstream and procyclic forms. The luciferase activity was measured 24 h after electroporation with equimolar amounts of each plasmid DNA. The luciferase activity of the different constructs did not show significant differences in either developmental stage (data not shown), as previously reported [14,43], confirming that a chromosomal context is necessary for developmental control of the ES promoter.

To obtain stable transformants lacking these ES promoter sequences, constructs containing the phleomycin-resistance gene (*ble*) and 300 bp of

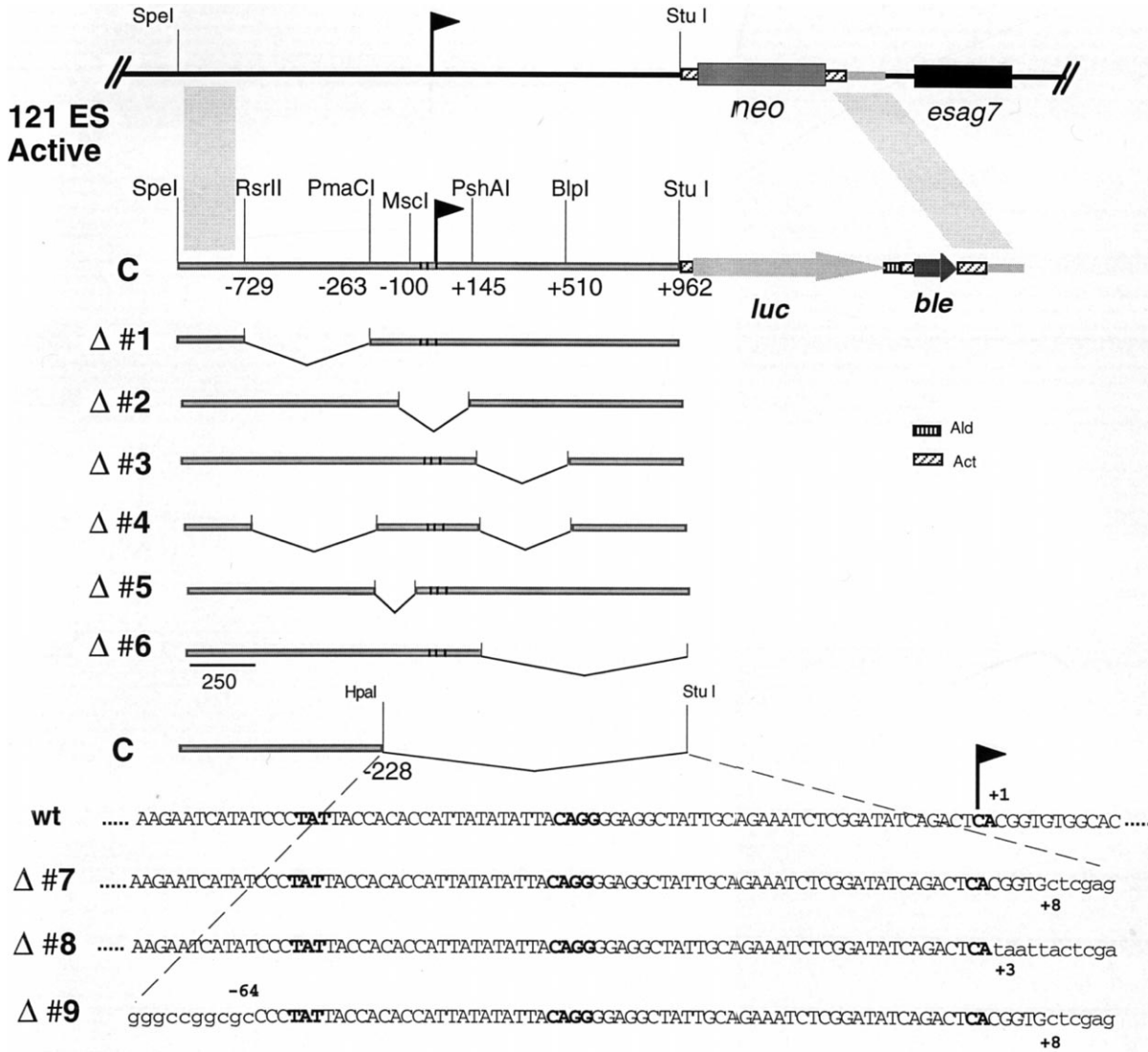

Fig. 3. Deletion analysis of the ES promoter region in situ. The ES promoter region is shown with the restriction sites used to generate the six first deletions, represented by thin v-shaped lines. Deletions 7 and 8 were obtained by PCR of the ES promoter sequences shown, which were ligated immediately upstream of the *luc–ble* cassette without any upstream deletion. Deletion # 9 is like D # 7, but the sequences upstream of the position −64 from the start site were changed to the sequences indicated in small letters. The sequence changes are indicated in lower case in comparison to the wild type sequence shown in upper case. The numbers indicate the position with respect to the start site considered here as the A, marked with a flag. Bold letters indicate boxes 1 and 2, and the transcription start site [21].

pGEM downstream of the luciferase gene were targeted to the 121 ES containing the *neo* cassette. The presence of the pGEM fragment, downstream of the *neo* cassette, provides a unique target sequence that, together with the 5′ region of the ES promoter (sequences downstream of *Spe*I site), will produce the desired replacement (Fig. 3). Recombinant clones could be tested for their sensitivity to G418 before performing DNA analysis. We selected transformants at a low phleomycin concentration (1 μg ml^{-1}), to be able to obtain resistant clones even if the target ES would have

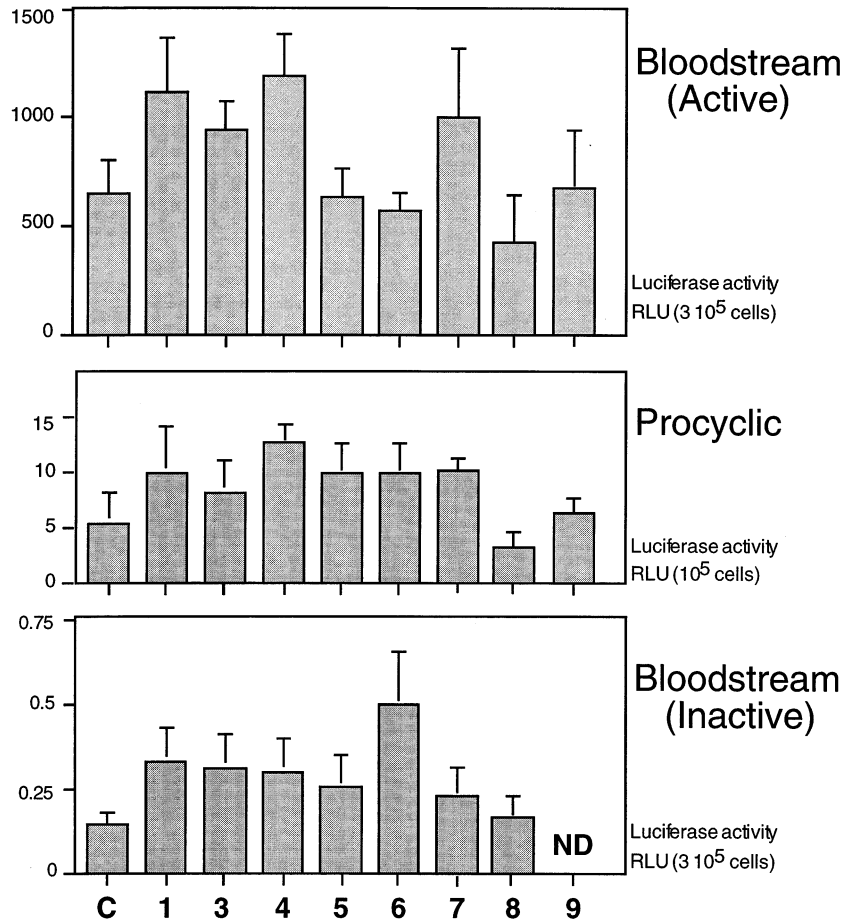

Fig. 4. Reporter activities of the deletion series in stable transformants. The luciferase activity for each deletion is shown on the right. The values were plotted as means of two independent clones (except deletions # 5 and # 8 for which only one clone was obtained) and measured at least three times. Error bars indicate the standard deviation of all measurements for each deletion. The clones containing the deletions described in Fig. 1 were differentiated to procyclic forms as described before [39]. After 2 weeks of differentiation, they were assayed for luciferase activity. The differentiation was done independently two times for each clone and luciferase measured two times. The reporter activities of one clone containing each of the deletions after ES-inactivation, in bloodstream forms, were measured three times.

switched off. Several independent electroporations were carried out, with the deletion series and with the control containing the entire unmodified ES promoter sequences. As expected, no phleomycin-resistant clone was obtained using Δ # 2, which lacks the ES promoter. The transformant clones were analyzed by Southern analysis using the luciferase coding region and *neo* as probes, demonstrating that the *neo* target had been replaced by the new *luc–ble* cassette (data not shown). Digestion with two restriction enzymes, whose sites flank the *luc* cassette and the *Spe*I site in the ES, was performed to determine if the deletions were properly generated. The size of the double digested fragments confirmed that the intended deletion was incorporated (data not shown).

The transformant clones, in which the deletions were successfully generated, were analyzed by indirect immunofluorescence using a specific antibody against Vsg121, together with DAPI as a nuclear stain. For every trypanosome clone, at

least 99% of the cell population expressed Vsg121. To analyze the expression levels of the reporter gene in these transformants, three independent luciferase assays were carried out (Fig. 4). No dramatic differences were detected. A 2-fold increase was seen in the clones with the deletions #1, #4, and #7. The rest of the clones exhibited expression levels similar to the control. To confirm this result, the *luc* mRNA levels were quantified using a phosphorimager and normalized to tubulin in Northern blots. The *luc* mRNA abundance correlated with the previously determined luciferase enzymatic activities (data not shown). Therefore, none of these deletions significantly affects ES promoter activity when the ES is actively transcribed. Since the bloodstream-form ES undergoes a developmental down-regulation when cells are differentiated to the procyclic form, we investigated whether these deletions affected developmental repression of the ES promoter. One clone containing each deletion described above was simultaneously differentiated to the procyclic form. The procyclic forms were assayed for luciferase activity 2 weeks after differentiation. When compared to the activities of the control clones, the deletion series showed no significant differences (Fig. 4). We also analyzed the reporter activity during the process of differentiation at days 5 and 10 post-induction, without detecting differences in the developmentally regulated repression of the ES promoter in clones lacking these sequences.

Clones in which the 121 ES was switched off were selected in Vsg121-immunized mice and assayed for luciferase activity. No significant differences were detected compared to the control (Fig. 4). We also analyzed whether the first set of deletions (Δ# 1, 3, 4, 6) affected the switching frequency. We selected 121 ES in situ re-activation using phleomycin (50 μg ml^{-1}). We calculated the frequency of in situ activation of the 121 ES containing these deletions versus the control. No significant differences were detected in the activation frequency, which fell in the expected range of 2.3×10^{-6} to 8×10^{-7}. DNA from one clone containing each deletion was analyzed after the consecutive switch off and on. No rearrangement was detected at the promoter region (data not shown).

3.4. Inactive ES showing intermediate promoter activities

While analyzing numerous clones that had switched off the 121 ES and were sensitive to 50 μg ml^{-1} of phleomycin, we obtained two switched clones from the control and some clones containing deletions that gave intermediate luciferase activities about 100-fold higher than a totally inactive ES. These clones did not express enough Vsg121 to be detected in vivo by 121 antibodies in mice or in vitro by immunofluorescence (based on counts of approximately 500 parasites). Chromosome localization of the luciferase gene as well as long range restriction mapping failed to identify any rearrangements or duplications of the cassette (data not shown). The steady-state luciferase and *vsg* mRNAs in these clones were analyzed (Fig. 5). Quantitation of the signals showed a correlation of mRNA and luciferase activities in the intermediate versus totally active ES. When *vsg*121 linked to the *luc-ble* cassette was used as a probe, a band was detected only in switched clones that showed intermediate luciferase activity. However, all the switches derived from 0R7 showed a strong *vsg*221 signal representative of full 221 ES activation. While 0R7s2 and 0R7s4 have properly activated the 221 ES, these clones show residual transcription of the 121 ES. This situation seems to be very stable, since the luciferase activity remained constant during 4 weeks of periodic measurements. To exclude the possibility that this intermediate activity could have been the result of a low proportion of cells that had re-activated the target ES, we recloned 0R7s2 and analyzed the luciferase activities of several clones after a short time. These clones gave the same luciferase activities as before (between 20 and 40 RLU). Since these activities might represent an intermediate step in the activation process, we measured the activation frequency of the 121 ES in these clones. We found no difference in the frequency of 121 ES activation in the clones showing an intermediate activity. The activation frequency was obtained by calculating the number of clones able to grow in the presence of a high dose of phleomycin (50 μg ml^{-1}). Two of the switches obtained were confi-

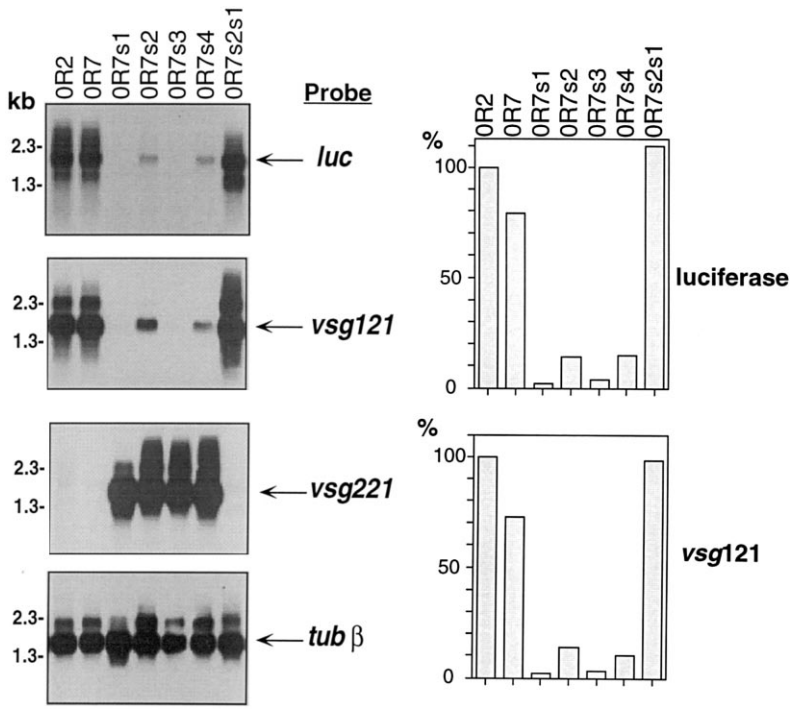

Fig. 5. Northern analysis of intermediate ES promoter activities.Total RNA (4 μg) was isolated from the indicated clones and separated in a gel. The membrane was hybridized with the probes shown on the right. The transcript of interest is indicated with an arrow (other RNA bands correspond to alternative or intermediate splice products). The phosphorimager quantitation of the RNAs (normalized to tubulin message) is shown to the right.

rmed to have properly activated the 121 ES (for example 0R7s2s1 in Fig. 5). To investigate whether the intermediate activity was present in some cells from a clone showing an apparently totally inactive ES, we selected clones resistant to intermediate levels of phleomycin (10 μg ml^{-1}).

We invariably obtained resistant clones with intermediate activity that did not present detectable Vsg121 on the surface but still expressed the Vsg221. These clones were obtained with a frequency of 10^{-5} and their luciferase activity displayed the same values as those of the intermediate clones described above (between 20 and 40 RLU). Northern analysis also showed a low abundance of the *vsg*121 transcript (data not shown). Thus, these intermediate activities not only occur naturally, after in vivo selection against the expressed Vsg121, but can also be isolated by selection with low levels of phleomycin. The same result was obtained with the original transformant clone described in Fig. 3, in which the *neo* marker gene is located downstream of an inactive 121 ES promoter. These G418-selected clones were able to provide intermediate levels of the *neo* transcript from the 121 ES while the 221 ES was still active. These experiments confirmed previous observations (M. Navarro and G. Cross, unpublished results) that it appears to be impossible to have two ESs fully active in one cell.

Although the intermediate ES promoter activity resulted in at least 15% of the *vsg*121 mRNA compared to a totally active ES (Fig. 5), these switches did not show a mixed Vsg coat by immunofluorescence, which explains why they did not have any disadvantage when grown in vivo in a Vsg121-immunized mouse. To estimate, in a more sensitive way, the amount of Vsg121 expressed in the intermediate clones, we performed a Western analysis using known amounts of pure Vsg121 in the same gel (Fig. 6). It was only possible to detect some Vsg121 in the intermediate clones

after a long exposure. Comparison with purified Vsg121 (25 and 2.5 ng) showed that approximately 10 ng of Vsg121 was expressed per 10^6 cells in the intermediate clones. 10^6 parasites contain approximately 800 ng of Vsg. Comparison of the ratios of *vsg*121 and *vsg*221 mRNA (0.15) and protein (0.013), suggests that the minor *vsg* is expressed less efficiently than the one derived from the authentic active ES. Isolation of Vsg-depleted membranes and soluble Vsg from one of the clones suggests that the poorly expressed Vsg121 was GPI-anchored and located on the membrane.

4. Discussion

We have performed an extensive in situ deletion analysis of the ES promoter region from −729 to −65 upstream and from +3 to +962 downstream of the transcription initiation site. Previous work [44,45] had suggested that sequences immediately downstream of the ES promoter could mediate the attenuation detected in procyclic forms. Other Pol I promoters in higher eukaryotes

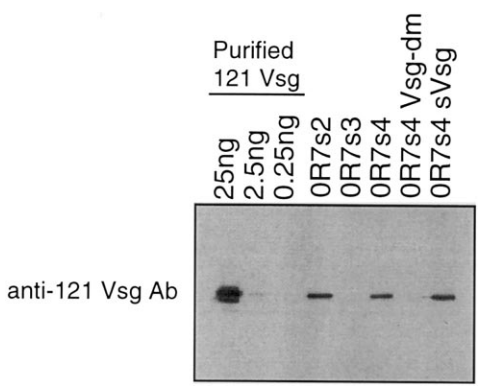

Fig. 6. Western analysis of clones showing intermediate reporter activities. Purified 121Vsg was loaded onto a SDS-PAGE gel in the indicated amounts. For 0R7s2 and 0R7s4 (intermediate clones) and 0R7s2, 10^6 cells were loaded per lane The same number of cells from 0R7s4 were used to isolate Vsg-depleted membranes (Vsg-dm) and soluble Vsg (sVsg) in a microscale of the protocol described previously [51]. The 121Vsg was detected using a 121Vsg rabbit antiserum that was depleted of cross-reacting antibodies. The Western blot was developed using the ECL technique (Amersham) and then exposed to film for 2 h.

[46] and in *T. brucei* [47] have DNA elements in the proximity of the core promoter that play important roles in its activity and/or regulation. Surprisingly, the complex regulation of the bloodstream-form ES, which allows the parasite to undergo antigenic variation and escape the host immune system, as well as the developmental down regulation of all ESs in the insect form, is not dependent on the sequences immediately surrounding the ES promoter. Thus, it seems that the 70-bp sequence, defined previously by transient assays [21] and in this work in situ, is able to provide proper ES regulation. Although we cannot rule out the possibility of other regulatory sequences farther upstream or downstream of the ES promoter, their existence seems unlikely, as the first of the ES-associated genes (*esag*7) is located downstream and tens of kilobases of 50-bp repeats are located a few hundred basepairs upstream of the region studied.

We have described the in situ ES promoter activities of a single clonal cell line in all of the transcriptional states that the ES undergoes in the *T. brucei* life cycle. The reporter activity was three orders of magnitude higher in the active ES compared to the inactive ES, demonstrating the high degree of regulation that assures the unique expression of a single ES in bloodstream form. However, the analysis of multiple switched-off clones has allowed us to detect intermediate activities. All these clones (inactive and intermediate) were obtained after selection in vivo against the expressing Vsg. When we initially isolated these clones, we took into consideration the possibility that they were a mixed population, containing cells that had switched back to the previously active ES (targeted with the *luc-ble* cassette), an event that we had often observed [15,41]. When these cells were recloned, however, they gave intermediate activities, confirming that the intermediate activity was not because of a mix of cell types. These results show that a second ES may be transcribed at a low level, in addition to the real active ES. These could partly account for the detection, by RT-PCR, of transcripts from multiple inactive ES promoter regions [48] and *vsgs* other than the active ES *vsg* in bloodstream-form trypanosome cDNA libraries [49]. However, our

data suggest that this weakly transcribed *vsg* is expressed very inefficiently, suggesting that other mechanisms may work in association with transcriptional regulation to assure that the active ES is the only one that is productively expressed, leading to a single major Vsg on the surface. On the other hand, when any of the antigenic variants containing the reporter gene, in either active, inactive or intermediate states, were differentiated to procyclic forms, all gave the same reporter activity.

The recognition of specific DNA sequences by transcription factors requires chromatin accessibility that can be detected by the preferential cleavage of nucleases [19]. We have shown the presence of a prominent hypersensitive site within an inactive core ES promoter by progressive treatment with micrococcal nuclease in permeabilized cells. This result suggests that the inactive ES in bloodstream forms contains transcription factors that are poised and ready to recruit the Pol I holoenzyme when it is activated. There is evidence from other systems that basic transcription factors like TFIID are present on some Pol II promoters that are inactive but inducible (reviewed in Ref [50]). The lack of activator-repressor sequences close to the ES promoter, as described in this work, is consistent with the idea that conformational changes (induced by unknown *trans*-activators) in an inactive ES core-promoter transcription-factor complex allow efficient pre-initiation complex assembly or isomerization to an active state. We did not detect significant differences in the degree of nucleosome packing between active or inactive chromatin at the promoter region (Fig. 2 and data not shown). Since previous studies have shown that there are no differences between the active and inactive nucleosome pattern within the *vsg* telomeric region using micrococcal nuclease [20], we can conclude that there are probably no important differences in chromatin packing along the ES or between active and inactive states in bloodstream forms. These data suggest the absence of general (promoter-independent) chromatin-changing mechanisms in the inactive ES, while favoring a hypothesis that includes promoter dependent mechanisms (protein interactions within the transcriptional complex) in the activation process. Taken together, these results suggest the existence of an activation mechanism that assures the highest transcription of a unique ES rather than a mechanism that merely silences all ESs in bloodstream forms.

The finding that a low level of transcription can be detected in some inactive ES promoters also supports the hypothesis of a 'ready to go' transcriptional complex able to generate, with a low frequency, transcription in other than the totally active ES. However, the expression of even low levels of a formerly expressed Vsg on the surface of the parasite would be selected against by the host immune system. We detected a low level of Vsg121 in the clones that showed intermediate activities, suggesting that post-transcriptional events, probably linked to the full ES activation mechanism, may target the mRNA for proper export and concomitant translation. This suggests a tentative model in which the active ES is located in a unique environment in the nucleus that enables not only the full activation and physical exclusion of all inactive ESs, but also enhances the appropriate targeting of the messages through an exclusive pathway out of the nucleus.

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