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Bloodstream Form-specific Up-regulation of Silent *VSG* Expression Sites and Procyclin in *Trypanosoma brucei* after Inhibition of DNA Synthesis or DNA Damage*

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The African trypanosome *Trypanosoma brucei* transcribes the active variant surface glycoprotein (VSG) gene from one of about 20 *VSG* expression sites (ESs). In order to study ES control, we made reporter lines with a green fluorescent protein gene inserted behind the promoter of different ESs. We attempted to disrupt the silencing machinery, and we used fluorescence-activated cell sorter analysis for the rapid and sensitive detection of ES up-regulation. We find that a range of treatments that either block nuclear DNA synthesis, like aphidicolin, or modify DNA-like cisplatin and 1-methyl-3-nitro-1-nitrosoguanidine results in up-regulation of silent ESs. Aphidicolin treatment was the most effective, with almost 80% of the cells expressing green fluorescent protein from a silent ES. All of these treatments blocked the cells in S phase. In contrast, a range of toxic chemicals had little or no effect on expression. These included berenil and pentamidine, which selectively cleave the mitochondrial kinetoplast DNA, the metabolic inhibitors suramin and difluoromethylornithine, and the mitotic inhibitor rhizoxin. Up-regulation also affected other RNA polymerase I (pol I) transcription units, as procyclin genes were also up-regulated after cells were treated with either aphidicolin or DNA-modifying agents. Strikingly, this up-regulation of silent pol I transcription units was bloodstream form-specific and was not observed in insect form *T. brucei*. We postulate that the redistribution of a limiting bloodstream form-specific factor involved in both silencing and DNA repair results in the derepression of normally silenced pol I transcription units after DNA damage.

African trypanosomes including *Trypanosoma brucei* evade immune attack during chronic infections by periodically switching a variant surface glycoprotein (VSG)[1] coat (1–4). The predominant VSG is encoded by a gene transcribed from 1 of about 20 telomeric *VSG* expression sites (ES). Switching VSG coats is mediated by DNA rearrangements moving a new *VSG* into the active ES from a repertoire of hundreds of silent *VSG* genes and pseudogenes in chromosome internal tandem arrays or at telomeres. Alternatively, a switch can be mediated by a transcriptional switch between ESs.

The large polycistronic ES transcription units contain an assortment of expression site-associated genes (*ESAG*s) in addition to the telomeric *VSG* (reviewed in Ref. 5). Although the basic ES structure is conserved, there is variation in exactly which *ESAG*s are present, as well as their number and order (6). Switching between the polymorphic *ESAG*s present in different ESs can allow the trypanosome to adapt to life in different hosts. This has been best investigated for the polymorphic *ESAG6* and *-7* genes encoding transferrin receptor subunits (7–9) and the serum resistance-associated gene conferring human serum resistance (10, 11). The polycistronic ESs are regulated as domains flanked upstream by extensive arrays of 50-bp repeats (12). Exogenous promoters integrated upstream of the 50-bp repeat arrays escape the transcriptional control operating on the downstream ES (13). In bloodstream form *T. brucei,* ES control is not sequence-specific (12, 14), does not appear to involve a repressed chromatin state (15, 16), or require homologues of genes involved in yeast telomere position effect, despite having a superficial resemblance to this phenomenon (17).

The multiple ESs are transcribed in a mutually exclusive fashion (reviewed in Ref. 18). Stable maximal activation of two ESs does not appear possible (19). Selection for double ES expressors using selectable markers inserted immediately downstream of different ES promoters results in trypanosomes that have one ES maximally active and another ES partially up-regulated, or that rapidly switch between two different ESs (19, 20). Possibly these rapidly switching trypanosomes alternate between a privileged subnuclear location that has been called an expression site body (ESB), a pol I transcriptional body specific to bloodstream form *T. brucei* (21). Location in an ESB transcription/RNA processing factory may give the active ES access to limiting factors necessary for transcription elongation and polyadenylation (22).

In contrast, in insect form *T. brucei* all ESs appear to be down-regulated to a great extent, although not as tightly as in the bloodstream form (23). Silencing is mechanistically different, as it is promoter sequence-specific and appears to involve repressed chromatin (15). However, it is unlikely that the telomeric location of ESs is critical for silencing in this life cycle stage, as ES promoters on circular bacterial artificial chromosomes (BACs) are as effectively silenced as those in the genome (24).

In order to investigate the machinery mediating ES down-regulation in bloodstream form *T. brucei*, we constructed reporter *T. brucei* strains with *GFP* inserted immediately behind

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[1] The abbreviations used are: VSG, variant surface glycoprotein; BAC, bacterial artificial chromosome; BrdUrd, 5-bromo-2′-deoxyuridine; DFMO, difluoromethylornithine; ES, expression site; ESAG, expression site associated gene; ESB, expression site body; FACS, fluorescence-activated cell sorter; GFP, green fluorescent protein; eGFP, enhanced GFP; MNNG, 1-methyl-3-nitro-1-nitrosoguanidine; pol I, polymerase I.

the promoter of silenced ESs. We attempted to disrupt the silencing machinery using various chemical treatments. Fluorescence-activated cell sorting (FACS) analysis allowed rapid and sensitive detection of ES derepression. We find that treatments inhibiting DNA synthesis or causing DNA damage result in a block in S phase and a concurrent up-regulation of silent ESs. In addition, up-regulation of procyclin transcript was also observed. Procyclin is transcribed from RNA polymerase I transcription units that are normally down-regulated in the bloodstream form. Surprisingly, this up-regulation of transcripts from normally silent sites is life cycle-specific, as there was no evidence for up-regulation of silenced ESs in insect form *T. brucei* after comparable treatments. We postulate the presence of a limiting bloodstream form-specific factor involved in both silencing and DNA repair. Redistribution after DNA damage could result in the derepression of normally silenced pol I transcription units.

EXPERIMENTAL PROCEDURES

T. brucei Transformants and Culturing Conditions—All trypanosomes used were *T. brucei* 427, and bloodstream form variants were all derived from *T. brucei* 427 VSG221a (25). Bloodstream form *T. brucei* was maintained at 37 °C in HMI-9 medium with the addition of 10% fetal calf serum and 10% Serum Plus (JRH Biosciences) (26). In order to ensure population homogeneity of the VSG coat expressed, trypanosome transformants with drug markers in the active VSG ES were continuously maintained on the appropriate drug selection pressure to prevent ES switching. The *T. brucei* RP2X-1 transformant was described previously (14). *T. brucei* HNI has a hygromycin gene inserted immediately behind the promoter of the 221 ES and a neomycin gene inserted behind the promoter of the VO2 ES and was described previously (27).

T. brucei 221GP1(221+) has a *GFP* gene in the active 221 ES and was made by replacing the hygromycin gene in the active 221 ES of *T. brucei* HNI(221+) with the 221GP1 construct containing *GFP* and the puromycin resistance gene. These trypanosomes were subsequently selected on G418 to select for trypanosomes that had activated the neomycin gene marked VO2 ES and silenced the *GFP*, resulting in *T. brucei* 221GP1(VO2+).

T. brucei VO2GP1(VO2+) has *GFP* in the active VO2 ES and was made by modifying *T. brucei* HNI(VO2+) by replacing the neomycin gene in the active VO2 ES with the VO2GP1 construct containing the *GFP* and blasticidin genes. These trypanosomes were subsequently selected on hygromycin to select for trypanosomes that had activated the hygromycin gene marked 221 ES and silenced the VO2 ES producing *T. brucei* VO2GP1(221+). The hygromycin resistance gene was subsequently replaced by a gene encoding puromycin resistance to create *T. brucei* VO2GP2(221+).

Insect form *T. brucei* 427 was maintained at 27 °C in SDM-79 medium with the addition of 10% fetal calf serum (28). The insect form *T. brucei* transformants (RPX1-1, ESX1-1, ESX1-2, and rDES1-1) were described previously (23) and were maintained on 25 μg ml^{-1} hygromycin.

Transfection Constructs—The 221GP1 construct has a puromycin resistance gene excised from construct pBS-Pur (gift of Isabel Roditi, University of Berne) (29) and inserted between tubulin intergenic regions containing splice and polyadenylation sites (23). The *GFP* gene is *eGFP* (Clontech) flanked upstream by the tubulin 3′ splice site present on a tubulin intergenic region, and downstream by the 221 *VSG* untranslated region and polyadenylation signal. The downstream flanking sequences are on a 612-bp fragment amplified by PCR using *Pwo* polymerase (Roche Applied Science) from a genomic clone of the 221 *VSG*[2] using the following primers: 221VSG3445s, 5′-TTTCCCCCCTCAAATTTCCCC-3′, and 221VSG4057as, 5′-CGAAAAATTAAGATTCAAAACCACGG-3′. The 1.3-kb 5′ 221 ES target fragment was amplified from the H25N7 BAC containing the 221 *VSG* ES (GenBank™ account number AL671259) (6) using PCR with *Pwo* polymerase and primers as follows: HNES481s, 5′-TTAAGCTTTCTAACACTTCCTTTTTG-3′, and VO228551as, 5′-AACCTCAATGGACGAAGGAG-3′. Integration of the construct was 216 bp downstream of the transcription start site. The 714-bp 3′ 221 ES target fragment was isolated from the H25N7 BAC using primers 221–73592s 5′-ACGAAGAGCAGGGGTGCAAC-3′ and HNES4244as 5′-GCTTCATCTGC-TGGTCGTCTTC-3′.

The VO2GP1 construct was analogous to the 221GP1 construct, and only the puromycin resistance gene was replaced by a blasticidin resistance gene from construct tubBSRtub (gift of P. Borst, Netherlands Cancer Institute, Amsterdam), and the target fragments were amplified by PCR from the N19B2 BAC containing part of the VO2 ES (GenBank™ account number AL671256) (6). The 5′ target fragment was amplified using the HNES481s and VO228551as primers listed above. The 3′ target fragment was amplified using primers VO2–118336s 5′-CTCTAGTGAGCGTATTTTAGAGG-3′ and HNES 4244as.

T. brucei Transformation—Transfection was performed using mid-log phase bloodstream form trypanosomes, which were washed and then resuspended at 5×10^7 ml^{-1} in cytomix without glutathione or ATP (30). 2.5×10^7 cells were electroporated with 10 μg of linearized DNA using a Bio-Rad Gene Pulser II with a single pulse of 1.5-kV and 25-microfarad capacitance in 0.2-cm cuvettes (Bio-Rad). After ~6 h recovery in HMI-9 medium, cells were distributed over 24-wells plates at densities between 7×10^4 and 3×10^5 cells ml^{-1}. Selection was with 0.2 μg ml^{-1} puromycin (Sigma) or 2 μg ml^{-1} blasticidin (Invitrogen). After 5–7 days on selection transformants were expanded for further analysis.

ES Switching—ES switching was performed using drug selection on agarose plates (31). *T. brucei* 221GP1(221+) with the puromycin gene in the active 221 ES was maintained in the presence of 0.2 μg of puromycin ml^{-1} (Sigma). To switch to the VO2 ES, 1×10^5 to 1×10^7 trypanosomes were spread on agarose plates containing 5 μg of G418 (Invitrogen) ml^{-1}. Colonies were counted after 9–10 days to determine the switching frequency (average of 4.4×10^{-6} determined on three plates) and analyzed by PCR to check for retention of the previously active 221 ES (27). *T. brucei* VO2GP1(VO2+) was switched in an analogous fashion; only these trypanosomes were maintained on 2 μg of blasticidin ml^{-1} and selected on 5 μg of hygromycin ml^{-1} to select for activation of the 221 ES. The frequency of 221 ES activation was 3×10^{-6} (average of four plates). Again PCR was used to monitor for retention of the VO2 ES.

T. brucei Chemical or DNA Damage Treatments—Mid-log phase cultures were pelleted and resuspended in fresh medium to remove drug selection pressure. Bloodstream form *T. brucei* was resuspended at 5×10^5 cells ml^{-1}, and insect form *T. brucei* was resuspended at 5×10^6 cells ml^{-1}. Cultures were treated with the appropriate chemical for 24 h for bloodstream form *T. brucei* unless stated otherwise in the figure legends or 48 h for insect form *T. brucei*. Alternatively, bloodstream form *T. brucei* was exposed to UV or γ-radiation and then recovered for 24 h. Aphidicolin, MNNG, berenil, pentamidine, suramin, trichostatin A, difluoromethylornithine (DFMO), and pCPT-cAMP were all from Sigma, and ethidium bromide from BDH. Cisplatin was a gift of Adrian Begg and Ben Floot (Netherlands Cancer Institute, Amsterdam) and rhizoxin a gift of Keith Gull.

Growth curves were initially performed with each of the different substances, and concentrations were chosen that flattened cell growth in a comparable fashion. Concentrations used are as follows: aphidicolin (30 μM) (32), MNNG (2 μg ml^{-1}) (20, 33), berenil (1 μM) (34), pentamidine (1 μM) (34), suramin (400 nM), trichostatin A (2 μg ml^{-1}), DFMO (difluoromethylornithine) (200 μM) (35), pCPT-cAMP (1 mM) (36), ethidium bromide (8 μM) (37), cisplatin (4 μM) (38), and rhizoxin (10 nM) (32).

UV irradiation was performed with 3840 J/m^2 UV light (254 nm) using a UV cross-linker (Stratagene). Cells were in 25 ml of tissue culture dishes with the lid removed during exposure (adapted from Ref. 39). γ-Irradiation was performed using a ^{137}Cs source (Gravitron RX30/55M, Graviner Manufacturing, Gosport, Hampshire, UK). Trypanosomes in HMI-9 medium were placed at a proximity to the cesium source so that the dose rate was 4.4 gray/min for a total dose of 160 gray. After removal from the exposure to UV or γ-radiation, cells were recovered for 24 h. The number of double-strand DNA breaks introduced into the *T. brucei* genome was estimated using agarose blocks containing *T. brucei* transfected with BACs of ~150 kb (24). Introduction of a single double-strand break into these large circles results in linearization. We estimated that 160 gray irradiation resulted in the introduction of ~370 double-strand breaks into the *T. brucei* genome.

FACS Analysis—Derepression of *GFP* was analyzed by FACS using a BD Biosciences FACSCalibur with an excitation wavelength of 488 nm. After treatment and recovery, cells were washed once in PSG (60 mM Na_2HPO_4, 3 mM NaH_2PO_4, 44 mM NaCl, 56 mM glucose) and resuspended at 10^6 cells ml^{-1} for analysis. Data were analyzed using CellQuest software version 3.3. To correct for fluorescence of the chemical used, *T. brucei* HNI, which lacks *GFP,* was also treated and analyzed by FACS, and any fluorescence in the FL-1 channel was subtracted from the final values. In addition, values were corrected by subtracting background fluorescence of untreated cells containing silenced *GFP*.

[2] T. Isobe and G. Rudenko, unpublished data.

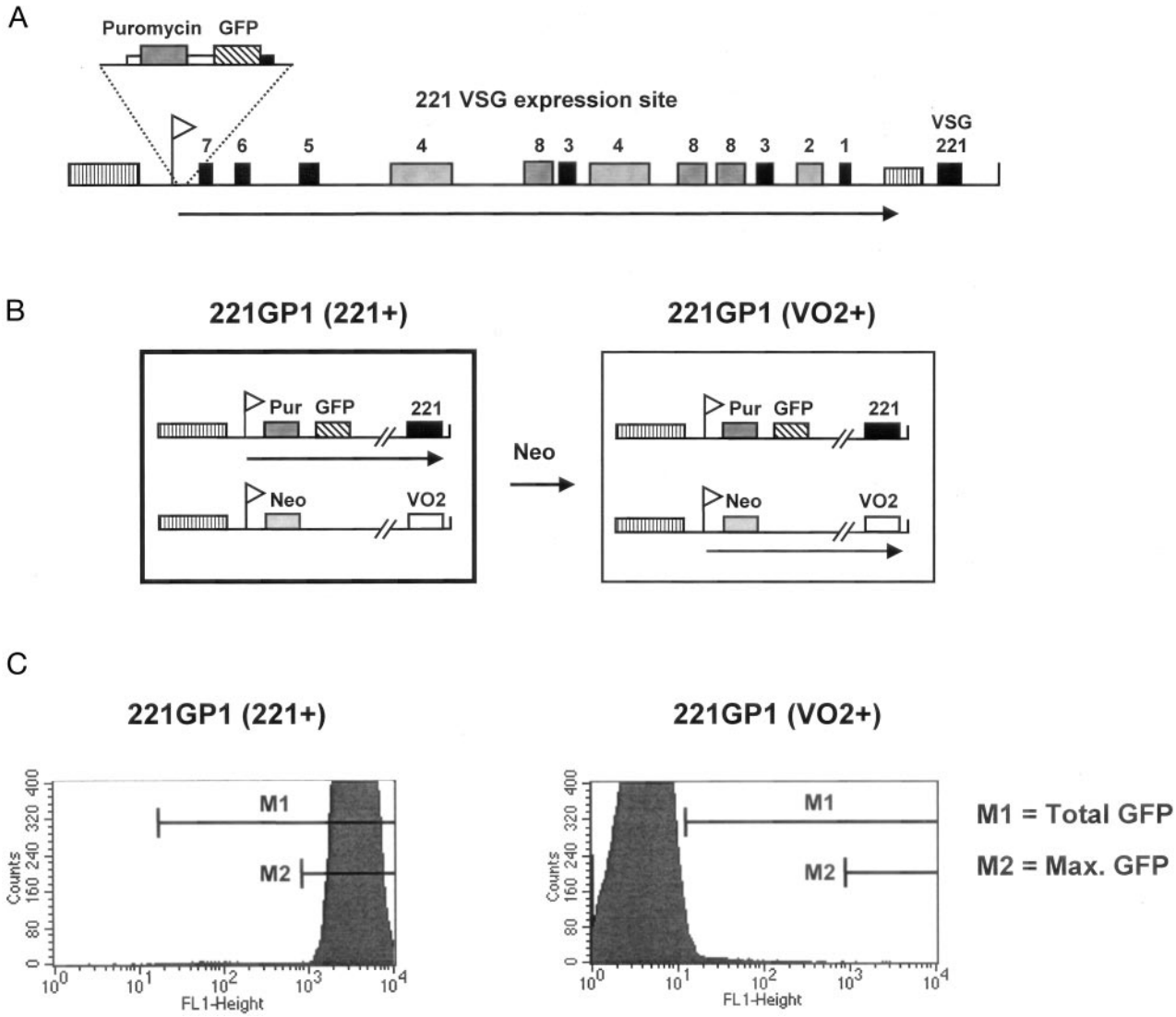

FIG. 1. **Creation of the *T. brucei* 221GP1(VO2+) reporter cell line with *GFP* in the silenced 221 *VSG* expression site.** *A*, integration of the 221GP1 construct containing a puromycin resistance gene (*Pur*) (*gray box*) and green fluorescent protein gene (*striped box*) immediately downstream of the promoter of the active 221 *VSG* ES (6). The puromycin gene is flanked by a splice site and polyadenylation signal from tubulin (*white boxes*). The *GFP* gene is flanked upstream by a tubulin splice site and downstream by the 221 *VSG* untranslated region and polyadenylation signal (*black box*). The *white flag* indicates the ES promoter and the *filled boxes* indicate various *ESAG*s and the 221 *VSG* gene. Simple sequence repeats upstream of the promoter (50-bp repeats) and upstream of the *VSG* gene (70-bp repeats) are indicated with *vertically striped boxes.* Transcription is indicated with an *arrow*. *B*, schematic showing silencing of the *GFP* marked 221 *VSG* ES. Trypanosomes are indicated with *boxes* containing schematics of the different *VSG* ESs. Transcription of the active ES is indicated with an *arrow*. 221GP1(221+) transformants expressing the 221 *VSG* expression site were subsequently selected on neomycin to select for trypanosomes that had switched to the VO2 *VSG* ES: 221GP1(VO2+). Further labeling is as in *A. C*, FACS analysis of GFP expression in 221GP1 trypanosomes with an active (*left*) or silent (*right*) 221 *VSG* ES. Fluorescence intensity in the FL-1 channel is shown on the *x* axis and number of cells on the *y* axis. The gates used to calculate the percentage of cells expressing GFP are shown, with *M1* representing total GFP (*i.e.* any cells expressing GFP above background levels) and *M2* representing maximum GFP expression.

For cell cycle analysis cells were stained with propidium iodide as described previously (40). Briefly, after treatment and recovery, cells were fixed in 70% methanol (10^6 cells ml^{-1}) and incubated at 4 °C overnight. Cells were washed in cold phosphate-buffered saline, resuspended in 1 ml of phosphate-buffered saline containing 10 μg ml^{-1} propidium iodide and 10 μg ml^{-1} RNase A, and incubated at 37 °C for 45 min. Detector FL2-A and an AmpGain value of 1.75 were used.

RNA Analysis—Total RNA was isolated from bloodstream form *T. brucei* treated for 24 (bloodstream form) or 48 h (procyclic form) with various chemicals in the absence of drug selection pressure. RNA was isolated from ~3 to 5 × 10^7 cells using an RNeasy RNA isolation kit (Qiagen). 5 μg of total RNA was electrophoresed in formaldehyde gels and blotted according to Ref. 41. Northern blots were hybridized with probes radiolabeled by random priming using the Megaprime DNA labeling system (Amersham Biosciences). The probe for eGFP is a 719-bp SalI/NotI fragment from the eGFP plasmid (Clontech). The probe for the 221 *VSG* is an 800-bp fragment of the 221 *VSG* corresponding to positions 122–925 in the sequence (GenBank™ account number X56762). The probe for the VO2 *VSG* is a 600-bp EcoRI/HindIII fragment from the VO2 *VSG* cDNA.[3] The tubulin probe is a 700-bp HindIII/EcoRI fragment. The procyclin probe is the entire CPT4 cDNA (42). The procyclin probe can be expected to hybridize with transcript from both the EP and GPEET procyclin variants. Quantitation was performed with a Bio-Rad PhosphorImager.

RESULTS

DNA Damage Results in the Up-regulation of GFP Marked Silent ESs—We constructed *T. brucei* strains with *GFP* immediately downstream of the promoter of the inactivated 221 or VO2 *VSG* ESs. As the ES promoters were silent, these strains allowed us to assay for disruption of the ES silencing machin-

[3] K. Sheader and G. Rudenko, unpublished data.

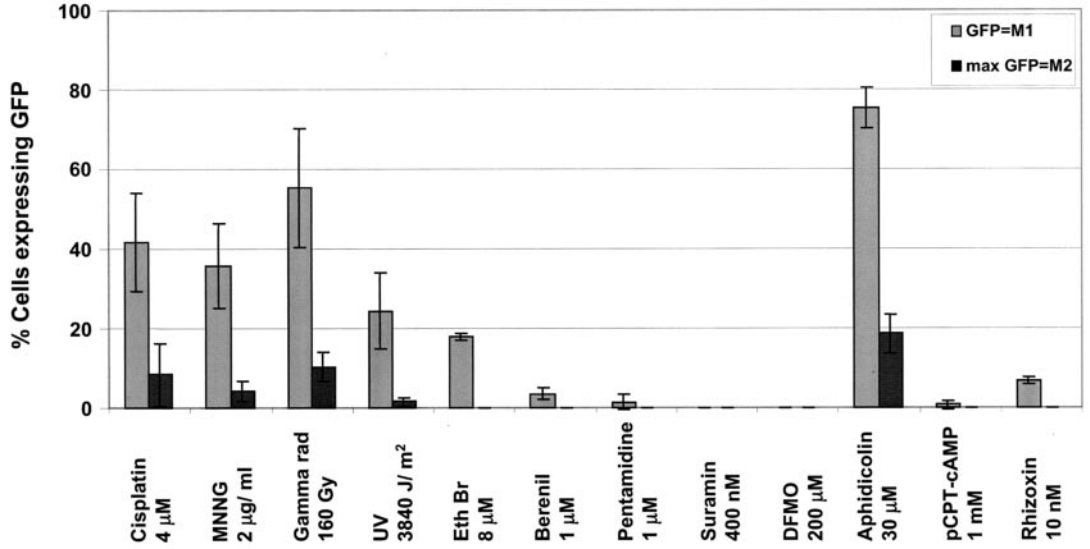

FIG. 2. **Up-regulation of the silent 221 ES after inhibition of DNA synthesis, DNA modification, or DNA damage.** 221GP1(VO2+) cells were treated with the agents indicated at concentrations that flattened cell growth in a comparable fashion (see "Experimental Procedures"). Cells were treated for 24 h with the exception of the γ-irradiation and UV irradiation, where cells were exposed to the doses indicated and then recovered for 24 h. The percentage of cells expressing GFP or maximum GFP (gates *M1* or *M2*, respectively, see Fig. 1*C*) as measured by FACS analysis is indicated. In all cases 200,000 cells were analyzed. The values shown have been corrected by subtracting background levels of GFP expressed in untreated cells. Corrections have also been made for fluorescence of the chemical used in the treatments, by subtracting the fluorescence values obtained by treating *T. brucei* HNI which does not contain *GFP* (27). The results shown are the average of three independent experiments. The standard deviation is indicated with *error bars*.

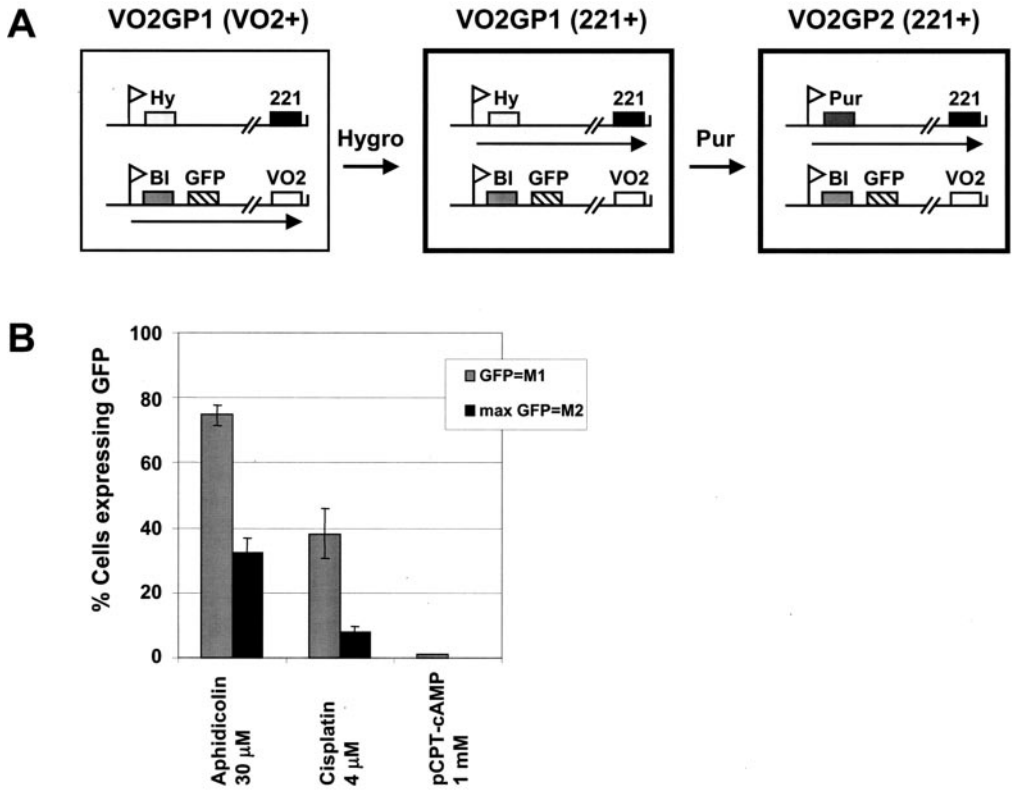

FIG. 3. **Up-regulation of the silent VO2 ES after DNA modification or inhibition of synthesis.** *A*, schematic showing the integration of a blasticidin resistance gene (*Bl*) and a *GFP* gene into the active VO2 ES and subsequent silencing. These cells subsequently had the hygromycin (*Hy*) resistance gene in the active 221 ES replaced by the puromycin (*Pur*) resistance gene. Labeling is as described in Fig. 1. VO2GP1(VO2+) cells were maintained on blasticidin. Hygromycin selection was used to select for cells that had switched to the 221 ES, VO2GP1(221+). The hygromycin gene in the active 221 ES was subsequently replaced by the puromycin resistance gene to produce the cells used in the derepression experiments, VO2GP2(221+). *B*, GFP expression after up-regulation of the VO2 ES after treatment of VO2GP2(221+) cells with the chemicals shown for 46 h. The figure is labeled as described in Fig. 2.

ery. We introduced a puromycin resistance gene linked to *GFP* into the active 221 *VSG* ES of *T. brucei* HNI(221+) (27) (Fig. 1*A*). The resulting transformant *T. brucei* 221GP1(221+) had the hygromycin gene located downstream of the 221 *VSG* ES promoter of *T. brucei* HNI(221+) replaced by a puromycin gene. As this modified variant of *T. brucei* HNI contains a

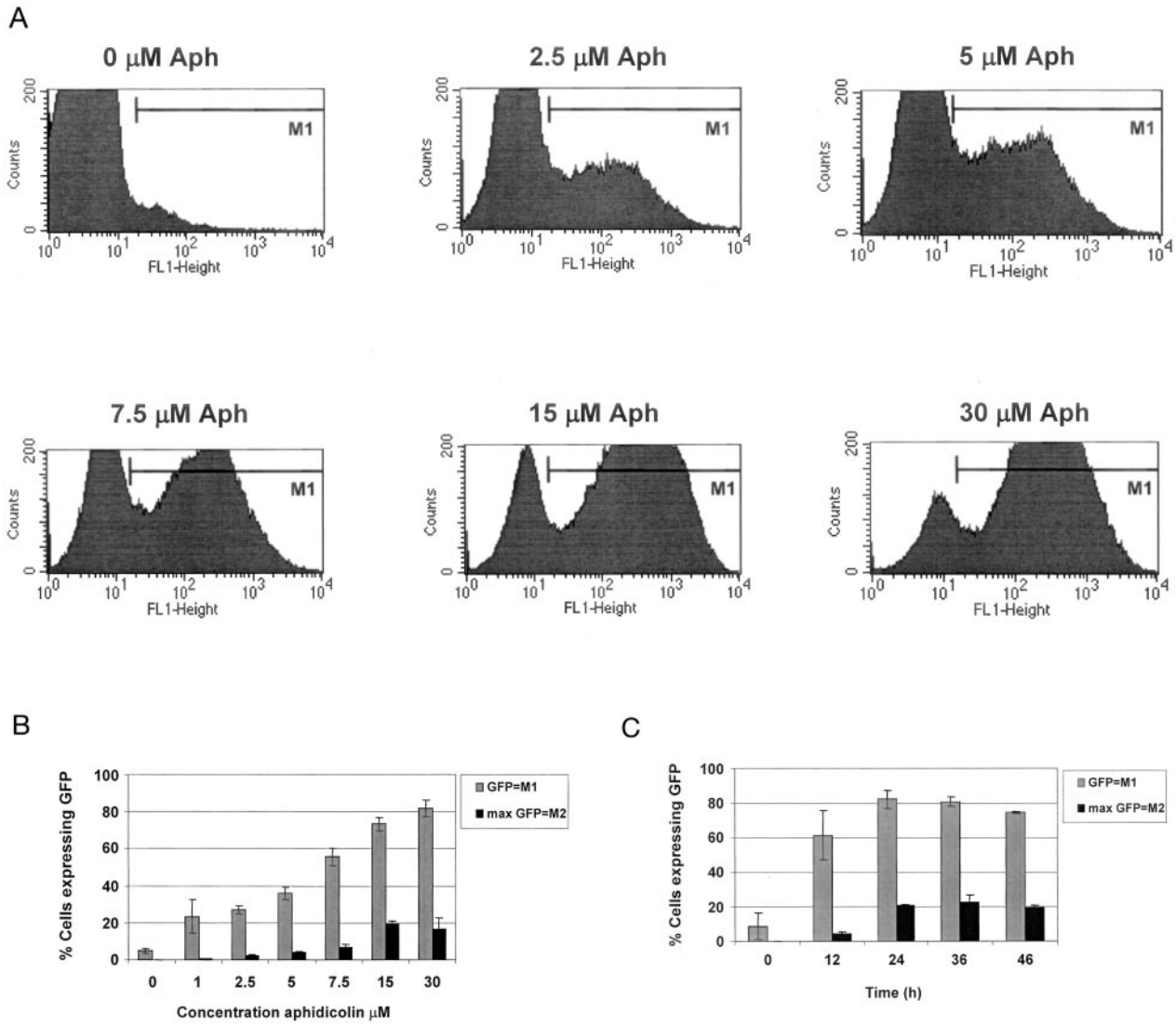

FIG. 4. **ES up-regulation after treatment with increasing amounts or length of time with the DNA synthesis inhibitor aphidicolin.** *A*, increased ES up-regulation after incubation of 221GP1(VO2+) cells with increasing concentrations of aphidicolin (*Aph*). FACS analysis measuring GFP expression of cells treated for 24 h with increasing concentrations of aphidicolin as indicated. *M1* shows the gate containing cells expressing GFP above background (see Fig. 1*C*). 200,000 cells were analyzed. The figure is labeled according to Fig. 1*C*. *B*, graphical representation of the FACS traces shown in *A*. Gates *M1* and *M2* (total and maximal GFP expression) are as indicated in Fig. 1*C*. Data were corrected for fluorescence of the aphidicolin by subtracting fluorescence of treated HNI cells that do not contain a *GFP* gene (as in Fig. 2). This is an average of two experiments with standard deviation indicated with *error bars*. *C*, ES up-regulation after incubation of 221GP1(VO2+) cells for increasing lengths of time with 30 μM aphidicolin. This is an average of two experiments with standard deviation indicated with *error bars*. Corrections for background fluorescence were made as in *B*.

neomycin gene located downstream of the silent VO2 *VSG* ES promoter, selection with G418 allowed us to screen for reactivation of the VO2 *VSG* ES, producing the reporter strain *T. brucei* 221GP1(VO2+) (Fig. 1*B*). *T. brucei* expressing *GFP* from an active *VSG* ES is more than 1000-fold brighter than background, allowing it to be very easily detected (Fig. 1*C*). FACS analysis of the *T. brucei* 221GP1(VO2+) trypanosomes with a silenced *GFP* provided a very sensitive and rapid assay system for measuring a change in expression of the silent *VSG* ES.

We incubated the *T. brucei* 221GP1(VO2+) reporter strain with a range of chemicals in order to screen for those resulting in the up-regulation of silent *VSG* ESs. First, we tested the histone deacetylase inhibitor trichostatin A, which generally causes derepression of silenced transcription units in a wide range of experimental systems due to disruption of repressed chromatin (reviewed in Ref. 43). Although trichostatin A suppressed growth of bloodstream form *T. brucei*, it had no detectable effect on derepression of the silent 221 *VSG* ES (result not shown). As there is no evidence that silenced ESs in bloodstream form *T. brucei* have a more repressed chromatin structure than active ESs (15, 16), this result is not surprising. Although trichostatin A did not cause up-regulation of silent ESs, a range of treatments resulting in DNA modification or damage did (Fig. 2).

Effective treatments that resulted in high levels of up-regulation of normally silent *VSG* ESs included DNA-modifying agents. Cisplatin treatment results in the introduction of a bulky cisplatin adduct into DNA (44), and MNNG methylates DNA (45). Treatment of bloodstream form *T. brucei* with both of these chemicals resulted in up to 40% of the cells expressing GFP (Fig. 2). Treatment with UV light also resulted in DNA modification in the form of thymidine dimers. This treatment was also effective at producing the up-regulation of *GFP*, as was the intercalating agent ethidium bromide. DNA damage in the absence of DNA modification was also effective in inducing up-regulation of *GFP* transcript. The introduction of double-strand DNA breaks with a cesium source also resulted in very high levels of silent *VSG* ES up-regulation, with more than 50%

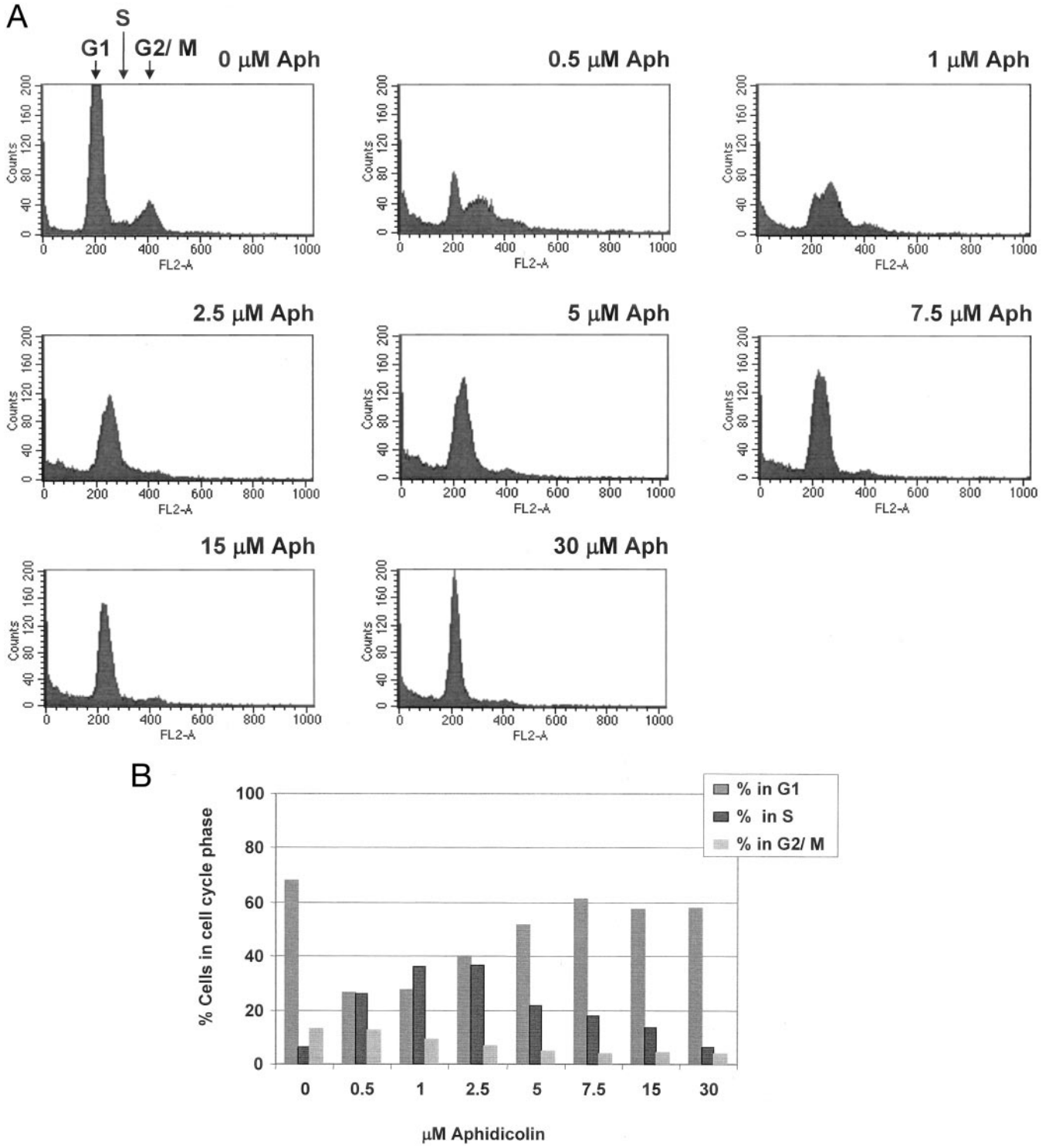

FIG. 5. ***T. brucei* treated with increasing concentrations of aphidicolin arrest at the G_1/S phase of the cell cycle.** *A*, *T. brucei* HNI cells (27) were treated with increasing concentrations of aphidicolin (*Aph*) for 24 h and stained with propidium iodide to measure DNA content. FACS analysis was performed on 20,000 cells with the number of cells (*y axis*) fluorescing in the FL2-A channel (*x axis*) indicated. In the untreated population (0 μM aphidicolin) the cells in the G_1, S, or G_2/M phases of the cell cycle are indicated with *arrows*. *B*, graphical representation of *A* with the percentage of cells in different phases of the cell cycle indicated with *bars*.

of the cells expressing GFP (Fig. 2).

In contrast, berenil and pentamidine selectively induced double-strand breaks in the kinetoplast DNA rather than nuclear DNA (34). Treatment with these drugs did not result in significant ES derepression. Treatment with the metabolic inhibitor suramin (46) or the ornithine decarboxylase inhibitor DFMO (35, 47) also had no detectable effect, despite inhibiting *T. brucei* cell growth in a comparable fashion to the other treatments (see "Experimental Procedures" for details). It is therefore likely that modification or damage of nuclear DNA is the key factor in the up-regulation of silent ESs that we observed.

Up-regulation of Silent ESs Correlates with a Block in S Phase—Treatment of *T. brucei* with DNA-modifying agents resulted in a block in DNA synthesis, as observed by measuring incorporation of BrdUrd in treated cells (result not shown) (48).

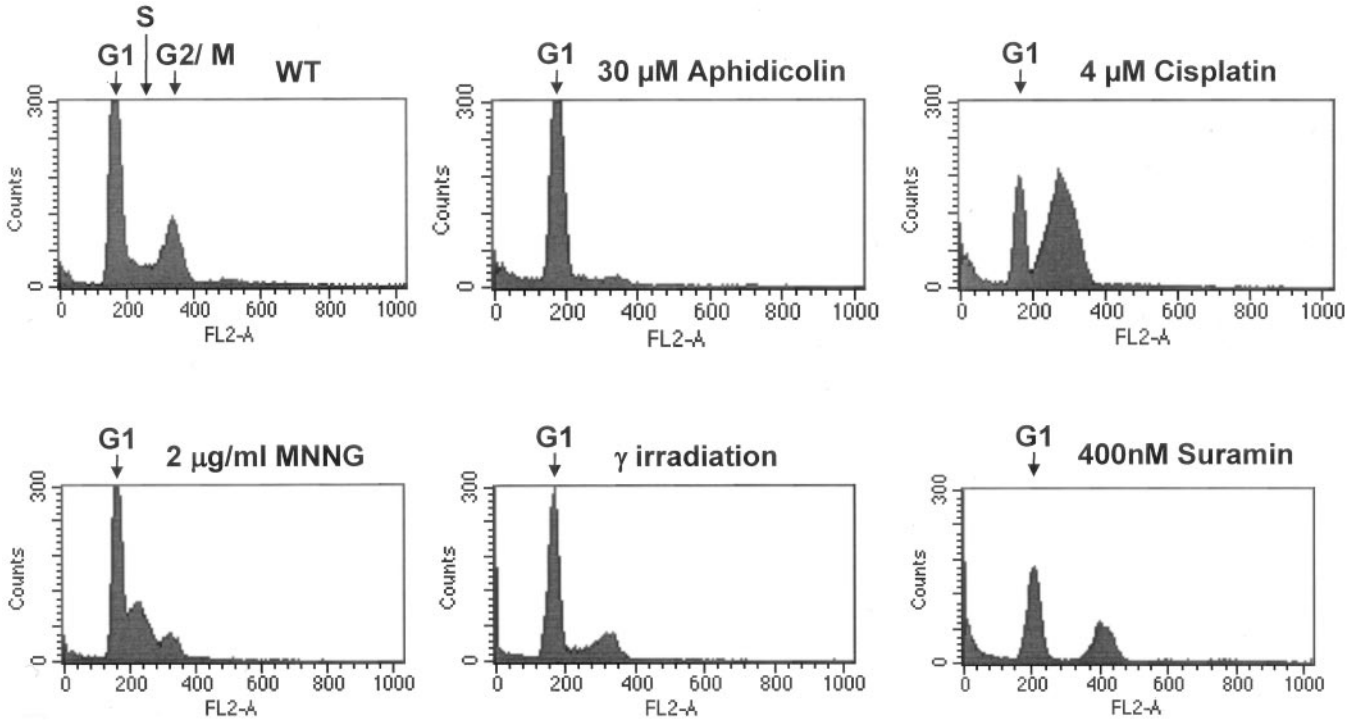

FIG. 6. **Cell cycle arrest in S phase or in the G_1/S phase transition in *T. brucei* exposed to treatments that damage DNA or block DNA synthesis.** *T. brucei* HNI was treated for 24 h with the substances indicated with the exception of γ-irradiation, where cells were treated with 160 gray and allowed to recover for 24 h. Cells were stained with propidium iodide to measure DNA content. 20,000 cells were analyzed in each experiment. The figure is further labeled according to Fig. 5*A*. *WT*, wild type.

In order to block nuclear DNA synthesis without introducing DNA damage, we incubated bloodstream form *T. brucei* with aphidicolin, which selectively binds and inhibits nuclear DNA polymerase α (49, 50). Aphidicolin has been used previously to stall trypanosomes at the G_1/S phase transition (32, 51). Treatment of bloodstream form *T. brucei* 221GP1(VO2+) with 30 μM aphidicolin resulted in very striking up-regulation of the silenced *GFP* (Fig. 2). Almost 80% of the cells expressed GFP, with almost 20% of the cells expressing GFP at maximal levels (*dark bar* in Fig. 2) (see Fig. 1*C* for description of the gates used).

As up-regulation of silent ESs was observed after treatments that resulted in a block in nuclear DNA synthesis, we investigated if this could also be observed if *T. brucei* was stalled in the G_1/G_0 phase of the cell cycle. DNA synthesis is inhibited in stumpy form *T. brucei*, which is a nonreplicating form stalled in G_1/G_0. pCPT-cAMP can induce this cell cycle block, also in the monomorphic *T. brucei* 427 strain used in this study (36, 52). Although incubation of *T. brucei* 221GP1(VO2+) in 1 mM pCPT-cAMP completely blocked cell growth and resulted in a block in DNA synthesis as measured by monitoring BrdUrd incorporation (result not shown), there was no significant ES derepression (Fig. 2). This indicates that stalling in S phase could be a critical factor, rather than simply blocking DNA synthesis.

Finally, we tested the effect of the mitotic inhibitor rhizoxin. Silenced telomeres in yeast are present in clusters at the nuclear periphery, and activation involves movement away from the perinuclear region (53, 54). The microtubule polymerization inhibitor rhizoxin inhibits mitosis in trypanosomes (32). Treatment of procyclic *T. brucei* with rhizoxin has been shown to result in the disruption of telomere clusters at the nuclear periphery (55). However, treatment of our *T. brucei* GFP reporter strain with rhizoxin did not result in significant up-regulation of the ES (Fig. 2).

This up-regulation of a normally silent ES was not specific to the 221 *VSG* ES. We constructed the reporter line *T. brucei* VO2GP2(221+), where a construct containing the blasticidin gene linked to *GFP* was downstream of a silenced VO2 *VSG* ES promoter (Fig. 3*A*). As expected, treatment of this cell line with either aphidicolin or cisplatin resulted in significant up-regulation of this ES, which was not observed after treatment with pCPT-cAMP (Fig. 3*B*).

Up-regulation of the 221 *VSG* ES in *T. brucei* 221GP1(VO2+) increased as the amount of aphidicolin used for treatment was raised between 0 and 30 μM (Fig. 4, *A* and *B*). In a time course using 30 μM aphidicolin, GFP expression was extensive at 12 h (60% of the cells GFP-positive) and essentially maximal at 24 h (about 80% of the cells GFP-positive) (Fig. 4*C*). As the doubling time of the *T. brucei* 221GP1(VO2+) cells is about 7 h, the extensive up-regulation observed at 12 h is compatible with most of the ES derepression occurring during one cell division. The percentage of cells scoring as maximally GFP-positive increased between 12 and 24 h, presumably due to accumulation of GFP protein. We attempted washing aphidicolin-treated cells to see if treatment was reversible, and if high levels of up-regulation of silent ESs resulted in increased ES switching. However, we were not able to recover viable cell lines from cells treated with aphidicolin at concentrations that resulted in significant up-regulation.

As expected for an inhibitor of nuclear DNA synthesis, incubating *T. brucei* 221GP1(VO2+) in increasing amounts of aphidicolin resulted in a block in S phase (Fig. 5*A*). An accumulation of cells in S phase was detectable using even small amounts of aphidicolin (0.5–1 μM) (Fig. 5, *A* and *B*). As the amount of aphidicolin used for treatment increased, the block became increasingly tighter, resulting in cells that don't enter S phase but stall at the G_1/S phase transition as observed previously (32, 51). The G_2/M peak disappeared at these increasing concentrations, presumably as cells that had passed S phase progressed further through the cell cycle, accumulating in G_1. Treating *T. brucei* with DNA-modifying agents like cis-

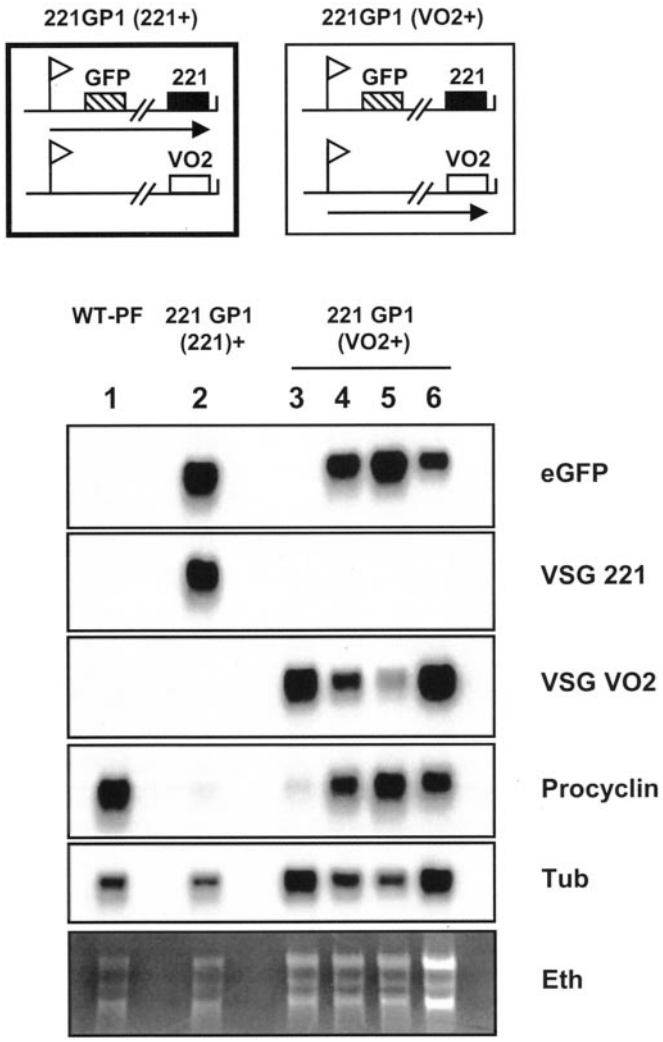

FIG. 7. **Up-regulation of normally silent pol I transcription units in 221GP1(VO2+) cells treated with agents modifying DNA or blocking DNA synthesis.** Schematics of the 221GP1(221+) and 221GP1(VO2+) cells are indicated at the *top* and labeled according to Fig. 1*B*. Northern blot analysis of RNA isolated from treated cells is indicated below. RNA from wild type procyclic *T. brucei* is shown in *lane 1*. RNA from *T. brucei* 221GP1(221+) with a *GFP* gene in an active ES is shown in *lane 2*. RNA from 221GP1(VO2+) cells is shown in *lanes 3–6*, where cells were untreated (*lane 3*) or treated with 4 μM cisplatin (*lane 4*), 30 μM aphidicolin (*lane 5*), or 2 μg ml^{-1} MNNG (*lane 6*) for 24 h. The blot was hybridized with the probes indicated on the *right of the panels*: GFP, VSG221, VSGVO2, procyclin, and tubulin (*Tub*). The ethidium stain (*Eth*) of the gel is shown at *bottom* to indicate the amount of RNA loaded per lane.

platin and MNNG also resulted in an accumulation of cells in S phase (Fig. 6). This is presumably because the *T. brucei* DNA damage repair machinery recruited to sites of DNA damage triggers an S phase cell cycle checkpoint, as has been described in other organisms (reviewed in Refs. 56 and 57).

Bloodstream Form-specific Partial ES Transcription—Up-regulation of the silenced 221 *VSG* ES resulted in transcript levels of about 20–30% of an active ES, but transcripts did not extend as far as the 221 *VSG* itself (Fig. 7). Possibly, stalled replication forks accumulating in the treated cells interfere with transcription of the large (~60 kb) 221 *VSG* ES (6, 39, 58). Down-regulation of VO2 *VSG* transcript from the active ES itself was also observed (Fig. 7). Unexpectedly, there also appeared to be general up-regulation of other repressed RNA polymerase I transcription units. Not only *VSG* ESs but also procyclin, which is down-regulated in bloodstream form *T. brucei,* was greatly up-regulated (Fig. 7). Procyclin mRNA levels were up-regulated to about 30–50% of the normal level in procyclic trypanosomes (42, 59–60). As our probe recognizes both the EP and GPEET procyclin variants (see "Experimental Procedures"), we do not know if one of these procyclin variants was preferentially up-regulated. Amounts of tubulin transcript were slightly decreased after treatment (Fig. 7). Although *VSG* ESs are polymorphic, their global architecture is roughly similar (6). In Northern blot hybridizations using probes extending along the *VSG* ES transcription unit, transcripts appeared to be particularly up-regulated near the ES promoter. For example, the transcript for *ESAG6/7* and *ESAG5* was up-regulated but not for *ESAG3* (Fig. 8). Because the *ESAG* probes used were not specific for the *ESAG*s in the 221 ES, we were not able to determine from which up-regulated ESs these transcripts were derived.

Surprisingly, this up-regulation of repressed RNA polymerase I transcription units after DNA modification or inhibition of DNA synthesis was specific to bloodstream form *T. brucei* and was not observed in procyclic trypanosomes. ESs are all down-regulated to a great extent in insect form trypanosomes, although repression is mechanistically different to the bloodstream form (15, 23, 61). Procyclic *T. brucei* transformants containing a hygromycin gene behind an ES promoter (ESX1–1 in Ref. 23) or an rDNA promoter in an ES (RPX1–1 in Ref. 23) were treated with aphidicolin, cisplatin, and MNNG. No up-regulation of these marker genes was observed after these chemical treatments (Fig. 9*A*). Similarly, when these *T. brucei* transformants plus a transformant containing an ES promoter in the rDNA spacer (rDES1–1 in Ref. 23) were incubated in 30 μM aphidicolin, no up-regulation of these marked promoters was observed (Fig. 9*B*) despite evidence for the induction of a stringent block in G_1/S as has been observed previously (32) (Fig. 9*C*).

DISCUSSION

We show that treatment of bloodstream form *T. brucei* with agents that cause a block in nuclear DNA synthesis or DNA damage results in the up-regulation of silenced *VSG* ESs. The extent of up-regulation increased with the amount of aphidicolin used and correlated with the tightness of the S phase block. Up-regulated silent ESs were only partially transcribed, with transcription extending past *ESAG5* but terminating before the telomeric *VSG*. In addition to inactive ESs, other RNA polymerase I transcription units like procyclin, which are normally silenced in bloodstream form *T. brucei*, were also up-regulated. In contrast, no up-regulation of silent ESs was observed in procyclic *T. brucei* after treatments resulting in a comparable block in S phase.

In our experiments, we find the most likely scenario is that normally repressed pol I transcription units have become derepressed, resulting in increased pol I transcription of normally silent locations. Formally, we have not shown that the up-regulated *VSG* ESs and procyclin loci are transcribed by pol I rather than pol II. However, we find it implausible that the promiscuity of pol II initiation in *T. brucei* would necessarily increase after treatments resulting in a block in S phase.

One initial explanation for the general up-regulation of normally silenced ESs was that the treatments used had severely perturbed the bloodstream form nuclear architecture. In bloodstream form *T. brucei*, RNA polymerase I is normally present in high concentrations in the nucleoli and the ESB (21). Blocking DNA synthesis could have resulted in the disintegration of these subnuclear compartments, resulting in the transcription of sequences that might normally not have access to pol I. There was some disruption of the normal nuclear architecture after aphidicolin treatment, in *in vitro* transcription experiments with BrUTP, and α-amanitin in permeabilized cells (re-

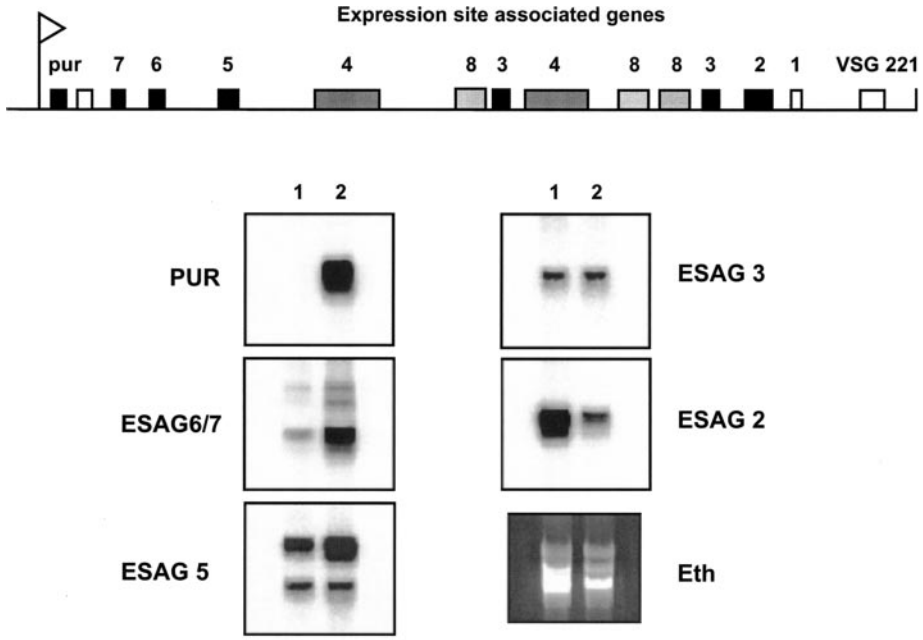

FIG. 8. **Aphidicolin treatment results in up-regulation of ES transcripts near the ES promoter region.** A schematic showing the 221 ES in the 221GP1(VO2+) trypanosomes is indicated at the *top* with the various *ESAG*s represented by *filled boxes.* The promoter is indicated with a *flag,* and the puromycin gene with a *black box* (*pur*). The downstream *GFP* gene is indicated with a *white box.* RNA from untreated 221GP1(VO2+) cells (*lanes 1*) was compared with cells treated with 30 μM aphidicolin for 24 h (*lanes 2*). The blot was hybridized with the probes indicated on the side: puromycin (*PUR*), *ESAG*s *6/7, 5, 3,* and *2.* The ethidium-stained gel (*Eth*) is shown at *bottom*.

sults not shown) (21, 62, 63). However, α-amanitin-resistant transcription foci corresponding to the nucleolus and the ESB could still be detected in bloodstream form *T. brucei* treated with aphidicolin (result not shown). Although transcription of up-regulated ESs was likely to be taking place outside of the ESB, experimental sensitivity was not high enough to establish this unequivocally.

Partial activation of ESs in bloodstream form *T. brucei* has been achieved before in experiments targeting drug resistance genes into "silent" ESs, although the levels of transcription were much lower than we show here (19, 64). These low levels of transcription were "erased" after activation and subsequent inactivation of the partially active ES, implying the resetting of an epigenetic state (19). In addition, Ulbert *et al.* (20) investigated ES control in *T. brucei* strains with three ESs marked with drug resistance genes. Different combinations of drug selection pressure resulted in either of the following scenarios: an unstable rapidly switching state existing between any two of three marked ESs. Alternatively, clones arose with one maximally active ES and other ESs partially active at a very low rate. However, by using MNNG treatment, novel *T. brucei* lines were isolated that had higher partial activation of two different marked ESs in addition to the active ES. Levels of derepression were about 10% compared with the active state.

Finally, derepression of silent ESs was shown in bloodstream form *T. brucei* treated with BrdUrd, a thymidine analogue that cannot be converted to the modified nucleotide J (65). ES derepression was interpreted as a consequence of reduction in J, as incubation with BrdUrd resulted in a 12-fold decrease in the levels of J. However, overproduction of J by incubation with hydroxymethyldeoxyuridine also resulted in derepression of silent sites, making it possible that perturbance of the DNA structure was the critical factor rather than the amount of J itself (65). Although incubation of our *T. brucei* GFP reporter strain with up to 400 μM BrdUrd resulted in silent ES up-regulation, levels were not as striking as with the other treatments shown here (results not shown).

Silencing in different experimental organisms has been shown to require transition through S phase of the cell cycle for the establishment of silenced chromatin (66–69). However, although this silencing is S phase-dependent, it does not always require DNA replication itself (70–72). The common feature of all of the treatments we performed that resulted in up-regulation of silent ESs is that they caused either a block in nuclear DNA synthesis by inhibiting DNA polymerase α like aphidicolin, or resulted in DNA damage, which typically triggers an S phase checkpoint, as has been extensively documented in other organisms (56, 57).

In *T. brucei* there are life cycle-specific differences in cell cycle checkpoints. Procyclic trypanosomes can undergo cytokinesis despite a block in S phase or mitosis, but in bloodstream form *T. brucei* inhibition of mitosis inhibits cytokinesis but not further rounds of DNA replication (32, 40). No cyclin homologue has yet been identified in *T. brucei* that is involved in S phase transition in the bloodstream form. We find that incubation with even very low amounts of aphidicolin resulted in cells starting to accumulate in S phase. Stalled replication forks are a potent signal for S phase checkpoint activation, which blocks progression through the cell cycle allowing DNA damage to be repaired (discussed in Ref. 56). However, the amount of ES up-regulation that we observed increased with the concentration of the DNA polymerase inhibitor aphidicolin used for treatment. We therefore find it unlikely that the up-regulation we observe is a direct consequence of stalling in S phase, and is more likely to be correlated to the number of stalled replication forks, which would be expected to increase as the concentration of aphidicolin increases. We cannot exclude that the stalled replication forks impact on RNA polymerase processivity. It is unknown if *T. brucei* RNA polymerases fall off at stalled forks, making themselves subsequently available for reinitiation at normally silent promoters.

In contrast, we prefer a model whereby a limiting factor involved in both silencing and DNA repair has been redistributed in our treated bloodstream form *T. brucei*, resulting in derepression. There is precedent for chromatin proteins involved in transcription repression and present in limiting con-

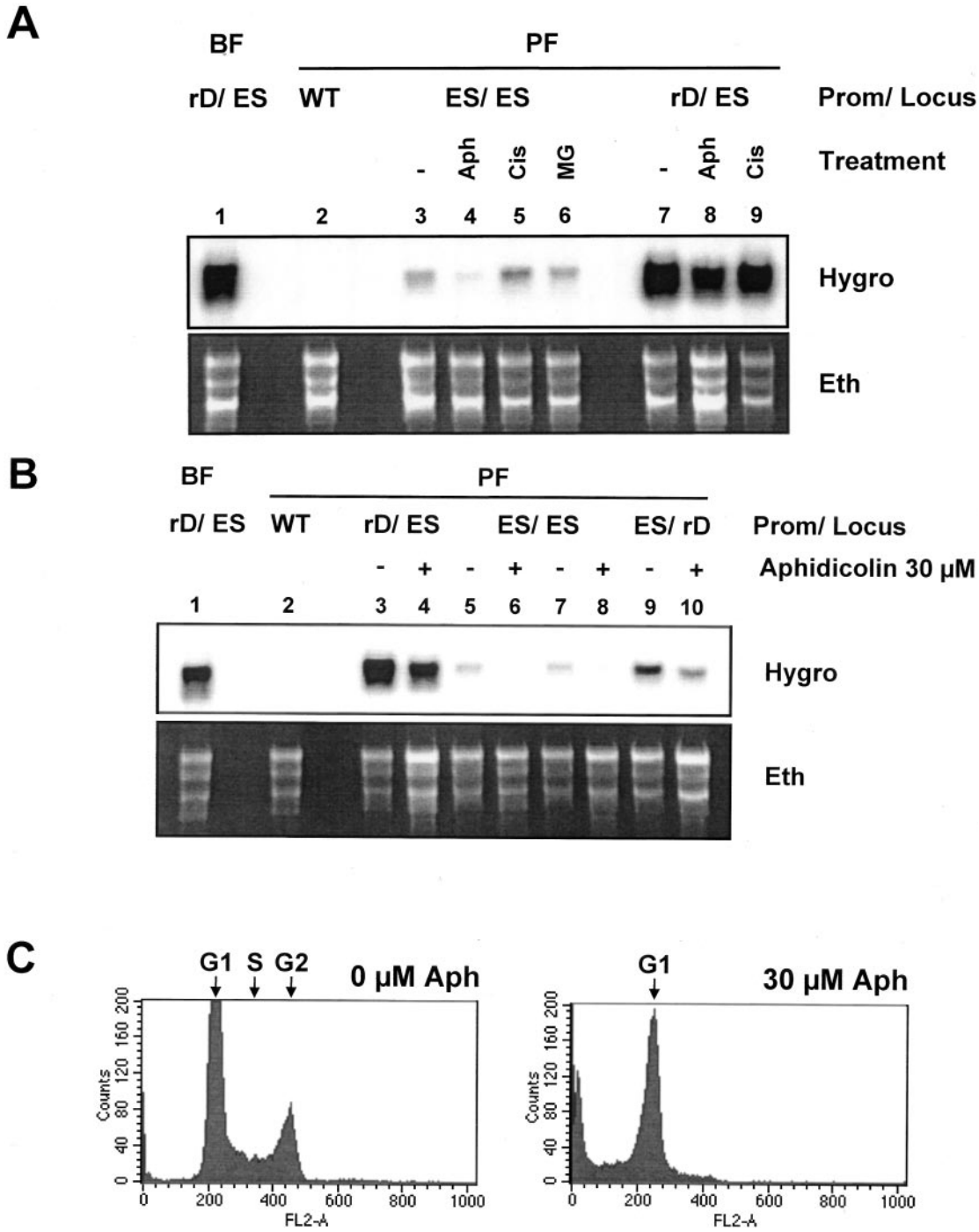

FIG. 9. **Insect form *T. brucei* treated with chemicals resulting in DNA modification or a block in DNA synthesis does not show up-regulation of *VSG* expression sites.** *A*, Northern blot analysis of treated insect form *T. brucei* transformants with ESs marked with a hygromycin resistance gene. *Lane 1* has RNA from bloodstream form (*BF*) *T. brucei* with an rDNA promoter (*PF*) directing transcription of a hygromycin resistance gene in the active 221 *VSG* ES (*rD/ES*) (transformant RP2X-1 in Ref. 14). *Lane 2* has RNA from wild type (*WT*) insect form *T. brucei*. *Lanes 3–6* have RNA from insect form *T. brucei* transformants with a hygromycin resistance gene directed by an ES promoter (*Prom*) in an ES (*ES/ES*) (ESX1–1 in Ref. 23). *Lanes 7–9* have RNA from insect form *T. brucei* with an rDNA promoter in an ES-driving hygromycin gene transcription (*rD/ES*) (RPX1–1 in Ref. 23). Cells were either untreated (−) or treated with 30 µM aphidicolin (*Aph*), 4 µM cisplatin (*Cis*), or 2 µg ml^{-1} MNNG (*MG*) for 48 h before RNA isolation. The blot was hybridized with a probe for the hygromycin gene (*Hygro*). A panel showing an ethidium stain of the gel is shown (*Eth*). *B*, aphidicolin treatment of insect form *T. brucei* does not result in up-regulation of *VSG* ESs. RNA from a bloodstream form transformant with an rDNA promoter directing transcription of an active ES (*rD/ES*) (RP2X-1) (*lane 1*) and wild type insect form *T. brucei* (*lane 2*) was compared with insect form *T. brucei* transformants incubated in the presence (+) or absence (−) of 30 µM aphidicolin. *Lanes 3* and *4* have RNA from insect form *T. brucei* with an rDNA promoter in an ES (RPX1-1 in Ref. 23); *lanes 5–8* have RNA from insect form *T. brucei* with an ES promoter in different ESs (ESX1-1 (*lanes 5* and *6*) and ESX1-2 (*lanes 7* and *8*) in Ref. 23. *Lanes 9* and *10* have RNA from insect form *T. brucei* transformants containing an ES promoter directing transcription of a hygromycin gene inserted into an rDNA spacer (*ES/rD*) (transformant rDES1-1 in Ref. 23). The blot was hybridized with a probe for the hygromycin gene (*Hygro*). An ethidium stain of the gel is shown (*Eth*). *C*, aphidicolin treatment of procyclic *T. brucei* results in stalling in the G_1/S phase of the cell cycle. *T. brucei* ESX1-1 transformants were treated with 30 µM aphidicolin for 24 h and then stained with propidium iodide to allow measurement of DNA content by FACS analysis. Similar results were obtained after a 48 h of treatment (not shown). 20,000 cells were analyzed. Cells in G_1, S, and G_2/M are indicated. The figure is further described as in Fig. 5*A*.

centrations being relocalized to sites of DNA damage in the cell, resulting in derepression of normally silent transcription units. For example, the Ku and SIR silencing proteins are normally sequestered at telomeres in *Saccharomyces cerevisiae* (54, 73, 74). However, after the introduction of DNA damage, these proteins are relocalized from the telomeres to DNA repair sites (75, 76), resulting in derepression of genes normally silenced by telomere position effect (77). A SIR2 homologue has been described recently in *T. brucei* (78) that plays a role in DNA repair and is enriched at telomeres. Although it appears unlikely that the telomere position effect plays a central role in ES down-regulation in bloodstream form *T. brucei*, one scenario is that the accumulation of stalled replication forks or DNA damage results in the relocalization of limiting silencing factors, resulting in derepression. This scenario would predict the presence of a bloodstream form-specific silencing factor, which is involved in both repression of silent ESs and procyclin as well as DNA repair and is present in limiting concentrations in the cell. This model would be compatible with the correlation observed between the degree of ES up-regulation and the amount of aphidicolin used for treatment.

The recruitment of DNA repair machinery to sites of DNA damage in repressed genes might have a secondary consequence of making these areas more accessible for transcription after the damage has been repaired. For example, treatments resulting in DNA damage or a block in DNA synthesis could result in the recruitment of DNA damage repair machinery to silenced sites, resulting in the displacement of factors blocking elongation of transcription. Blocking DNA synthesis with aphidicolin produces an accumulation of stalled replication forks and single strand DNA, which might also recruit proteins that are part of the DNA damage repair machinery.

A less likely scenario is that factors recruited to sites of DNA damage result in the stimulation of transcription. Some core factors have been shown to be involved in both transcription and DNA repair. Transcription factor TFIIH is a multisubunit complex that has been shown to be involved in RNA polymerase I and II transcription, DNA repair, and cell cycle control (reviewed in Ref. 79). TFIIH is not required for RNA pol I-mediated transcription initiation, but for a subsequent step like promoter clearance, transcription elongation, or reinitiation (80). Studies using GFP-tagged TFIIH showed that although TFIIH is normally involved in transcription, TFIIH rapidly relocates to repair sites after DNA damage (81). However, the argument that a factor involved in DNA repair stimulates transcription might be a bit farfetched, as it would presumably be more advantageous for a cell to induce silencing around the area of a DNA lesion rather than transcription. For example, the Ku and SIR proteins that are targeted to double-strand breaks are involved in transcription repression in yeast (75).

A striking feature of our results is that the up-regulation of normally silenced RNA polymerase I transcription units that we observe is bloodstream form-specific. We saw no evidence for increased transcription from down-regulated ESs in treated procyclic *T. brucei*. There are significant mechanistic differences in ES down-regulation in procyclic compared with bloodstream form *T. brucei*. In procyclic *T. brucei* there is evidence for a repressed chromatin state down-regulating ESs in a promoter sequence-specific fashion, which is absent in bloodstream form *T. brucei* (15, 23). The bloodstream form-specific nature of the up-regulation that we observe makes it plausible that bloodstream form-specific factors involved in both DNA repair and transcription play a role in the ES control necessary to mediate antigenic variation.

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