CONTROL OF METACYCLIC VSG GENE EXPRESSION IN THE LIFE CYCLE OF *Trypanosoma brucei*

Ben Wymer

Ph.D.

Wellcome Unit of Molecular Parasitology
Anderson College
University of Glasgow

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Summary

African trypanosomes evade the immune response in the bloodstream of mammalian hosts by antigenic variation, that is by continually switching their variant surface glycoprotein (VSG) coat on the cell surface. VSG genes are first expressed at the host-infective metacyclic stage in the salivary glands of the insect vector, the tsetse fly. At this stage each trypanosome expresses a single gene from a small specific subset of VSG genes (<=27 in *Trypanosoma brucei rhodesiense*). Unusually for trypanosomes the metacyclic VSG genes (M-VSG genes) are transcribed as short, monocistronic transcription units located at the ends (telomeres) of the largest set of trypanosome chromosomes. Previously, nuclear run-on analysis identified the promoter region of one metacyclic VSG gene, the ILTat 1.22 M-VSG gene, at a position 2.6-3.0 kb upstream of the VSG gene. The promoter region was shown to be active only at the metacyclic stage and silent at both the procyclic and bloodstream stages. This is the only gene analysed thus far in Kinetoplastida shown to be transcriptionally regulated: all other genes are, at least in part, under post-transcriptional control.

In this study a cloned restriction fragment containing the 1.22 M-VSG gene promoter was placed upstream of a copy of a chloramphenicol acetyltransferase (CAT) reporter gene in a plasmid vector and used in transient transfection of trypanosomes. In transient transfection of bloodstream trypanosomes the putative 1.22 M-VSG gene promoter was highly active although, previous studies had shown the same fragment to be inactive in transient transfection of procyclic trypanosomes (Graham and Barry, 1995). In order to determine what sequences within the promoter region were necessary for the observed activity of the promoter, a series of deletion mutants were prepared across the promoter region in a 5' to 3' direction and 3' to 5' direction. These deletion mutants were cloned into a CAT reporter plasmid and promoter fragment activity was determined by transient transfection of bloodstream trypanosomes. The deletion analysis indicated a region of 96 bp was necessary to maintain full promoter activity.

In vivo promoter analysis had shown that the 1.22 M-VSG gene promoter was inactive in bloodstream trypanosomes and it was possible that promoter activity in the transient transfection experiments was due to loss of regulation imposed by its normal chromosomal context. To study the effect of chromosomal location on the 1.22 M-VSG gene promoter, experiments integrating reporter constructs into the bloodstream trypanosome genome were conducted. First, bloodstream trypanosomes of stock EATRO 795 (from which the putative promoter fragment of the 1.22 M-VSG gene was isolated)

were adapted to axenic culture to allow transfection, cultivation and selection of stable transformants. Culture adapted bloodstream trypanosomes were phenotypically bloodstream forms which were highly virulent in mice and could differentiate, at least partially, to procyclic forms. For stable transformation two reporter constructs were used to integrate the promoter first to its endogenous expression telomere and second, to a chromosomal-internal position, the untranscribed spacer region of the ribosomal RNA gene transcription unit. In both cases the reporter construct contained a copy of the 1.22 M-VSG gene promoter directing transcription of a CAT gene. The promoter was flanked 5' by sequences homologous to the appropriate site of genomic integration. CAT gene expression was analysed in the cloned, transformed cells lines by CAT enzyme activity and Northern blot analysis. For the ribosomal untranscribed spacer region, nuclear run-on analysis showed that CAT gene transcription initiated at the 1.22 M-VSG gene promoter. The promoter was found to be up to 40 times more active at the ribosomal locus than when positioned at the 1.22 M-VSG gene expression telomere. Reduced activity of the 1.22 M-VSG gene promoter at the 1.22 M-VSG gene expression telomere suggests that one mechanism for down-regulation of the M-VSG gene promoter in bloodstream trypanosomes may be related to its telomere positioning.

Declaration

I declare that this thesis and the results presented in it are entirely my own work except where otherwise stated.

Ben Wymer

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Abbreviations

ATP	adenosine triphosphate
bp	base pair
ble	phleomycin resistance gene
BSA	bovine serum albumin
CAT	chloramphenicol acetyltransferase
cDNA	complementary deoxyribonucleic acid
cm	centimetre
cpm	counts per minute
DAPI	4,6-Diamidino-2-phenylindole
DEPC	diethylpyrocarbonate
dNTP	deoxynucleotide triphosphate
dpm	degradations per minute
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EATRO	East Africa trypanosomiasis research organisation
EDTA	ethylenediamine tetra-acetic acid
ES	VSG gene expression site
FD	faraday
FIGE	field inversion gel electophoresis
FITC	fluorescein isothiocyanate
g	gravity/gram
IPTG	isopropylthiogalactoside
ILTat	ILRAD *Trypanozoon* antigen type
J	joule
kb	kilobase
kD	kiloDalton
kDNA	kinetoplast deoxyribonucleic acid
l	litre
M	molar
Mb	megabase
mRNA	messenger ribonucleic acid
M-VSG	metacyclic variant surface glycoprotein
neo	neomycin phosphotransferase gene

PARP	procylic acidic repetitive protein
PBS	phosphate buffered saline
pBS	pBluescript
PCR	polymerase chain reaction
pH	acidity (neg.$\log_{10}$ molar [H^+])
poly A	polyadenlylated
psi	pounds per square inch
rDNA	ribosomal deoxyribonucleic acid
rNTP	ribonucleotide triphosphate
rpm	revolutions per minute
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulphate
tRNA	transfer ribonucleic acid
V	volt
VSG	variant surface glycoprotein
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
ZPFM	Zimmerman post fusion medium
ZPFMG	Zimmerman post fusion medium (plus glucose)

Chapter 1

Introduction

1.1 The African trypanosomiases

The diseases (trypanosomiases) caused by Salivarian trypanosomes affect many animal species, including man and his domestic livestock, in sub-Saharan Africa (Hoare, 1972). Sleeping sickness or human African trypanosomiasis initially infects the blood, lymph and other body fluids as well as tissues and organs such as the spleen. In the later stages of trypanosome infection, the central nervous system is usually infected and the patient becomes severely lethargic and is afflicted by bouts of uncontrollable sleep. If the disease is not treated, the patient finally falls into a deep coma and dies. Prior to parasite invasion of the nervous system, human African trypanosomiasis may be successfully treated with drugs such as suramin. Treatment of the later stages of the disease is more dangerous since arsenical drugs such as melarsoprol are used which lead to toxic poisoning in around 10% of treated patients. Around 25,000 new cases of human African trypanosomiasis are reported every year but this figure is derived from approximately 3 million people under surveillance, whilst more than 55 million people in 36 countries are at risk and the real number of people infected each year is probably between 250,000 and 300,000 (Molyneux *et al.*, 1996).

Human African trypanosomiasis in West Africa, is usually caused by *Trypanosoma brucei gambiense*. *T. b. gambiense* is transmitted between hosts by tsetse flies of the *Glossina palpalis* group which are prevalent in wet regions of West Africa, particularly in forested areas close to rivers (Baker, 1974). The number of parasites in the circulation of an animal infected with *T. b. gambiense* is very low and the primary transmission cycle of *T. b. gambiense* is thought to be Man-Fly-Man (Baker, 1974). In humans the infection is chronic usually lasting several years before the patient finally succumbs. Human African trypanosomiasis in East Africa is usually caused by *Trypanosoma brucei rhodesiense* which is transmitted by flies of the *Glossina morsitans* group. *G. morsitans* is more resistant to drier conditions and is prevalent in the vast areas of wooded savannah in East Africa where it feeds mostly on wild ungulates but also on man and his domestic livestock. *T. b. rhodesiense* infection of wild animals and domestic livestock may be chronic or even asymptomatic and the primary transmission cycle is thought to be Ungulate-Fly-Ungulate (Baker, 1974). However, infection of humans by *T. b. rhodesiense* results in an acute disease that can cause death within weeks of infection. The wild and domestic animals thereby act as reservoirs of the human disease (Hoare, 1972).

Apart from the direct effect of trypanosomiasis on man, trypanosomiasis of domestic animals, particularly cattle, causes great economic problems in sub-Saharan Africa. Up to one third of cattle on the African continent are at risk from trypanosomiasis caused by *T. b. brucei*, *T. b. rhodesiense* and particularly *T. congolense* which are all transmitted by tsetse flies (Molyneux *et al.*, 1996). Some species of Salivarian trypanosomes have evolved to be transmitted between hosts in the absence of the tsetse fly and have spread beyond the tsetse fly belt in sub-Saharan Africa. Therefore *T. equiperdum* that infects horses and is transmitted venereally, and *T. evansi* that infects camels and equines and is transmitted mechanically, are found not only in Africa but also in Asia and South and Central America (Molyneux and Ashford, 1983).

1.2 General classification of the trypanosomes

Trypanosomes are unicellular protozoan flagellates that belong to the family Trypanosomatidae which includes several important pathogens in addition to the African trypanosomes. Human pathogens include *Trypanosoma cruzi* which causes Chaga's disease and species of *Leishmania* that cause Human Leishmaniasis, both diseases predominantly occurring in South America. The general classification of trypanosomatids is shown in figure 1.2.1.

In the absence of a protozoan fossil record, such classification is made on the basis of cell morphology, life cycles and host ranges (Vickerman, 1994). The order Kinetoplastida is defined by the presence of the kinetoplast, a complex network of catenated circular DNA molecules that are contained in a specialised region of the single mitochondrion close to the basal body of the flagellum (Priest and Hadjuk, 1994a). The order Kinetoplastida is divided into two families, the Trypanosomatidae which are all uniflagellated obligate parasites, and the Bodonidae, which are biflagellated and may be free-living or parasitic. Parasitic bodonids mostly infect the gut, sexual organs and blood of fish whilst trypanosomatids parasitise a very diverse range of hosts including animals, plants and other protists (Vickerman, 1994). There are several trypanosomatid genera. The genus *Trypanosoma* parasitises all classes of vertebrates and most species are extracellular, living in the blood and body fluids of the host but some species (particularly *Trypanosoma cruzi*) live intracellularly in the muscle cells and other tissues. Trypanosomes are usually transmitted between hosts by cyclical development in an arthropod or leech vector. Organisms of the genus *Leishmania* are intracellular parasites that invade the mononuclear phagocytes of mammals and lizards, being transmitted by

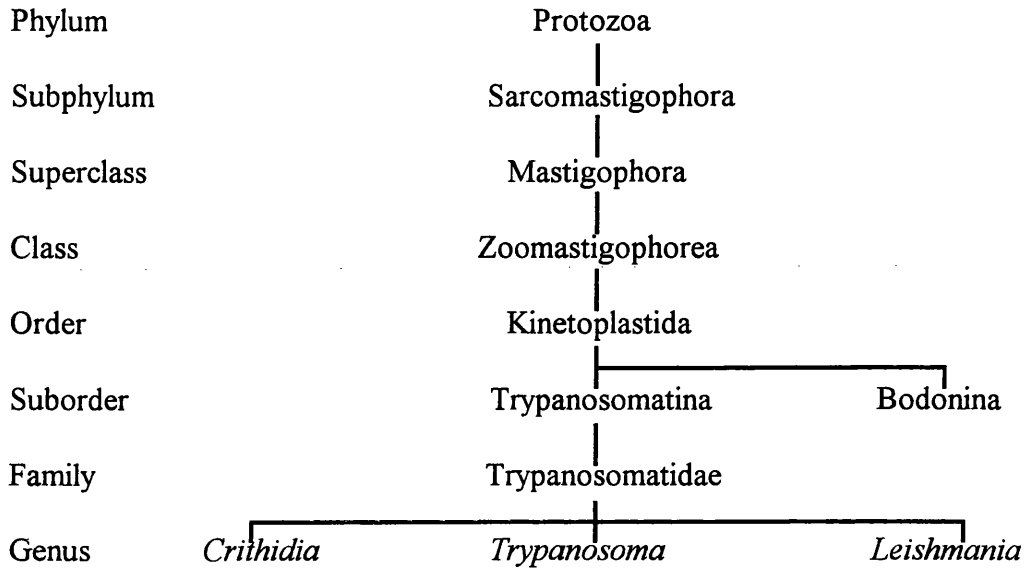


Figure 1.2.1 **The classification of the Kinetoplastida**. *Crithidia*, *Trypanosoma* and *Leishmania* are representative of many genera of the family Trypanosomatidae.

cyclical development in sandflies whilst sloths are infected by *Endotrypanum* species which are also transmitted by sandflies. A large range of flowering plants are infected by species of the genus *Phytomonas* that are transmitted between plants by cyclical development in various insect vectors (Vickerman, 1994).

These trypanosomatid genera (*Trypanosoma*, *Leishmania*, *Phytomonas* and *Endotrypanum*) are digenetic, that is they have life cycles that alternate between a vertebrate or plant host and an invertebrate vector. The remaining trypanosomatid genera (*Crithidia*, *Blastocrithidia*, *Herpetomonas*, *Leptomonas*, *Rhynchoidomonas* and *Proteomonas*) are monogenetic, being maintained in a single host and are mostly parasites of insects. The parasitic infection of monogenetic trypanosomatids is usually passed between hosts in contaminated faeces (Vickerman, 1994).

All trypanosomatids are thought to have evolved from a bodonid-like organism that may have been parasitic or free-living (Maslov and Simpson, 1995). Phylogenetic trees of the trypanosomatids have been constructed by comparing their ribosomal RNA sequences and using bodonids or other protists as an outgroup (Fernandes *et al.*, 1993). The phylogenetic trees suggest that the African trypanosomes were probably the first group of the trypanosomatids to evolve from an ancestral trypanosomatid (Fernandes *et al.*, 1993; Maslov and Simpson, 1995). These data also suggest that the African trypanosomes branched from the eukaryotic lineage at a very early stage, long before the division of eukaryotes into plants, animals and fungi (Sogin *et al.*, 1986; 1989; Fernandes *et al.*, 1993).

1.3 The life cycle of *Trypanosoma brucei*

The three subspecies of *Trypanosoma brucei*, *T. b. brucei*, *T. b. rhodesiense* and *T. b. gambiense*, are morphologically indistinguishable from each other (Hoare, 1972). These subspecies of *T. brucei* essentially undergo the same cyclical development in the tsetse fly (Hoare, 1972). Figure 1.3.1 is a schematic representation of the digenetic life cycle of *Trypanosoma brucei* and illustrates the two main life cycle stages, the bloodstream stage in the mammalian host and the procyclic stage in the insect vector. These life cycle stages are characterised by changes in the gross morphology of the cells, by changes to the mitochondrion and glycosomes that produce energy from oxidation of amino acids and glucose respectively, and by changes in the trypanosome surface membrane (Vickerman, 1985; Priest and Hadjuk, 1994a).

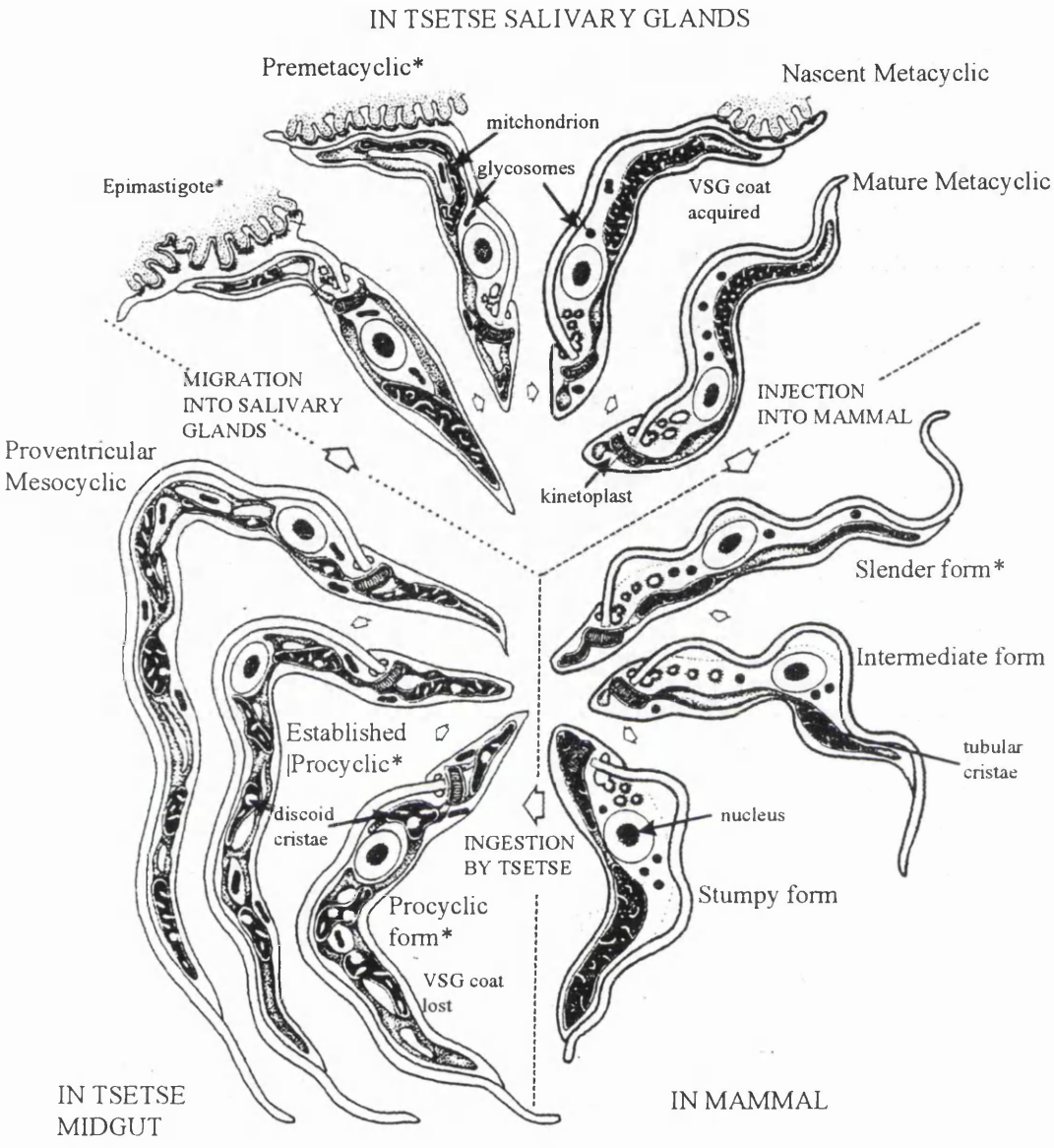


Figure 1.3.1. **Schematic representation of the life cycle of *Trypanosoma brucei*.** The mammalian bloodstream stages are shown on the right, the procyclic stages in the tsetse fly midgut on the left and the stages in the tsetse fly salivary gland at the top. The trypanosomes are represented in relative sizes during the developmental cycle. The diagram highlights the changes in the mitochondrion, glycosomes and the position of the nucleus and kinetoplast. Trypanosomes that express a VSG surface coat are shown in the right half of the diagram with a slightly thicker outline. * indicates that cell division occurs at these stages. After Vickerman, 1985.

The complex changes in trypanosome structure and ultrastructure during the life cycle reflect the very different conditions encountered by the trypanosome population in the fly vector and mammalian host. In the bloodstream of the mammalian host, long slender form bloodstream trypanosomes predominate during the exponential growth of the infection. These trypanosomes divide asexually by binary fission and can penetrate the walls of the blood vessels and lymphatics to enter connective tissue and late in the infection the central nervous system (Hoare, 1972). Long slender bloodstream trypanosomes contain prominent spherical glycosomes, organelles that are unique to kinetoplastids, that contain the glycolytic enzymes required for oxidation of glucose from the blood (Opperdoes and Borst, 1977). Pyruvate, the product of glycolysis, does not enter the citric acid cycle in the mitochondrion, but is excreted into the blood (Vickerman, 1985). The mitochondrion is therefore maintained in a repressed state in long slender bloodstream form trypanosomes, with an unbranched structure and no observable cristae (Priest and Hadjuk, 1994a). On the surface of all bloodstream form trypanosomes, and attached to the surface membrane, there is a dense surface coat that is 12-15 nm thick (Vickerman, 1969) and composed of variant surface glycoprotein (VSG) molecules (Cross, 1975). The VSG surface coat protects the trypanosome from non-specific lysis in the bloodstream and, through antigenic variation of the VSG molecules, allows the bloodstream trypanosome population to evade the specific cell-mediated immune response of the host (Barry and Turner, 1991; see 1.4).

During the course of a bloodstream infection, some long slender form bloodstream trypanosomes differentiate to the short stumpy form (Barry and Turner, 1991; Hesse *et al.*, 1995). Differentiation to the short stumpy form is characterised by shortening of overall cell length and an increase in the relative cell volume occupied by the mitochondrion which now develops tubular cristae (Vickerman, 1985). The short stumpy form does not divide and is not thought to switch its VSGs, though the surface coat is maintained (Vickerman, 1985). When ingested by a tsetse fly during feeding, bloodstream form trypanosomes are taken into the lumen of the midgut with the blood meal (Hoare, 1972). Here bloodstream trypanosomes differentiate to dividing procyclic trypanosomes. Short stumpy forms differentiate more readily to procyclic trypanosomes than long slender bloodstream trypanosomes *in vivo* (Wijers and Willet, 1960; Ashcroft, 1960) and *in vitro* (Ziegelbauer *et al.*, 1990) probably due to the partial activation of their mitochondrion (Vickerman, 1985).

In procyclic trypanosomes the mitochondrion is well developed, it is highly branched with many discoid cristae (Vickerman, 1985) and occupies up to 25% of the cell volume (Hecker, 1980). This is due to the switch from glucose as the major energy source, to oxidation of amino acids, especially proline, in the mitochondrion by means of the citric acid cycle (Bowman and Flynn, 1976). Conversely, the glycosomes that are used for glucose metabolism in bloodstream trypanosomes, become inactive in procyclic trypanosomes and change in shape from spherical to bacilliform (Vickerman, 1985). Under a light microscope, procyclic trypanosomes appear longer than bloodstream trypanosomes with the kinetoplast positioned closer to the nucleus and in a less posterior position (Vickerman, 1985; see Fig. 4.2.2.2). Following the triggering of differentiation from bloodstream forms to the procyclic form (primarily a shift in temperature from 37^0C to 26^0C), the surface coat of bloodstream trypanosomes is shed within 6 hours (Ziegelbauer *et al.*, 1993), synthesis of VSG stops (Overath *et al.*, 1983) and VSG gene mRNA is degraded (Pays *et al.*, 1993). In its stead, the procyclic trypanosomes express a surface molecule called procyclin or PARP (Roditi *et al.*, 1989; Ziegelbauer *et al.*, 1990) that may protect the trypanosome from the hostile environment of the tsetse fly midgut.

Within several days of establishing a mature infection in the tsetse fly midgut, procyclic trypanosomes slowly progress back towards the proventriculus and via the oesophagus and salivary tracts to the salivary glands (Hoare, 1972). In the salivary glands, the trypanosomes differentiate to dividing epimastigote forms that are attached to the epithelial cells of the salivary gland by tiny projections of their flagellum (flagellipodia) which interdigitate with the microvilli of the epithelial cells (Tetley and Vickerman, 1985). Epimastigote forms differentiate in the salivary glands to non-dividing metacyclic trypanosomes. Nascent metacyclic forms that remain attached to the epithelium, contain an unbranched mitochondrion and spherical glycosomes reminiscent of bloodstream trypanosomes (Vickerman, 1985). These nascent metacyclic forms also reacquire the VSG surface coat that is required for survival in the mammalian host (Tetley *et al.*, 1987). Finally, the nascent metacyclics detach from the salivary gland epithelium to yield the mature metacyclic form, remaining in the lumen of the salivary gland until the contents of the glands are injected into the mammalian host when the fly takes a blood meal (Tetley and Vickerman, 1985). Metacyclic trypanosomes are probably triggered to differentiate to bloodstream form trypanosomes, and resume cell division, when the temperature shifts from 26°C to 37°C.

1.4 The genetics of *Trypanosoma brucei*

DNA-DNA renaturation experiments showed that the haploid DNA content of *Trypanosoma brucei* was about 7 x 10^7 bp (Van der Ploeg *et al.*, 1989) of which about 68% is single copy, 12% highly repetitive and 20% moderately repetitive (Borst *et al.*, 1980). However, *Trypanosoma brucei* is probably diploid at all life cycle stages, though this cannot be verified by classical karyotyping as the chromosomes of kinetoplastids do not condense prior to mitotic division (Vickerman and Preston, 1970). Instead, studies of restriction fragment length polymorphisms (RFLPs) between alleles of housekeeping genes (Gibson *et al.*, 1985), analysis of isoenzymes (Tait, 1980; Tait *et al.*, 1989) and the DNA content of individual cells (Borst *et al.*, 1982) suggests that *Trypanosoma brucei* has a diploid genome.

Though individual trypanosome chromosomes cannot be identified by light or electron microscopy, they may be separated by pulsed field gel electrophoresis (PFGE) into approximately 20 bands (Van der Ploeg *et al.*, 1989). In *Trypanosoma brucei*, band 1 contains as many as 100 minichromosomes (50-150 kb), bands 2-6 contain chromosomes that are 200-430 kb in size and bands 7-15 contain chromosomes that are 680 kb-3 Mb in size. Bands 16, 17 and 19 correspond to chromosomes of size ~3 Mb, ~4.7 Mb and ~5.7 Mb respectively. Some chromosomal material remains in the slot of the pulsed field gel or close to it, and this is considered band 20 although it probably contains DNA maintained in the slot non-specifically (Van der Ploeg *et al.*, 1989). Southern blot analysis showed that of 18 trypanosome genes tested, all were located on the larger chromosomes in bands 7-19 (Gottesdiener *et al.*, 1990). Surprisingly, each of these genes were located on two different sized chromosomes (except the ribosomal RNA genes which were located on 4 chromosomes) and indicated that the chromosomes in bands 7-19 could be divided into at least 7, or as many as 10 homologous pairs (Gottesdiener *et al.*, 1990). Mostly the proposed chromosome homologues varied in size by about 20%, but some homologues (band 17 and 14, and band 16 and 11) varied in size by as much as 1.5 Mb (Gottesdiener *et al.*, 1990).

The ends of chromosomes in *Trypanosoma brucei* contain hexanucleotide telomere repeats of the sequence GGGTTA (Blackburn and Challoner, 1984; Van der Ploeg *et al.*, 1984a). The length of telomere repeat arrays at the ends of trypanosome chromosomes can vary between 2 and 26 kb (van Leeuwen *et al.*, 1996). The telomere repeat array usually increases at the rate of approximately 6 bp per generation (Van der

Ploeg *et al.*, 1984a) and the length of the telomere repeat arrays is periodically reduced by the loss of a large number of repeats (Bernards *et al.*, 1983; Van der Ploeg *et al.*, 1984a). At trypanosome chromosome ends there may also be larger subtelomeric repeats (29 bp in one instance; Van der Ploeg *et al.*, 1984a) that are directly upstream of the terminal hexanucleotide telomere repeats.

Low stringency hybridisation with the conserved C terminus of the VSG genes showed that these genes were located on 4 larger chromosomes (2 homologous pairs) as well as the minichromosomes (band 1) and intermediate chromosomes (bands 2-6) (Van der Ploeg *et al.*, 1989). Evidence suggests that trypanosomes may be aneuploid for minichromosomes and the intermediate chromosomes (Gottesdiener *et al.*, 1990) and furthermore, may be haploid for the majority of VSG genes including those contained on homologous chromosomes (Van der Ploeg *et al.*, 1982; 1984c; Borst, 1986).

Trypanosoma brucei divides asexually by binary fission at the bloodstream stage (the long slender form), procyclic stage and epimastigote stage (Vickerman, 1985). However, the diploid nature of the trypanosome genome and the presence of homologous chromosomes suggests that there is a haploid stage or that haploid gametes are formed at some point in the life cycle. Evidence for sexual reproduction in trypanosomes first came from analysis of isoenzymes in field isolates of *Trypanosoma brucei* (Tait, 1980). Later experiments involved the co-transmission of two cloned stocks of *Trypanosoma brucei* with different genotypes, through tsetse flies and the formation of hybrid progeny was confirmed by the inheritance of isoenzyme markers (Jenni *et al.*, 1986), molecular karyotypes, kDNA and human serum sensitivity (Gibson, 1989) and RFLPs (Turner *et al.*, 1990). The mechanism by which hybrid trypanosomes are generated has not yet been elucidated (see Sternberg and Tait, 1990 for a review) but genetic exchange is known to occur at some point during development in the fly (Turner *et al.*, 1990) and is unlikely to occur at the procyclic stage (Turner *et al.*, 1990). Lastly, the sexual cycle is not obligatory in trypanosomes and tsetse flies that are fed on two trypanosomes stocks, may produce metacyclic forms with parental genotypes as well as hybrid genotypes (Tait *et al.*, 1989; Turner *et al.*, 1990).

1.5 The VSG surface coat and immune evasion

At each life cycle stage, *Trypanosoma brucei* displays on its cell surface predominantly one protein species which is heavily glycosylated and attached to the cell membrane by a GPI anchor (McConville and Ferguson, 1993). At the procyclic and

epimastigote stages this protein is procyclin or PARP (Roditi *et al.*, 1989) and at the bloodstream and metacyclic stages this protein is the variant surface glycoprotein (VSG) (Cross, 1975; Vickerman, 1985). Approximately 10^7 VSG molecules (Cross, 1975) form a dense surface coat that covers the entire cell surface including the flagellum (Vickerman, 1969), and protects the parasite from non-specific and specific immune responses of the mammalian host (Vickerman, 1985). Non-specific immune responses are mediated by the complement alternative pathway, however this is not activated by VSG-coated trypanosomes (Ferrante and Allison, 1983). In the absence of a non-specific response to VSG-coated bloodstream trypanosomes, the mammalian host can only remove trypanosomes from the circulation by specific immune responses.

Specific immune responses to VSG-coated bloodstream trypanosomes are mediated by lymphocytes, particularly B cell lymphocytes, which are activated by trypanosome antigens. During a trypanosome infection, B cell lymphocytes are usually activated by the immunodominant VSG antigens and secrete anti-VSG antibodies which lyse trypanosomes and opsinise them for uptake by phagocytes. Because each trypanosome expresses only one VSG species (Cross, 1996), and within a bloodstream population there may be only one or a few dominantly expressed VSG species, anti-VSG antibodies produced against a dominantly expressed VSG leads to a relapse in the parasitaemia. However, the bloodstream population can escape the immune response by continually switching expression to antigenically distinct VSGs, a process of antigenic variation (Cross, 1990; 1996; Barry and Turner, 1991). The resulting infection is usually characterised by a series of peaks of parasitaemia in the blood which are neutralised by VSG-specific antibodies (Barry, 1986; Barry and Turner, 1991). Antigenic variation in the bloodstream trypanosome population continues in the host until the animal is overcome by the trypanosome infection or until the host raises antibodies against every VSG in the trypanosome repertoire and recovers from the infection. Unfortunately for the infected host, a bloodstream trypanosome population may express more than 100 antigenically distinct VSGs (Capbern *et al.*, 1977).

Switching expression from one VSG to another can occur at an overall rate of between 10^{-2} and 10^{-3} per trypanosome per generation in cloned lines of trypanosomes that have recently (within 100 generations) been transmitted through tsetse flies (Turner and Barry, 1989). However, some other trypanosome lines that have been adapted to growth in laboratory animals, switch their VSG coat at a much reduced rate (approximately 10^{-6} per trypanosome per generation, Turner and Barry, 1989; Cross,

1996). These laboratory-adapted lines are also very virulent, are generally unable to be transmitted through tsetse flies and usually produce acute infections in animals with very sharp peaks of parasitaemia (Barry and Turner, 1991; Cross, 1996). The highest rate of switching observed in fly-transmitted trypanosomes means that almost 1% of the trypanosome population can switch the VSG expressed every 6-10 hours (the doubling time for exponentially growing bloodstream trypanosomes). If trypanosomes switched to all available VSGs with equal frequency, then the entire trypanosome VSG repertoire would be very quickly expressed. Clearly then, it is necessary to present each of the VSGs to the host in a sequence such that the host can only acquire immunity to a small number of VSGs at a time. Experiments on animal trypanosome infections show that bloodstream trypanosomes do tend to express VSGs in an approximate order (Capbern *et al.*, 1977; Barry, 1986; Barry and Turner, 1991) that may be dependent on the position of the VSG gene in the genome (Thon *et al.*, 1990; see 1.6). The relatively high switching rate (10^{-2} per trypanosome per generation) suggests that switching does not occur by random mutation events but rather is driven by a specific mechanism or mechanisms (Borst and Greaves, 1987; Turner and Barry, 1989; see 1.6).

To be successful in evading the host immune response, a new VSG molecule must be functionally equivalent to its predecessor whilst exposing unique epitopes (antigenic determinants) to the host and concealing epitopes that are invariant between VSGs. There is no known biochemical activity associated with VSG molecules and so their only function is to fold correctly, target the surface membrane and form a dense surface coat (McConville and Ferguson, 1993). When released from the surface membrane by mechanical lysis, VSG molecules are associated in solution as homodimers and this is thought to be the form they adopt on the trypanosome surface (Auffret and Turner, 1981). The structure of the VSG dimer and its relationship with the trypanosome surface membrane is shown in figure 1.5.1. Each VSG monomer is approximately 60 kD in size (433-470 amino acids for *T. brucei*, Cross, 1990) and linked at the C-terminal residue to a GPI anchor in the surface membrane (Ferguson *et al.*, 1988). The GPI anchor targets the VSG molecules to the cell surface and allows a very high level of protein-packing at the trypanosome surface without compromising the fluidity of the surface membrane (McConville and Ferguson, 1993). The dense packing of VSG molecules is thought to be critical for the function of the surface coat: firstly, this prevents the large immunoglobulins of the host from recognising invariant surface molecules of the trypanosome, which are proposed to be situated between VSG

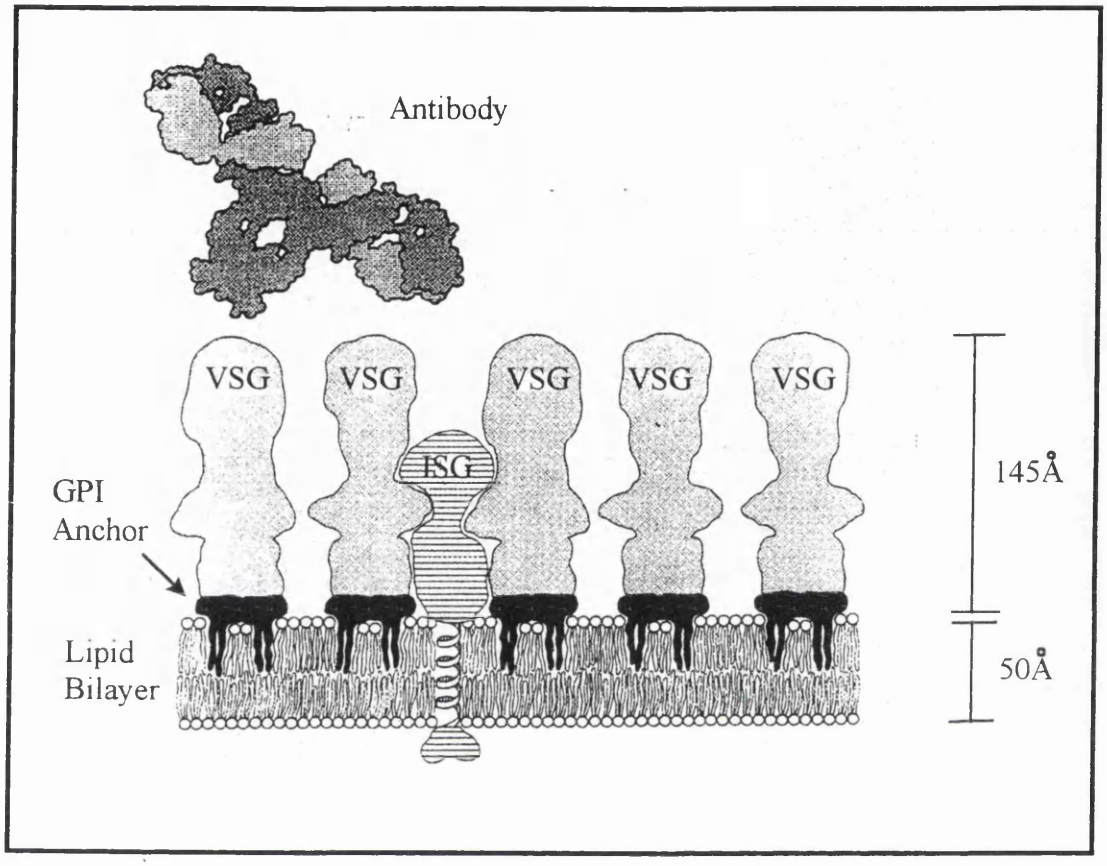


Figure 1.5.1. **A proposed arrangement of cell surface molecules in African trypanosomes**. The diagram shows the surface membrane lipid bilayer in cross-section. VSG dimers are shown anchored to the surface membrane by GPI anchors whilst an invariant surface glycoprotein is shown linked to the surface membrane by a membrane spanning α-helix. The ISG molecule does not extend beyond the VSG surface coat and therefore is unlikely to be detected by host antibody molecules shown in relative size in the diagram. The approximate sizes of each of the components of the surface membrane are shown on the right. After Overath *et al.*, 1994.

molecules and secondly, dense packing of the VSG molecules means that only a small region of each VSG molecule is exposed to the host's immunoglobulins (Overath *et al.*, 1994). The C-terminal domain, comprising about 20% of the VSG molecule, is relatively well conserved (Cross, 1984). Surprisingly, the N-terminal domain is almost totally unconserved in amino acid sequence (primary structure) and yet maintains an almost invariable tertiary structure with considerable conservation of secondary structure (Freyman *et al.*, 1990; Blum *et al.*, 1993). Only a small region of the N-terminal domain is exposed as epitopes to host immunoglobulins (Blum *et al.*, 1993) and so this high level of divergence in amino acid primary structure seems unnecessary, hypervariability of just 15 amino acid positions would be sufficient to present an almost unlimited number of different epitopes (Rossmann *et al.*, 1985). However, when trypanosomes in the bloodstream have been lysed, VSG epitopes of the N-terminal domain, that are usually concealed, may be exposed. These epitopes may activate lymphocytes which could elicit an effective immune response against the entire N-terminal region of a VSG molecule. If this is the case then it is necessary for the different VSG molecules to be highly variable in amino acid sequence over the entire N-terminal domain and not just the short sequence that is exposed to host immunoglobulins (Blum *et al.*, 1993).

In the circulation of the mammalian host, trypanosomes need to acquire various nutrients from the bloodstream. Small molecules such as glucose can penetrate the surface coat and are moved across the surface membrane by multi-membrane-spanning polypeptides, the trypanosome glucose transporters (Bringaud and Baltz, 1993). Ziegelbauer *et al.* (1992) proposed that these transporter molecules do not project a significant distance from the surface membrane and do not perturb the packing of the VSG surface coat. Therefore, the invariant glucose transporter remains undetected by host immunoglobulins and may be distributed across the entire cell surface (Ziegelbauer *et al.*, 1992). There are at least two examples of invariant surface proteins, of unknown function, which in live trypanosomes were unrecognised by host antibodies and have been shown to be distributed evenly across the trypanosome surface membrane (Ziegelbauer *et al.*, 1992). Endocytosis of macromolecules that cannot penetrate the surface coat poses a problem for the parasite since the necessary receptors would be invariant molecules projecting beyond the surface coat and would be accessible to host immunoglobulins. However, to solve this problem, endocytosis in bloodstream trypanosomes occurs exclusively at the flagellar pocket, a closed invagination of the cell membrane at the base of the flagellum (Borst, 1991b; Webster and Russell, 1993). At

the aperture of the flagellar pocket the flagellum is bound to the cell body by hemidesmosome-like junctions which prevents access of lymphocytes and phagocytes but allows access of macromolecules such as proteins and complex lipids (Borst, 1991b). The receptors for transferrin and the low density lipoprotein are both thought to be localised at the flagellar pocket and remain undetected by the host in live trypanosomes (Borst, 1991b). However, antibodies against flagellar pocket proteins are produced by the infected host probably when trypanosomes are lysed (Hobbs and Boothroyd, 1990) and these immunoglobulins, though very large, can penetrate the flagellar pocket (Borst, 1991b) and may prove successful in interfering with endocytosis (Olenick *et al.*, 1988).

1.6 The molecular basis of antigenic variation

There are as many as 1000 VSG genes (and pseudogenes) in the genome of *Trypanosoma brucei* (Van der Ploeg *et al.*, 1982). The majority of these genes are clustered in large tandem arrays at internal regions of chromosomes that are 1.5-5.7 Mb in size (Van der Ploeg *et al.*, 1982; Van der Ploeg *et al.*, 1989), though a large number are found at the very ends (telomeres) of some trypanosome chromosomes, most notably the minichromosomes (Van der Ploeg *et al.*, 1984c). However, VSG genes can only be expressed when located at special telomeric sites called expression sites (De Lange and Borst, 1982; Kooter *et al.*, 1987; Pays *et al.*, 1989a; Zomerdijk *et al.*, 1991b; see Cross, 1996 for review). Three of these expression sites have been mapped to chromosomes of 300 kb, 1.5 Mb and 3 Mb (Van der Ploeg *et al.*, 1989). It is estimated from hybridisation experiments with expression site sequences, that there are up to 20 bloodstream VSG gene expression sites in the diploid genome of *Trypanosoma brucei* (Cully *et al.*, 1985; Kooter *et al.*, 1987; Zomerdijk *et al.*, 1990; Navarro and Cross, 1996) each containing a single VSG gene (Cully *et al.*, 1985; Kooter *et al.*, 1987; Pays *et al.*, 1989a). Normally only one of these bloodstream VSG gene expression sites is fully active at any one time and therefore, each trypanosome expresses only one VSG (Cross, 1990; Rudenko *et al.*, 1995). The VSG gene is transcribed from an expression site promoter positioned approximately 45-60 kb upstream (Kooter *et al.*, 1987; Pays *et al.*, 1989a; Crozatier *et al.*, 1990; Zomerdijk *et al.*, 1990; Jefferies *et al.*, 1991). Other than the variation in size, expression sites are remarkably similar in sequence and structure, a schematic representation of a typical expression site is shown in figure 1.6.1.A. Between the expression site promoter and the VSG gene there are up to 10 expression site associated genes (ESAGs) which are co-transcribed with the VSG gene from the

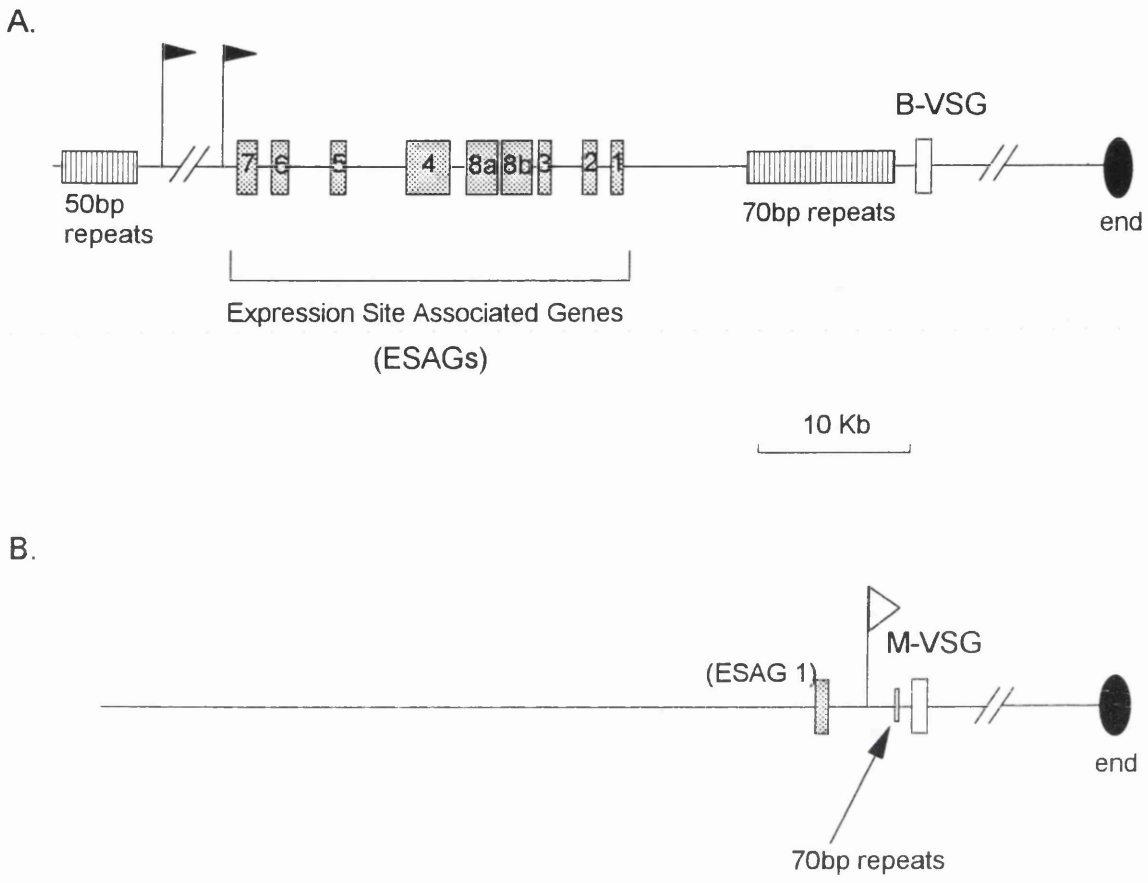


Figure 1.6.1. **A schematic representation of the consensus structrue of the bloodstream VSG gene expression sites and metacyclic VSG gene telomeres.** (A) In the bloodstream VSG gene expression site, the VSG gene (B-VSG) is co-transcribed with the expression site associated genes (ESAGs) from an expression site promoter (black flag). In some expression sites there is a second copy of the expression site promoter ~15 kb upstream of the first promoter which is flanked upstream by 50 bp repeats. Starting 1-2 kb upstream of the VSG gene there is a series of imperfect 70 bp repeats that is usually several kilobases long. The end of the chromosome is represented on the right of the diagram. (B) At the metacyclic VSG gene expression telomere the metacyclic VSG gene (M-VSG) is transcribed from the metacyclic VSG gene promoter (white flag) a short distance upstream. The metacyclic VSG gene is flanked by only a small number of 70 bp repeats. There are no ESAGs that are co-transcribed with the metacyclic VSG gene but at some metacyclic expression telomeres there is an ESAG 1-related gene or partial sequence of an ESAG 1 gene upstream of the metacyclic VSG gene promoter.

expression site promoter (Cully *et al.*, 1985; Kooter *et al.*, 1987; Johnson *et al.*, 1987; Pays *et al.*, 1989a; Crozatier *et al.*, 1990). ESAGs encode proteins of different functions (see Vanhamme and Pays, 1995 for a review) some of which have already been identified such as the transferrin receptor, a heterodimer of the protein products encoded by ESAG 6 and ESAG 7 (Schell *et al.*, 1991), and an adenylate cyclase encoded by ESAG 4 (Ross *et al.*, 1991). The protein product of ESAG 8 is somewhat unusual in that it encodes a nuclear protein (Lips *et al.*, 1996). The transferrin receptor, and possibly other proteins encoded by ESAGs, is only required at the bloodstream stage and the grouping of the ESAGs in the bloodstream VSG gene expression site may allow developmental expression of these genes at the bloodstream stage (Clayton, 1992). There is no evidence that ESAGs are involved in expression or function of the VSG surface coat, although most ESAGs probably encode membrane-associated proteins (Vanhamme and Pays, 1995).

Antigenic variation occurs when a bloodstream trypanosome switches expression from one VSG gene to another. This can happen in two main ways, either the active expression site transcribing the expressed VSG gene can be silenced and a another expression site, containing a different VSG gene, can be activated, or the trypanosome can change the VSG gene in the active expression site. The different mechanisms of VSG gene activation are summarised in figure 1.6.2 and are reviewed by Borst (1991a), Van der Ploeg *et al.* (1992) and Pays *et al.* (1994). The mechanism of activating a VSG gene *in situ* at a silent telomeric expression site, and concomitantly silencing the active expression site, remains obscure. At some expression sites there are two copies of the expression site promoter and recombination between these promoters, removing the intervening 13-15 kb of sequence, has been proposed as a mechanism for expression site inactivation (Gottesdiener *et al.*, 1991; 1992; Lodes *et al.*, 1993). However, DNA rearrangements were not observed within 1.4 kb upstream of the expression site promoter when another active expression site was inactivated (Zomerdijk *et al.*, 1990). In addition, chromosomal rearrangements were not observed when an expression site was silenced and reactivated (Rudenko *et al.*, 1995), suggesting that small or large scale DNA rearrangements are not necessary for activating VSG genes *in situ*.

A little more is known about the events which result in a new VSG gene replacing a resident VSG gene in an active expression site. The untranscribed VSG genes at chromosome-internal positions and at the telomeres of minichromosomes, termed basic copy genes, may only be transcribed and expressed by transfer to a

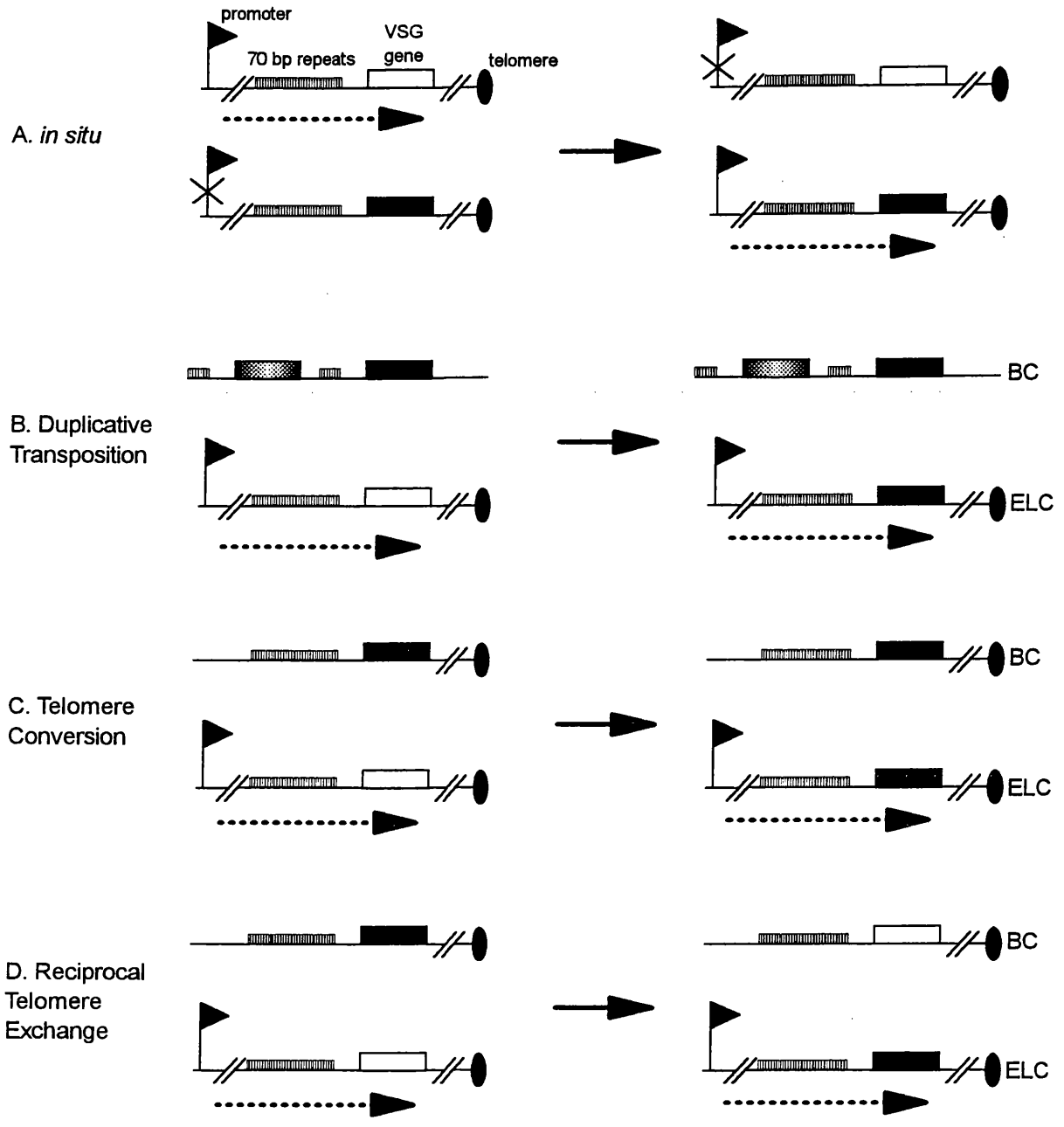


Figure 1.6.2. **Mechanisms of antigenic variation in African trypanosomes.** The diagram represents four known mechanisms of antigenic variation. (A) *in situ* activation of a VSG gene. An expression site transcribing one VSG gene is inactivated and another expression site transcribing adifferent VSG gene is activated. (B) Duplicative transposition, a basic copy VSG gene is copied to an active expression site, displacing the resident VSG gene. (C) Telomere conversion, a telomeric basic copy VSG gene is copied to an active expression site displacing the resident VSG gene. (D) Reciprocal telomere exchange, the telomeric region of an active expression site, including the VSG gene, is exchanged with a telomerecontaining another VSG gene. Black flag, expression site promoter; crossed black flag, inactivated promoter; dotted arrow; expression site transcription; open and filled boxes, VSG genes; repeated small boxes, 70 bp repeats; black oval, chromosome end; BC, basic copy; ELC, expression linked copy.

telomeric expression site (De Lange and Borst, 1982; see Van der Ploeg *et al.*, 1992 for review). Transfer of a silent VSG gene to an expression site may occur by 3 different mechanisms; 1) duplicative transposition, 2) telomere conversion, or 3) reciprocal telomere exchange (see Fig. 1.6.2). Duplicative transposition involves duplication of a basic copy VSG gene and transposition of the duplicated copy to an expression site replacing the resident VSG gene (Hoeijmakers *et al.*, 1980; Pays *et al.*, 1981). Transposition of the duplicated copy to the expression site is thought to occur by homologous recombination between common sequences flanking the basic copy VSG gene and the VSG gene at the expression site (Cross, 1990). Usually chromosome-internal VSG genes are flanked at the 5' end by a small number of copies (1-17) of imperfect 70 bp repeats that that are (A-T)-rich (Van der Ploeg *et al.*, 1982; Aline *et al.*, 1985). VSG genes that reside at a telomeric expression site are flanked at the 5' end by a large array of these 70 bp repeats that start 1-2 kb upstream of the VSG gene and may be several kilobases in length (Campbell *et al.*, 1984; Shah *et al.*, 1987). When a duplicated copy of a VSG gene is inserted into a expression site the 70 bp repeats are often thought to be the limit of recombination at the 5' end (Michels *et al.*, 1984). At the 3' end of the transposed VSG gene the limit of recombination is usually homology in the 3' coding sequence of the VSG gene or the 3' untranslated region (UTR) (Timmers *et al.*, 1987; Lee and Van der Ploeg, 1987). Telomere conversion is similar in mechanism to duplicative transposition except that a telomeric VSG gene rather than a chromosome-internal VSG gene is duplicated and transposed to an expression site (Bernards *et al.*, 1984). However, the limits of recombination may be as far as 40 kb upstream of the VSG gene (Borst, 1986) and may extend downstream to the telomeric repeats (Bernards *et al.*, 1984). The large number of minichromosomes, present only in species of trypanosomes that undergo antigenic variation, may function only to increase the number of telomeric VSG genes that are available for telomere conversion events (Van der Ploeg *et al.*, 1984c). Reciprocal telomere exchange appears to be a rare event and involves the complete exchange between telomeres of sequences that include a VSG gene (Pays *et al.*, 1985).

To date the timing and frequency of each of these mechanisms of antigenic variation during a natural bloodstream infection has not been determined. It is necessary for the bloodstream trypanosome to express the VSG gene repertoire in an approximate order so that the antigens are not presented to the host simultaneously (Cross, 1990; see 1.5). The variety of mechanisms by which VSG genes are activated may be fundamental

to sequential expression of VSG genes in the host (Pays, 1989; Thon *et al.*, 1990; Barry and Turner, 1991; Vanhamme and Pays, 1995). VSG genes that reside at telomeres, and are expressed by switching the active expression site or by telomere conversion, may be the first VSG genes to be expressed during the course of an infection (Michels *et al.*, 1984; Aline *et al.*, 1985), whilst chromosome-internal VSG genes, activated by duplicative transposition, may be expressed later in the infection (Michels *et al.*, 1984). However, the mechanism of duplicative transposition is very important means of antigenic variation during a long term infection as this is the only mechanism by which chromosome-internal VSG genes, that constitute the majority of VSG genes in the trypanosome genome, may be expressed.

1.7 The activation of metacyclic VSG genes

When bloodstream trypanosomes enter the tsetse fly midgut they rapidly differentiate to the procyclic form (Vickerman, 1985). This differentiation is accompanied by shedding of the VSG surface coat and acquisition of the procyclic stage-specific molecule, procyclin or PARP (procyclic acidic repetitive protein), which covers the cell surface (Roditi *et al.*, 1987; 1989; Mowatt and Clayton, 1988; Ziegelbauer *et al.*, 1990). PARP is expressed at all insect stages of the parasite until the epimastigote form differentiates to the metacyclic form in the salivary glands of the fly. At the metacyclic stage the trypanosomes reacquire the VSG surface coat (Tetley and Vickerman, 1985; Tetley *et al.*, 1987) but express only a limited number of VSGs; no more that 27 in *T. b. rhodesiense* (Turner *et al.*, 1988) and 12 in *T. congolense* (Crowe *et al.*, 1983), which is ~1-2% of the total VSG repertoire. Immunogold labelling and immunofluorescence using monoclonal antibodies or antisera against specific metacyclic VSGs (M-VSGs), labels different subsets of trypanosomes in the metacyclic population, indicating that each metacyclic trypanosome expresses a different metacyclic VSG (Hadjuk *et al.*, 1981; Barry *et al.*, 1983; Crowe *et al.*, 1983; Tetley *et al.*, 1987). Immunogold labelling of metacyclic trypanosomes in the salivary gland also shows that different metacyclic VSGs are activated simultaneously in adjacent cells whilst the metacyclic forms are still attached to the salivary gland epithelium, and therefore heterogeneity in the population is achieved as the VSG surface coat is expressed and is not due to antigenic variation (Tetley *et al.*, 1987). The presentation of a mixture of antigen types at the metacyclic stage may be necessary for metacyclic trypanosomes to infect hosts that are already pre-

immune to one or more of the metacyclic VSGs due to previous trypanosome infection (Barry *et al.*, 1990).

At the bloodstream stage, trypanosomes of one antigenic type are usually the result of clonal expansion from a single trypanosome expressing one VSG (Cross, 1990). However, the simultaneous activation of metacyclic VSGs in the salivary glands, combined with the fact that metacyclic forms do not divide, means that metacyclic trypanosome populations are polyclonal rather than clonal with respect to the surface coat expressed (Tetley *et al.*, 1987). Different metacyclic trypanosomes in the polyclonal population may have activated the same VSG by different events. Metacyclic VSGs are probably activated in a random manner (Tetley *et al.*, 1987), although the same metacyclic VSGs are expressed in similar proportions in different flies (Hadjuk *et al.*, 1981, Barry *et al.*, 1983; Turner *et al.*, 1986), suggesting that some metacyclic VSGs have a greater probability of being activated than others. The repertoire of metacyclic VSGs, and the proportions in which they are expressed at the metacyclic stage, remained almost constant during 10 sequential transmissions of a cloned stock of *T. brucei* through tsetse flies (Barry *et al.*, 1983). Similarly, in field isolates of *T. brucei* from the same serodeme (i.e. trypanosomes that have the same bloodstream VSG repertoire) that were isolated over an 18 year period, some metacyclic VSGs were expressed in most or all of the isolates following transmission through tsetse flies, though there was a significant drift in the metacyclic VSG repertoire over this period (Barry *et al.*, 1983).

What is the mechanism that activates metacyclic VSGs at the metacyclic stage? There are at least two lines of phenotypic evidence that suggest the mechanism of VSG activation at the metacyclic stage is distinct from the mechanisms of VSG activation at the bloodstream stage. Firstly, the VSG expressed by the bloodstream population when ingested by the fly, the ingested VSG, is not expressed at the metacyclic stage (Turner *et al.*, 1986). However, the ingested VSG is amongst the first bloodstream VSGs to be expressed after expression of the metacyclic VSGs (Hadjuk and Vickerman, 1981; Delauw *et al.*, 1985). This implies that the ingested VSG gene resides at a bloodstream expression site which is inactive at the procyclic and metacyclic stages, and is only reactivated in bloodstream trypanosomes. Secondly, the repertoire of metacyclic VSGs expressed at the metacyclic stage, was unaffected by the ingested VSG (Barry *et al.*, 1985; Turner *et al.*, 1986). Phenotypic evidence is supported by DNA analysis. Metacyclic VSGs that are expressed at the metacyclic stage may be re-expressed during the course of a bloodstream infection (Barry *et al.*, 1985; Cornelissen *et al.*, 1985). All

metacyclic VSG genes so far analysed are situated at telomeric sites (Lenardo *et al.*, 1984; Cornelissen *et al.*, 1985) and are activated at the bloodstream stage by duplication, probably to a bloodstream VSG gene expression site (Cornelissen *et al.*, 1985; Graham *et al.*, 1990). There are insufficient metacyclic trypanosomes produced in the salivary glands of the tsetse fly to allow direct molecular analysis of metacyclic VSG gene activation at the metacyclic stage. Instead, early bloodstream trypanosomes that are derived from metacyclic trypanosomes (metacyclic-derived trypanosomes) were analysed which maintain expression of metacyclic VSGs for up to 7 days after infection of the mammalian host (Barry *et al.*, 1979; Esser *et al.*, 1982). Lenardo *et al.* (1986) used a polyclonal population of metacyclic-derived trypanosomes in Southern analysis to suggest that there was no duplication of metacyclic VSGs when they were expressed at the metacyclic stage. However, this approach does not exclude many different duplication events in the polyclonal population which may result in a spectrum of restriction fragments containing the duplicated gene copy that might not be detected in Southern blot analysis. Graham *et al.* (1990) used Southern blot analysis of clonal populations of metacyclic-derived trypanosomes to show that duplication events were not involved in the activation of two metacyclic VSG genes, ILTat (for ILRAD *Trypanozoon* antigen type) 1.22 and ILTat 1.61, in these trypanosome populations. The clonal populations of metacyclic-derived trypanosomes were obtained from a model line of *Trypanosoma brucei* EATRO 795 which is unusual in that it is transmissible through tsetse flies but is also virulent in mice (Turner and Barry, 1989). From a single metacyclic trypanosome isolated from the fly salivary gland and injected into a mouse, a metacyclic-derived population of trypanosomes can be generated that is >90% homogenous for the metacyclic VSG gene expressed and is sufficiently large enough for molecular analysis within 7 days of infection, i.e. prior to switching to VSG gene expression from bloodstream VSG gene expression sites (Graham *et al.*, 1990). Immunofluorescence of clonal metacyclic-derived trypanosome infections from patency (about day 5) to day 7 showed that these populations remained >99% homogenous for a single VSG (Graham *et al.*, 1990). This suggested there were no switching events early in the bloodstream infection and therefore the mode of expression of metacyclic VSG genes in metacyclic-derived trypanosomes is likely to be the same as that of metacyclic trypanosomes. The results of Southern blot analysis of clonal metacyclic-derived trypanosome populations suggested that the metacyclic VSG genes, ILTat 1.22 and ILTat 1.61, were activated at the metacyclic stage *in situ* at their telomeric sites (Graham

et al., 1990). The expression of metacyclic VSG genes by duplication at the bloodstream stage, but *in situ* at the metacyclic stage, suggests that *in situ* activation of metacyclic VSG genes is a metacyclic stage-specific mechanism (Graham *et al.*, 1990).

The telomeric loci that contain metacyclic VSG genes, and from which they are expressed at the metacyclic stage, are located on the largest set of trypanosome chromosomes (>2 Mb) in all cases so far studied (Cornelissen *et al.*, 1985; Lenardo *et al.*, 1986). These metacyclic VSG gene expression telomeres are very different from the telomeric bloodstream VSG gene expression sites (see Fig. 1.6.1.B). The regions upstream of the metacyclic VSG genes at the 1.22 and 1.61 metacyclic VSG gene expression telomeres, which have been studied in this laboratory, are devoid of open reading frames for at least 15 kb (Barry *et al.*, 1990). There is only limited sequence homology to ESAG 1 and ESAG 2 upstream of the 1.61 M-VSG gene (Graham *et al.*, 1993) but metacyclic trypanosomes may express some ESAG-related genes from positions in the genome that are not closely linked to metacyclic VSG genes (Graham and Barry, 1991). There is very little sequence at these telomeres that is repetitive in the genome except for very short 70 bp repeat regions (Matthews *et al.*, 1990). Although the 70 bp repeats were the 5' limit of recombination when the 1.22 M-VSG gene was duplicated and expressed at the bloodstream stage (Matthews *et al.*, 1990), the lack of large regions of 70 bp repeats flanking the metacyclic VSG genes may prevent metacyclic telomeres from acting as acceptors of duplicated VSG genes, thereby ensuring the relative stability of the metacyclic VSG repertoire (Matthews *et al.*, 1990). Other metacyclic expression telomeres have been studied containing the MVAT4, MVAT7 and MVAT5 metacyclic VSG genes which contain no 70 bp repeats upstream of the VSG gene (Lenardo *et al.*, 1986; Alarcon *et al.*, 1994). Upstream of the MVAT4 and MVAT5 metacyclic VSG genes there is an ESAG 1 gene but this does not appear to be transcribed with the VSG gene (Alarcon *et al.*, 1994). The ESAG 1 sequence upstream of several metacyclic VSG genes may act as the 5' limit of recombination when a metacyclic VSG gene is duplicated and expressed at the bloodstream stage. Transcription of the metacyclic VSG genes is discussed in section 1.11.

1.8 The control of protein-coding gene expression in trypanosomes

Protein-coding genes in trypanosomes (and other kinetoplastida) are generally clustered in the genome. The gene arrays may consist of tandemly repeated genes, such as the hexose transporter genes (Bringaud and Baltz, 1993), or tandem arrays of genes

that encode very different proteins, such as the ESAGs and VSG genes of bloodstream expression sites (Pays *et al.*, 1989a). These arrays probably constitute long, polycistronic transcription units (see Graham, 1995 for review). Genes within a transcription unit are usually transcribed at the same or similar rates (Kooter *et al.*, 1987; Rudenko *et al.*, 1990), however the level of steady-state transcripts or gene products of adjacent genes in a transcription unit may be very different (Kooter *et al.*, 1987; Pays *et al.*, 1989a; Vijayasarathy *et al.*, 1990; Bringaud and Baltz, 1993). Therefore, in contrast to higher eukaryotes, control of gene expression in trypanosomes is largely post-transcriptional (see Clayton, 1992; Graham, 1995; Vanhamme and Pays, 1995 for reviews).

Figure 1.8.1 is a schematic representation of mRNA processing in trypanosomes. Processing of the nascent RNA transcripts occurs very rapidly after RNA synthesis and probably occurs co-transcriptionally such that polycistronic transcripts are not formed but monocistronic mRNAs are produced (Ullu *et al.*, 1993). Processing at the 5' end of each gene transcript involves cleavage of the nascent transcript and addition of a 39 nucleotide spliced leader sequence (Kooter *et al.*, 1984; see Fig. 1.8.1). The spliced leader (SL) sequence or mini-exon is probably added to the 5' end of all trypanosome mRNAs (Borst, 1986; Walder *et al.*, 1986) and is derived from a 141 nucleotide RNA intermediate transcript called the SL RNA. SL RNA is transcribed from tandem arrays of 1.35 kb repeats (Kooter *et al.*, 1984) that are located on 2 large trypanosome chromosomes (approximately 3.0 and 4.7 Mb; Gottesdiener *et al.*, 1990). The SL sequence is added to nascent RNAs by the process of *trans*-splicing which is similar in many respects to *cis*-splicing in higher eukaryotes (Agabian, 1990; Nilsen, 1994). 3' end processing of trypanosome nascent transcripts involves cleavage and poly(A) addition about which very little is known in trypanosomes (Vanhamme and Pays, 1995; see Fig. 1.8.1).

Trypanosome polycistronic transcription units are often densely packed with genes and there is usually an intergenic region of only 200-600 nucleotides between the sequences of the mature mRNAs (Clayton, 1992). In addition to the coding sequence, each mRNA usually contains a 5' untranslated region (5' UTR) between the coding sequence and the SL sequence, and a 3' UTR between the coding sequence and the poly (A) tail (Fig. 1.8.1). Evidence shows that 3' UTRs, the intergenic regions and the 5' UTRs contain the signals for polyadenylation and *trans*-splicing. Both *trans*-splicing and polyadenylation site specificities are signalled at least in part by tracts of polypyrimidine

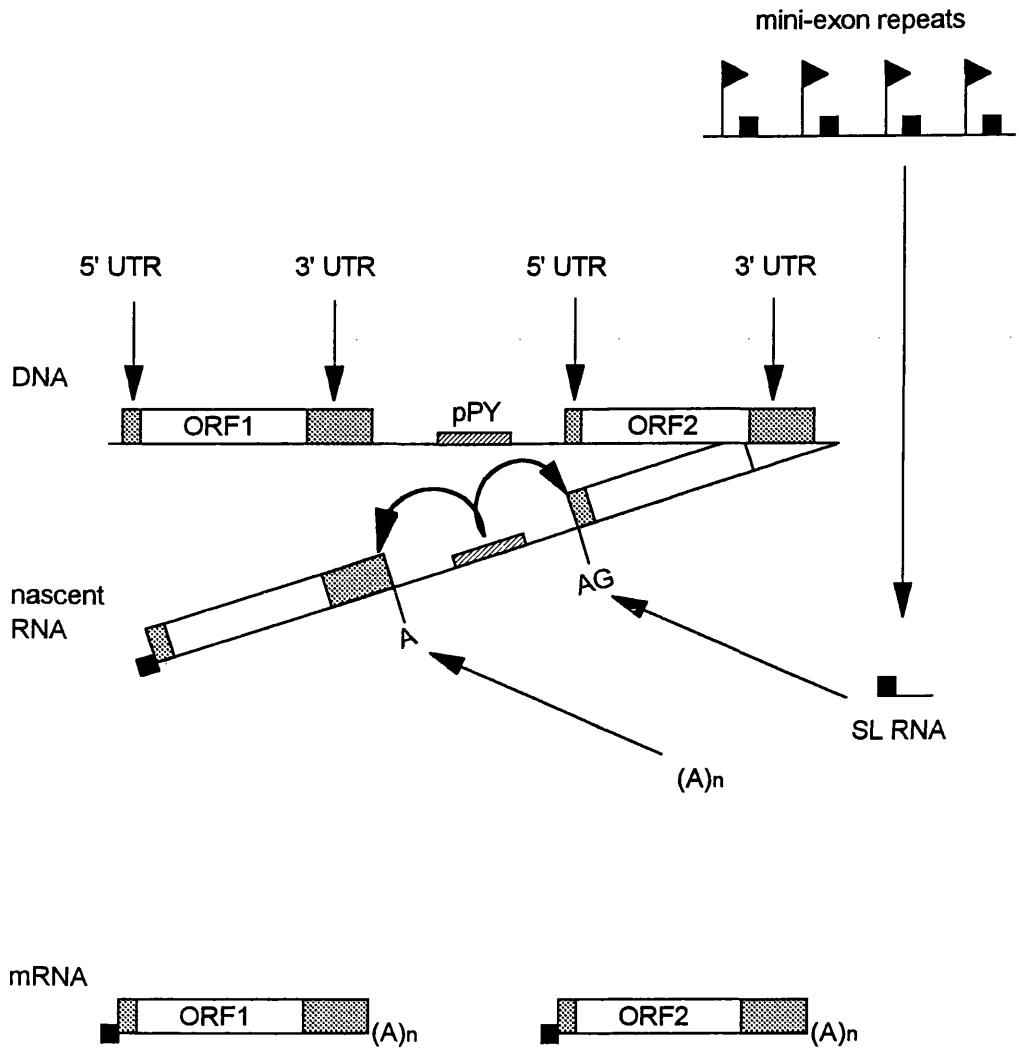


Figure 1.8.1. **RNA processing of nuclear protein-coding genes in trypanosomes.** Schematic diagram shows nascent RNA transcript of two protein-coding genes (ORF1 and 2) with polyadenylation at an A residue and *trans*-splicing of the spliced leader sequence (black box) at an AG dinucleotide. Polyadenylation and *trans*-splicing are directed by a polypyrimidine tract or tracts (pPY) in the intergenic region. The spliced leader sequence is derived from the spliced leader RNA (SL RNA) which is transcribed from the mini-exon repeats. Each mRNA is flanked by a 5' and 3' untranslated region (5' and 3' UTR). (A)n, poly (A) tail; black flag, mini-exon promoter.

nucleotides in the intergenic region. *trans*-splicing occurs within 50 bp downstream of a polypyrimidine tract and the SL sequence is usually added at an AG dinucleotide 30-70 bp upstream of the translational start codon (Agabian, 1990; Huang and Van der Ploeg, 1991; Matthews *et al.*, 1994; Schurch *et al.*, 1994). No consensus signal sequence for poly(A) addition has been observed in trypanosomes (Nilsen, 1994) but polyadenylation occurs at A residues, especially at repeated A residues, approximately 100-140 bp upstream of a polypyrimidine tract in the intergenic region, which when removed or mutated results in a reduced efficiency of poly(A) addition and a shift of the polyadenylation site upstream (Hug *et al.*, 1994; Matthews *et al.*, 1994; Schurch *et al.*, 1994). Interestingly, in transient transfection experiments using the intergenic regions of the tubulin gene array in *Trypanosoma brucei* (Matthews *et al.*, 1994), and the DHFR-TR locus in the related kinetoplastid *Leishmania major* (LeBowitz *et al.*, 1993), the same polypyrimidine tract is used to signal both accurate polyadenylation of the upstream gene and *trans*-splicing of the downstream gene. In *Trypanosoma brucei*, polyadenylation of tubulin transcripts was shown to be dependent on a functional *trans*-splicing machinery (Ullu *et al.*, 1993), and it has been proposed that the two processes may be coupled by common processing factors (LeBowitz *et al.*, 1993; Ullu *et al.*, 1993; Matthews *et al.*, 1994; see Fig. 1.8.1).

During its life cycle, *Trypanosoma brucei* is exposed to very different environmental conditions in the mammalian host and in the insect vector (see 1.3) and adjusts to new conditions by changing its pattern of gene expression. In particular, there is differential regulation of genes that encode the surface molecules at the bloodstream and procyclic stages (VSG and PARP respectively) and genes involved in energy metabolism (Clayton, 1992). For some stage-regulated genes the control of gene expression has been shown to be modulated, at least in part, by sequences in the 3' UTR. These include the genes for PARP and VSG (Hug *et al.*, 1993; Berberof *et al.*, 1995), the hexose transporter (Hotz *et al.*, 1995), aldolase (Hug *et al.*, 1993) and phosphoglycerate kinase (Blattner and Clayton, 1995). When the 3' UTR of a stage regulated gene is positioned downstream of a reporter gene, reporter gene expression may be up-regulated or down-regulated at the respective life cycle stages. For instance, PARP is expressed at the procyclic stage but not at the bloodstream stage (Roditi *et al.*, 1989). When the PARP gene 3' UTR is positioned downstream of a transcribed reporter gene, reporter gene expression is high when assayed in procyclic trypanosomes but low in bloodstream trypanosomes in relation to a non stage-regulated gene 3' UTR (Hug *et*

al., 1993; Berberof *et al.*, 1995). The converse is true of the VSG gene 3' UTR which is only expressed at the bloodstream stage (Berberof *et al.*, 1995). The 3' UTR does not affect the rate of transcription of the reporter gene (Berberof *et al.*, 1995), but does affect the level of reporter gene steady state transcripts (Berberof *et al.*, 1995; Hotz *et al.*, 1995). Unfortunately, in all these experiments the 3' UTR regions were used in the absence of the adjacent intergenic regions that have been shown to be involved in processing of RNA transcripts in trypanosomes (see above). However, the 3' UTR regions may control gene expression by increasing the stability of the mRNA at the life cycle stage when the gene is expressed, for example the VSG gene 3' UTR at the bloodstream stage (Berberof *et al.*, 1995), or by signalling rapid turnover of the mRNA at the life cycle stage when the gene is not expressed, for example the PARP gene mRNA at the bloodstream stage (Dorn *et al.*, 1991; Graham and Barry, 1996). Alternatively, the 3' UTR may affect RNA processing (Hotz *et al.*, 1995) or translation (Hehl *et al.*, 1994). These data indicate that post-transcriptional control of gene expression in trypanosomes can operate at several different levels.

Regulating gene expression at a post-transcriptional level may allow trypanosomes to differentiate rapidly to new life cycle forms when moving from one environment to another, i.e. from the bloodstream into the fly or from the fly back into the bloodstream. There is good evidence that in the transition from bloodstream trypanosomes to procyclic trypanosomes, post-transcriptional regulation of major surface gene expression is important: there is loss of VSG gene mRNA probably due to an increased rate of degradation (Ehlers *et al.*, 1987; Pays *et al.*, 1993), and accumulation of PARP gene mRNA perhaps through a combination of increased PARP mRNA stability and transcription (Roditi *et al.*, 1989; Dorn *et al.*, 1991; Graham and Barry, 1996). The stability of VSG mRNA at the bloodstream stage, and the PARP mRNA at the procyclic stage is likely to be regulated by protein factors (Ehlers *et al.*, 1987; Dorn *et al.*, 1991). The activity of these protein factors may be regulated by post-translational mechanisms such as phosphorylation and dephosphorylation, resulting in accumulation of PARP mRNA and loss of VSG mRNA as bloodstream trypanosomes differentiate to procyclic trypanosomes. There is analogous post-transcriptional control of cytochrome *c* reductase during differentiation from bloodstream forms to procyclic forms, the electron transport protein is probably required for mitochondrial activation at the procyclic stage (Priest and Hadjuk, 1994b). Such post-transcriptional regulation of stage-regulated

genes, which are necessary for successful adaptation to new conditions, is likely to be more rapid than control at the level of transcription initiation.

1.9 Transcription and transcription initiation in *Trypanosoma brucei*

Sites of transcription initiation in trypanosomes have been difficult to identify as trypanosome pre-mRNAs are rapidly *trans*-spliced following transcription, and primary transcripts are extremely short lived. This means that the identification of transcriptional start sites by RNase protection or primer extension is very difficult. However, by using techniques such as nuclear run-on reactions, primer extension, nuclease protection and low dose UV irradiation which inhibits transcription elongation and RNA decay (Coquelet *et al.*, 1991), the transcriptional start sites have been mapped of the ribosomal RNA gene repeat (White *et al.*, 1986), the PARP gene loci (Pays *et al.*, 1990; Rudenko *et al.*, 1990) and the bloodstream VSG gene expression sites (Pays *et al.*, 1989a; 1990; Zomerdijk *et al.*, 1990; Gottesdiener *et al.*, 1991). As in other eukaryotes, trypanosome ribosomal RNA gene repeats are clustered in tandem arrays in the genome (White *et al.*, 1986) which are located on 4 trypanosome chromosomes that are ~1-3 Mb in size (Gottesdiener *et al.*, 1990). Each repeat contains a copy of the 18S, 5.8S and 28S ribosomal RNA genes which are *cis*-transcribed from a transcriptional start site 1.2 kb upstream of the 18S RNA gene (White *et al.*, 1986; see 5.2.4). The PARP genes are positioned at 4 loci (PARP B1, PARP B2 and two PARP A loci), that each contain 2 or 3 PARP genes arranged in tandem (Mowatt and Clayton, 1987; Clayton, 1992) and are located on 4 different trypanosome chromosomes that are 2-6 Mb in size (Gottesdiener *et al.*, 1990). The PARP genes are transcribed in short polycistronic transcription units (Rudenko *et al.*, 1990) that also contain at least one other gene at the 3' end (Berberof *et al.*, 1991; Koenig-Martin *et al.*, 1992; Vanhamme and Pays, 1995). Transcription initiates ~86 bp upstream of the first PARP gene in the transcription unit (Brown *et al.*, 1992) and for 500 bp upstream of the transcriptional start site, the region that probably contains promoter sequences, there is a high degree of sequence homology between the 4 PARP loci (Clayton, 1992). Transcription of the bloodstream VSG gene expression sites has been described above (see 1.6). There are several expression sites in the genome and the putative promoter sequences of these expression sites are highly homologous (Zomerdijk *et al.*, 1990; Gottesdiener *et al.*, 1991; Jefferies *et al.*, 1991; Rudenko *et al.*, 1994).

In higher eukaryotes, sensitivity to the toxin α-amanitin is used to distinguish between transcription by the three classes of RNA polymerase, RNA polymerase I, II and III. RNA polymerase I is highly insensitive α-amanitin, RNA polymerase II is sensitive to low levels of α-amanitin and RNA polymerase III has an intermediate sensitivity to α-amanitin. Transcription of the trypanosome ribosomal RNA genes, the PARP genes, the bloodstream VSG gene expression sites and the metacyclic VSG genes is insensitive to high concentrations (1mg/ml) of α-amanitin (Kooter and Borst, 1984; Pays *et al.*, 1989a; Rudenko *et al.*, 1990; Graham and Barry, 1991), whilst the transcription of housekeeping genes, such as tubulin and actin, is highly sensitive to α-amanitin (Kooter and Borst, 1984; Amar *et al.* 1988; Rudenko *et al.*, 1992). This indicates that RNA polymerase I, which usually transcribes only the ribosomal RNA genes in eukaryotes, also transcribes the protein-coding genes of the PARP loci and VSG expression sites in trypanosomes. The trypanosome housekeeping genes are probably transcribed by RNA polymerase II (Rudenko *et al.*, 1992). Transcription of protein-coding genes in higher eukaryotes exclusively by RNA polymerase II is due to the addition of the trimethylguanosine (TMG) cap to the 5' end of each mRNA (probably necessary for stability and translatability of the mRNA) by an activity linked to the polymerase II complex (Jove and Manley, 1982). The modification of trypanosome mRNAs at their 5' end by *trans*-splicing (the spliced leader sequence is capped at its 5' end) rather than TMG capping, means that mRNA processing is not linked to transcription by RNA polymerase II and therefore, trypanosome protein-coding may potentially be transcribed by any RNA polymerase (Zomerdijk *et al.*, 1991a). Efficient mRNA synthesis and expression of protein-coding genes in trypanosomes can result from transcription initiated by the trypanosome ribosomal RNA gene promoter (Rudenko *et al.*, 1991; Zomerdijk *et al.*, 1991a; 1991c) or even transcription by bacterial RNA polymerases (Wirtz *et al.*, 1994). The transcription of PARP and VSG genes, the major surface antigens at the procyclic and bloodstream stages respectively, by RNA polymerase I may allow these genes to be transcribed at a higher rate than other protein-coding genes.

Transient transfection experiments using the CAT reporter gene, have shown that the putative promoter regions upstream of the transcriptional start sites were able to direct transcription of the reporter gene when located on an extrachromosomal element (Rudenko *et al.*, 1990; Zomerdijk *et al.*, 1990; 1991c; Jefferies *et al.*, 1991). Moreover, each of the promoters were roughly equally active in procyclic trypanosomes (Zomerdijk

et al., 1991c) and in bloodstream trypanosomes (Biebinger *et al.*, 1996). Deletion analysis and site directed mutagenesis of the promoter sequences showed that although the promoters for the PARP genes, ribosomal RNA genes and VSG gene expression site showed little sequence homology with each other, or with other known eukaryotic promoter sequences (Zomerdijk *et al.*, 1991c), they were similar in their arrangement of functional elements (Sherman *et al.*, 1991; Brown *et al.*, 1992; Janz and Clayton, 1994; Vanhamme *et al.*, 1995b; Pham *et al.*, 1996; see Vanhamme and Pays, 1995 for review). The core promoter for each of these transcription units is 70-80 bp in length with 2 short sequences of 5-10 bp that are essential for transcription initiation centred approximately at positions -60 and -35 bp (i.e. 60 and 35 bp upstream of the transcriptional start site). In the ribosomal promoter and bloodstream expression site promoter there were also one or two nucleotides at or close to the start site that were essential for transcription initiation (Zomerdijk *et al.*, 1991c). For the bloodstream VSG gene expression site promoter, the core promoter of 70 bp was sufficient for maximal promoter activity (Zomerdijk *et al.*, 1991c; Pham *et al.*, 1996). However, both the PARP promoter and the ribosomal promoter required sequences upstream of the core promoter for maximal promoter activity (Zomerdijk *et al.*, 1991c; Brown *et al.*, 1992). For the PARP promoter this region, called the upstream control element (UCE), consists of a sequence of no more than 10 bp centred at -135 bp (Brown *et al.*, 1992), whilst for the ribosomal promoter, the upstream sequence was positioned approximately 200 bp upstream of the transcriptional start site (Zomerdijk *et al.*, 1991c). These features, in particular the upstream control element, are similar to those of other eukaryotic ribosomal RNA gene (RNA polymerase I) promoters (Sollner-Webb and Tower, 1986; Haltiner Jones *et al.*, 1988; Kulkens *et al.*, 1991; Jacob, 1995). The functional elements of these trypanosome promoters may also be interchanged so that highly efficient hybrid promoters can be formed from the ribosomal promoter and PARP promoter (Janz and Clayton, 1994) or ribosomal promoter and bloodstream expression site promoter (Vanhamme *et al.*, 1995b) further indicating that the PARP and bloodstream expression site promoters are functionally related to the ribosomal promoter.

1.10 Stage-regulated transcription in *Trypanosoma brucei*

The fact that the PARP and bloodstream expression site promoters were equally active when assayed in transient transfection of procyclic and bloodstream trypanosomes, suggests that these promoters are not life cycle stage-regulated (Zomerdijk *et al.*, 1991c;

Biebinger *et al.*, 1996). However, when transcription initiation from the PARP promoter and expression site promoter was assayed *in vivo* at the procyclic and bloodstream stages, regulation of transcription initiation was observed. At the bloodstream stage, transcription initiation from the PARP promoter, although readily detectable, was 5-10 fold down-regulated *in vivo* when assayed by nuclear run-on or in stably transformed cell lines (Pays *et al.*, 1990; Vanhamme *et al.*, 1995a; Biebinger *et al.*, 1996). At the procyclic stage, transcription initiation from the expression site promoter was reduced by at least 50% when assayed by nuclear run-on (Pays *et al.*, 1990). Initially, the reduced activity of the bloodstream expression site promoter in procyclic trypanosomes was attributed to just one expression site promoter (Pays *et al.*, 1990). However, subsequent analysis of cDNAs derived from primary transcripts homologous to sequences close to the site of transcription initiation, showed that most or all of the expression site promoters were active in procyclic trypanosomes at a low level (Rudenko *et al.*, 1994). Furthermore, a low level of transcription initiation was observed from the expression site promoter at the expression site in stably transformed procyclic trypanosomes (Rudenko *et al.*, 1994). Therefore, expression site promoters and PARP promoters are similarly active in transient transfection experiments but differentially active *in vivo* in bloodstream and procyclic trypanosomes. This suggests that transcription initiation from these promoters may be up-regulated or down-regulated at their respective life cycle stages by the chromosomal context of the expression site and PARP loci.

In addition to transcription initiation, transcription elongation is also controlled in a life cycle stage-dependent manner. Though reduced, transcription initiation from the expression site promoter does occur in procyclic trypanosomes, however the VSG genes that reside at the 3' end of the expression sites are not transcribed because transcription elongation is attenuated within 1 kb of the transcriptional start site (Pays *et al.*, 1989b; 1990; Rudenko *et al.*, 1994). Conversely, transcription is progressively reduced through the PARP loci in bloodstream trypanosomes suggesting transcription elongation is also attenuated in this case (Vanhamme *et al.*, 1995a). These data show that control of expression of PARP and VSG is due to regulation of transcription initiation and transcription elongation as well as post-transcriptional mechanisms.

1.11 Transcription of the metacyclic VSG genes

Metacyclic VSG genes are stage regulated in the trypanosome life cycle. The transcription of the 1.22 and 1.61 M-VSG genes was examined *in vivo* by nuclear run-on

experiments using nuclei from cloned metacyclic-derived trypanosomes that express either the 1.22 or 1.61 M-VSG gene. These experiments indicated that transcription of the metacyclic VSG genes initiated only a short distance upstream (Graham and Barry, 1995). In the case of the 1.22 M-VSG gene the transcriptional start site was located within a 426bp restriction fragment 2.6-3.0 kb upstream of the VSG gene (Graham and Barry, 1995). Assuming the promoter that directs transcription initiation is located directly upstream of the transcriptional start site, the promoter for the 1.22 M-VSG gene is likely to within or just upstream of this 426 bp fragment. However, transcription of the 1.22 M-VSG gene and the region of the transcriptional start site could not be detected in nuclei prepared from bloodstream or procyclic trypanosomes, suggesting that the expression of the metacyclic VSG genes is controlled at the level of transcription initiation in the trypanosome life cycle (Graham and Barry, 1995). The control of metacyclic VSG gene expression at the level of transcription initiation is unusual in *Trypanosoma brucei* as most genes are controlled post-transcriptionally (see 1.8). Those promoters that have been identified in trypanosomes were shown to be active at both the procyclic and bloodstream stages (see section 1.9). The metacyclic VSG genes may therefore be the first examples of genes that are controlled in the life cycle of *Trypanosoma brucei* exclusively at the level of transcription initiation.

1.12 Aims of the project

By nuclear run-on analysis a putative promoter region of 426 bp had been identified in *Trypanosoma brucei* that initiates transcription of the 1.22 M-VSG gene at the metacyclic stage. The aim of this work was to analyse how transcription of the 1.22 M-VSG gene was down-regulated in the bloodstream stages of *Trypanosoma brucei*. This project sought to:

1) Assay the putative 1.22 M-VSG gene promoter in transient transfection experiments in bloodstream trypanosomes.

2) Sequence the putative promoter region and perform deletion analysis of the promoter to define what elements were necessary and sufficient for promoter activity.

3) To establish an axenic culture system for bloodstream forms of the non-laboratory adapted trypanosome line of EATRO 795.

4) To use cultured bloodstream trypanosomes in stable transformation experiments to investigate the role of chromosome positioning in regulation of metacyclic VSG gene promoter.

Chapter 2

Materials and Methods

2.1 Plasmids

All fragments of DNA were cloned into pBluescript SK(-) or KS(-) (Stratagene). Plasmid pHD52 (a gift from C. Clayton, Heidelberg) was derived from pGEM3 (Promega).

2.2 Growth and transformation of *E. coli*

Supercompetent cells of XL-1 Blue MRF' (Stratagene) or INVαF' (Invitrogen) *E. coli* strains were transformed with ampicillin resistant plasmids by heat shock at 42^{0}C according to the manufacturer's instructions. Transformed *E. coli* containing pBluescript plasmids were selected by growth on L-agar plates containing ampicillin (50 μg/ml) and X-gal (0.0002%) and IPTG (2.5 μg/ml) for blue/white selection. Single colonies which were ampicillin-resistant and could not include β-galactosidase expression due to DNA insertion were selected for subsequent growth, analysis and extraction of DNA.

E. coli genotypes:

1) XL-1 Blue MRF' Δ[mcrA0 183 Δ(mcrCB-hsdSMR-mrr) 173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac {F' proAB lacI^{q}ZΔM15 Tn10 (Tet^r)]

2) INVαF' F' endA1 recA1 hsdR17(r_k^-,m_k^+) supE44 thi-1 gyrA96 relA1 ϕ801acZΔM15Δ (lacZYA-argF)U169

2.3 Preparation of plasmid DNA from *E. coli*

Plasmid DNA was prepared from single colonies of transformed *E. coli* by alkali lysis (Sambrook *et al.*, 1989). For all plasmids that were used for transient transfection of trypanosomes, alkali lysis was followed by sedimentation of supercoiled plasmid DNA in a equilibrium gradient of caesium chloride (1.55 g/ml) in a Beckman Ti70.1 rotor at 49,000 rpm for 16 hours at 25^{0}C (Sambrook *et al.*, 1989). Other plasmids were prepared from *E. coli* using Qiagen maxipreps according to the manufacturer's instructions. To screen colonies of transformed E. coli for those that contain the correct plasmid, DNA was prepared from small volumes of bacterial culture (3ml) established from single colonies using Promega minipreps according to the manufacturer's instructions. These plasmid preparations were then analysed by restriction digests or sequencing.

2.4 Manipulations of plasmid DNA

DNA restriction digests were performed using enzymes and buffers from Gibco BRL and ligations were performed using T4 DNA ligase and ligase buffer from Gibco BRL according to the manufacturer's instructions. The ends of linear plasmid DNA were dephosphorylated when necessary, to prevent self-ligation by treatment with calf intestinal alkaline phosphatase from Boehringer Mannheim according to the method of Sambrook *et al.* (1989). Blunting of DNA overhanging ends after restriction digestion was performed using the Klenow fragment of DNA Polymerase I for 5' overhanging ends and T4 DNA Polymerase for 3' overhanging ends. Both enzymes were from Gibco BRL and the blunting reactions performed according to standard protocols (Sambrook *et al.*, 1989). Deletions of plasmid DNA were performed using an ExoIII/S1 nuclease deletion kit from Pharmacia and deletions and ligations carried out as specified in the manufacturer's instructions. Recircularised plasmids containing the deletions were sequenced to determine the exact position of each deletion.

2.5 Sequencing of plasmid DNA

Sequencing reactions were performed on double-stranded plasmid DNA using the Sequenase kit from USB. [α-^{35}S]-dATP (Dupont/NEN) was incorporated into elongation reactions according to the manufacturer's instructions using primers from the Sequenase kit (USB) such as the T7 primer or primers designed against novel sequence of the 1.22 M-VSG gene promoter region. Reporter plasmids for transient transfection were checked by sequencing using a primer (T3actinSA: AGCGGAGACTGCAATGC) that anneals to the actin gene 5' UTR (which flanks the CAT gene in all transient transfection reporter constructs) and initiates the sequencing reaction in the direction of the promoter region (see Fig. 3.2.3.1). Sequencing products were separated on 6% polyacrylamide gels containing 8% urea prepared in a BRL vertical electrophoresis apparatus. The gels were run in 1X TBE buffer (Sambrook *et al.*, 1989) at a constant 65 W. Following electrophoresis the gels were fixed in 10% methanol/10% acetic acid, and blotted to Whatman 3MM paper before vacuum drying with a Bio-Rad dryer. Gels were exposed to Kodak X-ray film and read manually.

2.6 Electrophoresis of DNA in agarose gels

Horizontal agarose gels were prepared using electrophoresis grade agarose from Gibco BRL and run in 1X TBE buffer (Sambrook *et al.*, 1989). Gels were stained with

ethidium bromide at 0.5 μg/ml, visualised by UV transillumination and photographed with a polaroid camera or using a Stratagene CCD camera and imager. 1 kb DNA size markers from Gibco BRL were loaded on each agarose gel and DNA samples were loaded using sample loading buffer containing 15% Ficoll (Sambrook *et al.*, 1989). DNA fragments were excised from agarose gels and extracted using the Qiagen gel extraction kit according to the manufacturer's instructions.

2.7 Trypanosomes

All experiments were conducted with a virulent line of *Trypanosoma brucei rhodesiense* EATRO 795 (Turner and Barry, 1989) which was isolated from a cow at Uhembo, Kenya in 1964. The model trypanosome line described here is a cloned bloodstream trypanosome line of EATRO 795 that expresses the surface antigen ILTat (ILRAD Trypanozoon antigen type) 1.2 (Turner and Barry, 1989).

EATRO 795 procyclic trypanosomes were cultured in SDM-79 medium (Gibco BRL) containing 10% foetal calf serum (Gibco BRL) and 7.5 μg/ml haemin at 27°C. Procyclic trypanosomes were passaged 1 in 10 with fresh medium every 2 days. Bloodstream trypanosomes were cultured at 37°C according to the conditions detailed in section 2.11.

2.8 Cryopreservation

Procyclic and bloodstream trypanosomes were cryopreserved by similar methods. 5-10 ml of trypanosome culture in the mid-log phase of growth were centrifuged at 800 g for 10 minutes at room temperature. The cell pellet was resuspended in approximately 1 ml of remaining medium and 660 μl of cells transferred to a plastic screw cap tube (Nunc) containing 330 μl of 22.5% glycerol in trypanosome culture medium (HMI-9 [Hirumi and Hirumi, 1989] for bloodstream trypanosomes and SDM-79 [Brun and Schonenberger, 1979] or DTM [Overath *et al.*, 1986] for procyclic trypanosomes). The tubes were packed in cotton wool in a polystyrene box and frozen overnight at -70°C before transferring to liquid nitrogen.

2.9 Transient transfection of bloodstream trypanosomes

For transient transfection experiments, the EATRO 795 model trypanosome line was grown in CFLP mice (Bantin and Kingman Ltd) and the blood from an infected mouse used to infect female Wistar rats (Bantin and Kingman Ltd). Rats infected with

bloodstream trypanosomes of the model trypanosome line were exsanguinated by cardiac puncture using a syringe containing 0.2 ml of 2% sodium citrate. The parasitaemia was usually between 2.5 x 10^8 and 1 x 10^9 trypanosomes/ml. The whole blood was centrifuged at 1000 g for 10 minutes at room temperature and the buffy coat layer of trypanosomes above the blood cells was transferred to 20 ml of sterile Zimmerman post-fusion medium containing glucose (ZPFMG; 132mM NaCl, 8 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.5 mM $MgAc_2$, 0.9mM $CaAc_2$, 1% glucose). The trypanosomes were counted and centrifuged at 600 g for 10 minutes at room temperature, the supernatant was aspirated and the pellet resuspended at 1 x 10^8 trypanosomes/ml in fresh ZPFMG. 0.5 ml of trypanosomes in ZPFMG were electroporated with 10 μg of supercoiled plasmid that had been ethanol precipitated and resuspended in sterile water. Electroporation was carried out in a new cuvette (0.4 cm width) for each transfection using a Bio-Rad electroporator set at 25 μFD and 1.5 KV. Each transfection was pulsed just once and the 0.5 ml of trypanosomes transferred to a T25 flask containing 10 ml of HMI-9 medium (at 37^{0}C) and the trypanosomes left to recover for 5 hours at 37^{0}C before assaying for CAT enzyme activity. The cells were counted in each flask prior to assay.

2.10 Assay for CAT enzyme activity

Trypanosomes were pelleted at 1500 g for 10 minutes at room temperature. The supernatant was removed with an aspirator and the trypanosomes resuspended in 1 ml of sterile PBS before transferring to microfuge tubes and spinning in a microfuge at 10,000 rpm for 25 seconds. The supernatant was removed with an aspirator and the pellet resuspended in 160 μl of sterile CAT resuspension buffer (0.11 mM Tris-HCl pH 7.8, 1 mM EDTA, 1 mM DTT). Each microfuge tube was snap frozen in a dry ice/ethanol bath and thawed in a 37^{0}C water bath then snap frozen and thawed a second time. The samples were put on ice after the second thaw and pelleted at 10,000 rpm in a microfuge at 4^{0}C for 3 minutes before the supernatant was saved to new microfuge tubes and heated to 65^{0}C for 6 minutes to denature trypanosome acetylases. After heating, the samples were put on ice for 10 minutes, then frozen at -20^{0}C and assayed for CAT enzyme activity the following day.

The assay for CAT enzyme activity is based on the phase-extraction method of Seed and Sheen (1988). A stock solution of 50 mM chloramphenicol (Sigma) was prepared (80 mg of chloramphenicol in 5 ml of 100% ethanol) and this stock solution

was diluted to 0.5 mM by adding 10 µl to 990µl of water. For each trypanosome lysate sample, 20 µl of 0.5 mM chloramphenicol solution, 2 µl [ring-3,5-^{3}H] chloramphenicol (Dupont/NEN) and 2 µl of 25 mM buturyl CoA (Pharmacia) was added and mixed in a microfuge tube and 24 µl of the combined mixture added to each trypanosome lysate sample and mixed thoroughly. The reactions were incubated at 37^{0}C for 2 hours. After 2 hours the reactions were stopped by adding 400 µl of xylene and vortexing twice for approximately 6 seconds. The samples were spun in a microfuge at 10,000 rpm for 2 minutes and the upper xylene layer removed to a new tube containing 200 µl of CAT extraction buffer (10 mM Tris-HCl pH 7.5, 1mM EDTA). The samples were extracted twice more with 200 µl of CAT extraction buffer (followed by vortexing and microfuging) and 30 µl of the final xylene layer (approximately 300 µl in volume) removed for counting in a LKB scintillation counter using the ^{3}H channel.

2.11 Adaptation of EATRO 795 bloodstream trypanosomes to axenic culture

EATRO 795 bloodstream trypanosomes of the model line expressing ILTat 1.2 were grown in CFLP mice (Bantin and Kingman Ltd), passaged into female Wistar rats (Bantin and Kingman Ltd) which were exsanguinated by cardiac puncture once the parasitaemia reached a level of between 2 x 10^8 and 1 x 10^9 trypanosomes/ml. The blood was centrifuged at 1000 g for 10 minutes at room temperature and the buffy coat layer above the blood cells removed and placed in HMI-9 medium (Hirumi and Hirumi, 1994) at 37^{0}C. The cells were counted and the concentration of trypanosomes adjusted to 2 x 10^5 cells/ml in 5 ml of HMI-9 medium in a T25 flask. The flask was maintained vertical with a closed cap in a 37^{0}C incubator in the absence of CO_2. Each day 2.5 ml of the culture medium was removed and replaced with fresh medium irrespective of the trypanosome concentration. After 8 days the culture was below 2.5 x 10^3 cells/ml at which point the trypanosomes were no longer passaged and left at 37°C. 10 days after passaging was stopped, a trypanosome population grew up to approximately 1 x 10^6 cells/ml. The trypanosomes were passaged every 2 days by removing all the medium from the flask and replacing with fresh medium.

2.12 Preparation of trypanosomes for light and electron microscopy

Smears of trypanosomes were made on glass microscope slides and fixed by immersion in methanol at -20^{0}C for 5 minutes before being Giemsa-stained (Sigma) according to the manufacturer's instructions.

To prepare trypanosomes for sectioning for transmission electron microscopy, 50 ml of bloodstream trypanosomes in axenic culture at a density of approximately 1 x 10^6 cells/ml were harvested by centrifugation at 800 g for 10 minutes at room temperature. The supernatant was removed and the cell pellet was fixed according to the method of Sabatini, Bensch and Barrnett (1963). Fixation was with 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 for 30 minutes at 20^{0}C then the glutaraldehyde was thoroughly removed with 3 x 5 minute washes with phosphate buffer followed by fixation with 1% aqueous osmium tetroxide in 0.1 M phosphate buffer at pH 7.4 for 1 hour. The trypanosome pellet was thoroughly washed with 3 x 10 minute washes with distilled water and stained with 0.5 % uranyl acetate solution for 30 minutes in the dark. The sample was dehydrated in a series of ethanol/water washes to absolute ethanol before embedding in Spurr's resin (Spurr, 1969). The resin was baked at 80^{0}C for 2 days and the sample sectioned using a diamond knife.

2.13 Immunofluorescence of trypanosomes

Indirect immunofluorescence was performed with an antibody that reacts against the PARP surface coat of procyclic trypanosomes. The primary monoclonal antibody (#418) was a gift from Dr. C. M. R. Turner, Biochemical Parasitology, University of Glasgow. Smears of trypanosomes were made on glass microscope slides that had been coated with poly-L-lysine by immersion of slides in 100 μg/ml poly-L-lysine solution and allowed to dry. The trypanosome smears were fixed by immersion in methanol at -20^{0}C for 5 minutes and rehydrated by immersion in PBS for 5 minutes. Once dry, small reaction circles were made on each slide using a Texpen ball point paint marker and 30 μl of the antibody diluted 1:500 in PBS was added to each reaction circle. The primary antibody was allowed to react for 45-90 minutes at 25^{0}C in a humid chamber. The slides were then washed in PBS (3 x 10 minutes) and incubated with the secondary antibody (rabbit anti-mouse immunoglobulin conjugated with FITC [Sigma]) diluted 1:50 in the PBS under the same conditions as for the primary antibody. Following incubation with the secondary antibody the slides were washed in PBS (3 x 10 minutes) and then mounted in Mowiol containing p-phenylenediamine. If required 1 μl of DAPI solution (10 μg/ml) was added to each reaction circle during the secondary antibody incubation.

Indirect immunofluorescence of bloodstream trypanosomes was performed with primary monoclonal antibodies or antisera against the VSG surface coat by Kathleen

Milligan at Biochemical Parasitology, University of Glasgow essentially as described above.

2.14 Transmission of trypanosomes through tsetse flies

Teneral female tsetse flies (*Glossina morsitans morsitans*) were supplied by Dr. Ian Maudlin at the Tsetse Research Laboratory, Bristol. The flies used in transmission experiments were adult *Glossina morsitans morsitans* that were placed individually in small aerated plastic tubes (the flies were stunned at 4^0C) before feeding. Flies were fed on defibrinated sheep's blood which was prepared by centrifugation at 1500 g at 4^0C in the presence of 2% citrate and the plasma removed by aspiration. The plasma was replaced with an equal volume of serum-free HMI-9 medium and centrifuged again at 1500 g to pellet the blood cells. For and infective feed, 10 ml of blood cells were added to 10 ml of axenic culture bloodstream trypanosomes in HMI-9 medium, 10 ml of foetal calf serum (Gibco BRL) and 3 ml of 600 mM sterile glucosamine. This trypanosome and blood cell mixture was poured onto a hot plate at 41^0C and overlaid with a sterile silicone membrane onto which approximately 50 flies were placed and allowed to feed. The flies were maintained in a 26^0C incubator at a humidity >75% and fed with 30 ml of defibrinated sheep's blood containing 60 mM glucosamine each Monday, Wednesday and Friday.

3 weeks after feeding with the infected bloodmeal, flies were allowed to probe on a glass microscope slide on a hot plate at 41°C and the salivary smears examined for the presence of metacyclic or proventricular form trypanosomes using phase contrast light microscopy. Thereafter the flies were probed every 2 days.

2.15 Stable transformation of bloodstream trypanosomes

Axenic culture bloodstream trypanosomes in 100 ml of HMI-9 (Hirumi and Hirumi, 1989) were harvested in their exponential phase of growth (between 5 x 10^5 and 1 x 10^6 trypanosomes/ml) and centrifuged at 800 g. The cell pellet was resuspended in 5 ml of ZPFMG at 37^0C and centrifuged again at 800 g before resuspending in 1 ml of ZPFMG at 37^0C. Aliquots of 0.5 ml of trypanosomes were each electroporated in a fresh cuvette (cuvette width of 0.4 cm) at 25 μFD and 1.5 KV. Each aliquot was pulsed twice with plasmid DNA that had been digested with restriction enzyme, ethanol precipitated and resuspended in sterile water. Following electroporation the trypanosomes were allowed to recover in 10 ml of HMI-9 medium overnight.

Transformed trypanosomes were selected with phleomycin in liquid culture at the concentrations specified in Chapter 5. Stock phleomycin was made up at 10 mg/ml in sterile water.

2.16 Cloning of bloodstream trypanosomes in axenic culture

Axenic culture bloodstream trypanosomes were cloned either by serial dilution or by plating trypanosomes on agarose plates. Serial dilution of trypanosomes was carried out in 96 well plates. 200 µl of HMI-9 medium was added to each of the 96 wells and 200 µl of medium containing approximately 1000 trypanosomes was added to the top left well of the grid. From this well, trypanosomes were serially diluted 1:2 (i.e. 200 µl of trypanosomes in 200 µl of HMI-9 medium) from the top to bottom in the first column of wells and then serially diluted 1:2 from left to right in each row of wells. This means that each population of trypanosomes can be diluted by more than 1:250,000 in increments of dilution of 1:2. Following dilution the plates were sealed and incubated at 37^0C for 5-7 days after which wells containing growing trypanosomes were apparent from the red to orange colour change of the medium. Trypanosome colonies could also be readily observed using an inverted microscope. Trypanosome clones were picked from the last well in a serial dilution that produced a trypanosome population.

Cloning of bloodstream trypanosomes in axenic culture on agarose plates was carried out according to the method of Carruthers and Cross (1992). A 10X suspension of agarose (for 10 plates; 1.3g of low melting point agarose and 20 ml of water) was autoclaved with a magnetic stir bar and then immediately placed in a 50^0C water bath with 180 ml of HMI-9 medium. Once equilibrated to 50^0C the medium was added to agarose under sterile conditions and mixed using the magnetic stir bar before dispensing 20 ml to petri dishes (100 mm diameter) also under sterile conditions. Once set the agarose plates were dried with their lids off under sterile conditions in a vertical laminar flow hood for exactly 1 hour. Between 100 and 10,000 trypanosomes were spread on each plate in 100 µl of medium using a sterile glass spreader and then the plates dried under the hood for 5 minutes before sealing and placing at 37^0C. Colonies of trypanosomes were observed after 3 days under the microscope and 5-7 days with the unaided eye. Colonies could be picked from the agarose plates using a sterile pasteur pipette and the trypanosomes resuspended in liquid medium. Trypanosomes were selected during cloning on the agarose plates by adding antibiotic at the required

concentration (see section 5.2.2) to the agarose medium mixture prior to pouring the plates.

2.17 Isolation of *T. brucei* genomic DNA, Southern blotting and hybridisation

Genomic DNA was isolated from *T. brucei* (usually 10^8 trypanosomes) using the Scotlab Nucleon II kit according to the manufacturer's instructions. Genomic DNA was digested in 200 µl of restriction buffer with 20 units of enzyme for 3 hours and a further 20 units of enzyme for 2 hours. Digested genomic DNA was ethanol precipitated and resuspended in 20 µl of TE buffer (Sambrook *et al.*, 1989) before loading on agarose gels (usually 0.7 %) and running overnight at 2 V/cm. Following ethidium bromide staining, gels were depurinated with 2 x 15 minute washes with 0.25 M HCl, denatured with 2 x 15 minute washes with denaturing solution (0.5 M NaOH, 1.5 M NaCl) and neutralised with 2 x 20 minute washes with neutralising solution (0.5 M Tris-HCl pH 7.5, 3 M NaCl). DNA was transferred from the gel to Hybond-N nylon membrane (Amersham International plc) by capillary action in 20X SSC (see Sambrook *et al.*, 1989) and following transfer overnight, DNA was UV cross-linked to the membrane using 120,000 µJ/cm^2 in a UV Stratalinker (Stratagene).

Field inversion gel electrophoresis (FIGE) was performed using a switchback pulse controller (Hoefer) and a 1% GTG agarose (Biometra) gel run in 0.5X TBE buffer. The gel was run at 150 V (5.2 V/cm) for 22 hours at 4^{0}C with a pulse time of 0.6-2.0. The equipment was set on reverse mode with a forward to reverse ratio of 3.0:1 and a run-in time of 10 minutes. Molecular size markers of 8-48 kb (Biorad) were included on each gel and stained with ethidium bromide (0.5 µg/ml). FIGE gels were depurinated, denatured and neutralised as above and then Southern blotted to Zeta probe nylon membrane (BioRad) in 20X SSC. Following blotting the nylon membrane was baked at 80^{0}C for 2 hours.

Southern blots were prehybridised for 2 hours in Pall membrane solution (5X Denhardts, 5X SSC, 0.2% SDS and 100 µg/ml herring sperm DNA) at 65^{0}C and hybridised in Pall membrane solution for 16 hours at 65^{0}C. Prehybridisation and hybridisation were carried out in rotating bottles in a hybridisation oven. Unless otherwise stated Southern blot hybridisations were washed twice with 3X SSC/0.1% SDS (2 x 15 minute washes) at 65^{0}C and twice with 0.1X SSC/0.1% SDS (2 x 15 minute washes) at 65^{0}C.

2.18 PCR analysis of genomic DNA

Genomic PCR reactions were performed on uncut genomic DNA samples prepared from trypanosomes as described in section 2.17. Each reaction contained 20 ng of uncut genomic DNA, 10 μM of each primer (Mprom: TGCGGAACTGCCGC TCATTGCACGTT, and Rprom: TAAAGAGCCAGAATGCACCCGCGCTG), 1mM of each nucleotide triphosphate (dATP, dCTP, dGTP, dTTP) and 2 units of *Taq* DNA polymerase (Applied Biosystems). The reactions were performed in 10 μl in thin-walled tubes that also contained 45 mM Tris. HCl (pH 8.8), 11 mM NH_4SO_4, 4.5 mM $MgCl_2$, 6.7 mM 2-β-mercaptoethanol, 4.4 mM EDTA pH 8.0 and 113 μg/ml BSA. Using a Stratagene Robocycle the DNA templates were denatured at 94^{0}C for 3 minutes before adding the *Taq* DNA polymerase (hot-start reactions) and 30 PCR cycles were performed: denaturation at 94^{0}C for 1 minute, annealing at 70^{0}C for 1 minute and elongation at 72^{0}C for 2 minutes.

2.19 Isolation of *T. brucei* total RNA, Northern blotting and hybridisation

Total RNA was isolated from *T. brucei* (usually 10^8 trypanosomes) using the TRIzol reagent from Gibco BRL according to the manufacturer's instructions. Total RNA (usually 5 μg per track) was vacuum dried and resuspended in 15 μl of Northern resuspension buffer (500 μl formamide, 166 μl formaldehyde, 100 μl 10X MOPS running buffer [Sambrook *et al.*, 1989], 230 μl water) before heating to 70^{0}C for 10 minutes and adding 3 μl of Northern loading buffer (15% Ficoll 400, 30% sucrose, 10mM sodium phosphate buffer pH 7.0, 0.06% bromophenol blue). RNA samples were loaded on a 100 ml 1% agarose gel containing 6.6% formaldehyde and run in 1X MOPS running buffer (Sambrook *et al.*, 1989) at 140 V for 2-3 hours. 0.5 μg of 0.24-9.5 kb RNA standard markers from Gibco BRL were also loaded on the gel. Following electrophoresis, the denaturing gel was washed in water (3 x 20 minute washes) to remove the formaldehyde and the blotted to Hybond-N membrane (Amersham International plc) as for the Southern blots. The Northern blots were UV crosslinked with a Stratalinker (Stratagene). RNA was stained on the blot by washing first with 2% SDS to remove the bromophenol blue, washing briefly in water and staining with 0.04 methylene blue in 0.5 M sodium acetate at pH 5.2 for 10 minutes. The blot was destained with 25% ethanol and the mobility of the RNA standard markers measured.

Northern blots hybridised with DNA probes were prehybridised at 42^{0}C for 2 hours in prehybridisation buffer (50% formamide, 5X SSC, 5X Denhardts, 50 mM sodium phosphate buffer pH 6.8, 0.1% SDS, 60 μg/ml herring sperm DNA) and then hybridised at 42^{0}C for 16 hours in hybridisation buffer (50% formamide, 5X SSC, 1X Denhardts, 20mM sodium phosphate buffer pH 6.8, 0.1% SDS, 10% dextran sulphate, 60 mg/ml herring sperm DNA). When Northern blots were hybridised with antisense RNA riboprobes, prehybridisation for 2 hours and hybridisation for 16 hours was carried out at 55^{0}C in antisense hybridisation solution (50% formamide, 5X SET [Sambrook *et al.*, 1989], 5X Denhardts, 0.5% SDS, 50μg/ml herring sperm DNA). All Northern hybridisations were carried out in rotating bottles in a hybridisation oven. Northern blots hybridised with DNA probes were washed at 65^{0}C with 3X SSC/0.1% SDS (2 x 15 minute washes) and 0.1X SSC/0.1% SDS (2 x 20 minute washes). Northern blots hybridised with antisense RNA riboprobes were washed at 55^{0}C with 3X SET/0.1% SDS (2 x 15 minute washes) and 0.5X SET/0.1% SDS (2 x 20 minute washes). When required, blots were stripped of hybridised probe by twice immersing in 0.1% SDS at 100^{0}C and left to cool to room temperature.

2.20 Preparation of DNA and RNA probes

DNA probes were prepared by random primer extension of double stranded DNA fragments purified from agarose gels using the Qiagen gel extraction kit. 25 ng of purified double stranded DNA was labelled to a specific activity of > 1 x 10^9 dpm/μg with [α-^{32}P]dCTP or [α-^{32}P]dATP (3000 Ci/mmole; Dupont/NEN) using the Prime-It random primer labelling kit (Stratagene). DNA probes were purified from unincorporated radionucleotides using Nuctrap push columns from Stratagene exactly as described in the manufacturer's instructions.

Antisense RNA riboprobes were prepared from a restriction enzyme digested double-stranded plasmid containing the CAT gene. The *in vitro* transcription reactions were carried out using the T3/T7 transcription kit from Stratagene using T3 RNA polymerase and incorporating [α-^{32}P]rUTP (3000 Ci/mmole; Dupont/NEN). The riboprobes were purified from unincorporated radionucleotides using Nuctrap push columns from Stratagene exactly as described in the manufacturer's instructions.

2.21 Preparation of trypanosome nuclei

Axenic culture bloodstream trypanosomes were grown in 1000 ml of HMI-9 medium to a density of approximately 10^6 cells/ml (10^9 trypanosomes in total). Bloodstream trypanosomes in axenic culture did not grow well in large flasks or containers, and therefore large numbers of trypanosomes were grown in a series of 20 flasks each containing 50 ml of medium. The trypanosomes were concentrated by low centrifugation (800 g) and resuspension in approximately 50 ml of medium. The trypanosomes were then disrupted in a Stanstead cell disruptor at 60 psi and again collected in approximately 50 ml. The disrupted trypanosomes were spun at 4500 rpm for 5 minutes at 4^0C to pellet the nuclei and resuspended in 20 ml of ice-cold (4^0C) nuclei wash buffer (20 mM Pipes pH 7.5, 15 mM NaCl, 60 mM KCl, 14 mM β-mercaptoethanol, 0.5 mM EGTA, 4 mM EDTA, 0.15 mM Spermine, 0.5 mM Spermidine, 0.125 mM PMSF). The nuclei were spun a second time at 4500 rpm at 4^0C and resuspended in 10 ml of cold wash buffer, and then spun a third time and resuspended in 5 ml of cold wash buffer. Finally, the nuclei were spun a fourth time at 4500 rpm at 4^0C and resuspended in 0.5 ml nuclei storage buffer (20 mM Tris-HCl pH 7.9, 75 mM NaCl, 0.5 mM EDTA, 0.85 mM DTT, 0.125 mM PMSF, 50% glycerol) and snap frozen in liquid nitrogen before storing at -70^0C.

2.22 Nuclear run-on reactions

Nuclear run-on reactions were performed after the method of Kooter and Borst (1984). The nuclei stored at -70^0C were thawed on ice and 250 µl (approximately 1 x 10^8 nuclei) of 500 µl removed and spun for 5 minutes at 12,000 rpm in a microfuge. The nuclei storage buffer was completely removed and the nuclei pellet resuspended in 70 µl of run-on reaction buffer (50 µl 2X elongation buffer, 13 µl water, 2 µl RNasin [Promega, 20 U/µl], 5µl creatine kinase [Sigma, 10 µg/µl]). 2X elongation buffer was made up fresh for each run-on reaction from 100 mM Tris-HCl pH 8.0, 50 mM NaCl, 100 mM KCl, 2 mM $MgCl_2$, 4 mM $MnCl_2$, 2mM DTT, 0.15 mM spermine, 0.5 mM spermidine, 10 mM creatine phosphate, 2 mM rGTP, 2 mM rATP, 2 mM rCTP, 20% glycerol. To each run-on reaction was added 25 µl of [α-^{32}P]rUTP (3000 Ci/mmole, 10 mCi/ml) and the reaction incubated at 37^0C for 5 minutes.

Following the run-on reaction total RNA was isolated from the nuclei preparation using TRIzol reagent (Gibco BRL) according to the manufacturer's instructions. The RNA pellet was resuspended in 50 µl of RNase-free water and purified from

unincorporated nucleotides using Nuctrap push columns (Stratagene). The run-on probe was heated at 70^0C for 10 minutes to denature any secondary structure, before adding to hybridisation solution of the smallest possible volume e.g. 1 ml. Small Southern blots of plasmid DNA (typically less than $20cm^2$) were UV crosslinked to Hybond-N membrane as before, prehybridised for 4 hours and hybridised for 2 days in plastic bags at 55^0C in run-on hybridisation buffer (3X SSC, 0.1% SDS, 10X Denhardts, 20 mM sodium phosphate buffer pH 6.5, 100 μg/ml herring sperm DNA, 50 μg/ml *E. coli* tRNA). The blots were washed following hybridisation with 3X SSC/0.1% SDS (3 x 20 minute washes) at 65^0C. The blots were not washed at higher stringency as low stringency washing gave sufficiently specific hybridisation (see Fig. 5.2.7.1).

Chapter 3

Functional Analysis of the 1.22 M-VSG Gene Promoter in Bloodstream Trypanosomes

3.1 INTRODUCTION

3.1.1 Transcriptional activation of metacyclic VSG genes

At the metacyclic stage of *Trypanosoma brucei*, each trypanosome expresses a single VSG from limited repertoire of no more than 27 VSG genes (Turner *et al.*, 1988). These VSG genes are activated at the metacyclic stage in a stochastic and polyclonal manner, giving a mixture of antigenic types in the metacyclic population in the tsetse fly salivary gland (Tetley *et al.*, 1987). Hajduk and Vickerman (1981) proposed that activation of a mixture of antigen types at the metacyclic stage would best be achieved by activating at random one of several metacyclic stage-specific promoters, each promoter controlling transcription of a single metacyclic VSG gene. In our laboratory the activation of two metacyclic VSG genes has been studied, ILTat (for ILRAD Trypanozoon antigen type) 1.61 and ILTat 1.22. The expression of these genes has been studied in a model trypanosome line of EATRO 795 that is transmissible through tsetse flies, is virulent in mice and maintains >90% homogenous expression of a metacyclic VSG gene for up to 7 days following infection with a single metacyclic trypanosome. These early bloodstream populations, or metacyclic-derived trypanosomes, have not yet switched to expression of VSG genes from bloodstream expression sites and are likely to express metacyclic VSG genes by the same mode of expression as metacyclic trypanosomes (see section 1.7). Both the ILTat 1.22 and ILTat 1.61 genes were found to be activated *in situ* in metacyclic-derived trypanosomes, i.e. the genes were not duplicated to a bloodstream VSG gene expression site (Graham *et al.*, 1990). Also there were no detectable DNA rearrangements within 8.5 kb upstream of the VSG gene (Graham *et al.*, 1990). The expression of metacyclic VSG genes *in situ* without DNA rearrangement events is consistent with transcriptional activation of the genes from their metacyclic expression telomeres.

3.1.2 Stage-regulated transcription of the ILTat 1.22 M-VSG gene

Nuclear run-on experiments were used to study the transcription of metacyclic VSG (M-VSG) genes *in vivo*. Insufficient numbers of metacyclic trypanosomes are generated at the metacyclic stage for molecular analysis, therefore metacyclic VSG gene activation was studied in clonal populations of metacyclic-derived trypanosomes. These are early bloodstream trypanosomes derived from metacyclic trypanosomes but still

expressing metacyclic VSG genes from metacyclic expression telomeres (Graham *et al.*, 1990; see section 1.7). Nuclei were isolated from metacyclic-derived trypanosomes expressing the ILTat 1.22 M-VSG gene *in situ*, and run-on *in vitro* with radiolabelled nucleotides that were incorporated into the nascent RNA transcripts. These radiolabelled nascent RNA transcripts were used to probe Southern blots containing restriction fragments of cloned, single copy sequences from the 1.22 M-VSG gene telomere (Graham and Barry, 1995). Firstly, the results of these experiments indicated that transcription of the 1.22 M-VSG gene was insensitive to α-amanitin at 500 mg/ml (Graham and Barry, 1991), suggesting the metacyclic VSG genes may be transcribed by the same polymerase as the bloodstream VSG genes (see section 1.9). Secondly, the pattern of hybridisation to the Southern blots localised transcription initiation of the 1.22 M-VSG gene to a 426 bp restriction fragment 2.6-3.0 kb upstream of the 5' end of the VSG gene. This indicates that, unusually for trypanosomes, the 1.22 M-VSG gene is transcribed in a monocistronic transcription unit as there are no open reading frames downstream of this transcriptional start site except the 1.22 M-VSG gene. Given that the promoters for protein-coding genes in trypanosomes (only 2 promoters have been identified) and higher eukaryotes are located at or immediately upstream of the transcriptional start site, the promoter for the 1.22 M-VSG gene is likely to be within or just upstream of this 426 bp restriction fragment. Immediately upstream of the transcriptional start site there is a transcriptional gap of at least 13 kb at the metacyclic expression telomere (Graham and Barry, 1995) which indicates that this fragment includes a true transcriptional start site rather than a site of re-initiation of transcription downstream of an upstream transcription unit.

In other nuclear run-on experiments, transcription could not be detected from the 426 bp restriction fragment in either procyclic or bloodstream trypanosome nuclei. This implies that transcription initiation from the putative 1.22 M-VSG gene promoter is restricted to the metacyclic and early bloodstream stages only (from 5-7 days post-infection), and indicates that metacyclic VSG genes are regulated at the level of transcription initiation during the trypanosome life cycle (Graham and Barry, 1995).

The putative promoter region of the 1.22 M-VSG gene (426 bp *Bam*HI to *Pst*I restriction fragment of pMT1.22BPs in Fig. 3.2.1.1), identified in nuclear run-on experiments of metacyclic-derived trypanosomes, was used in transient transfection

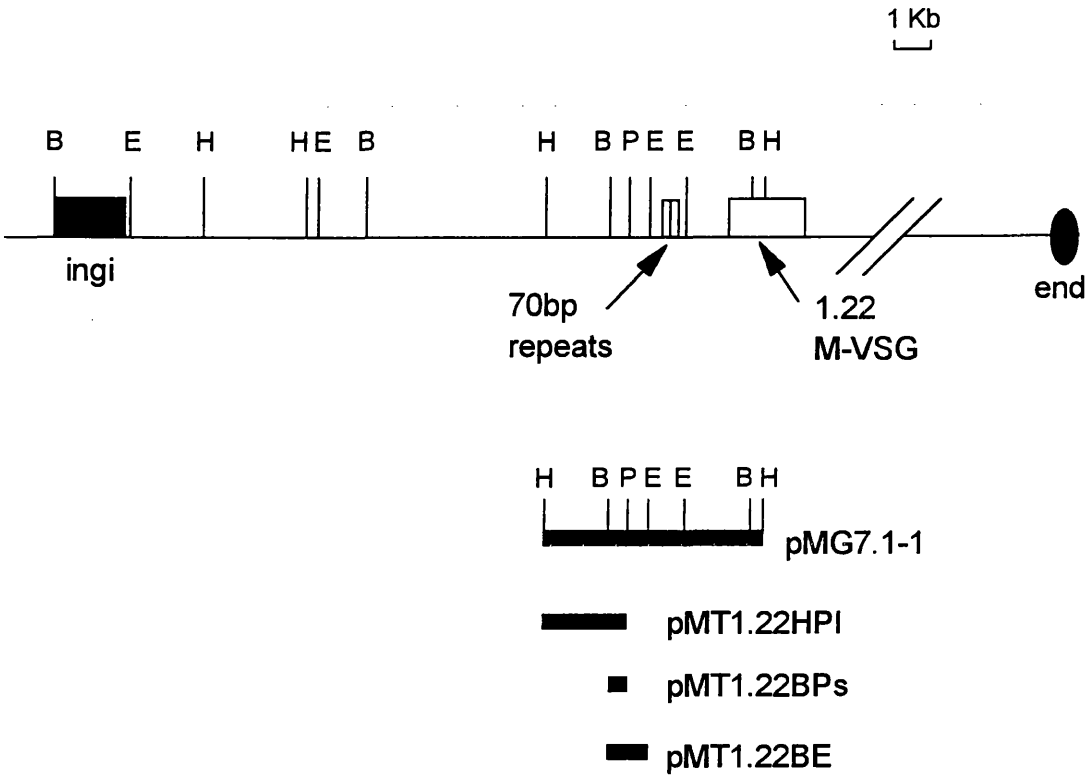


Figure 3.2.1.1. **Restriction map of the 1.22 M-VSG gene expression telomere.** The 1.22 M-VSG gene is located close to a chromosome end (black oval) and is flanked at its 5' end by just 2 copies of the 70 bp repeats. Approximately 16 kb upstream of the 1.22 M-VSG gene is sequence that resembles the ingi transposable element (ingi). Sequences upstream of the 1.22 M-VSG gene have been cloned into pBluescript and are represented underneath the restriction map as black bars. plasmid pMT1.22HPI contains a 1.6 kb *Hind*III-*Pst*I fragment and pMT1.22BPs contains a 426 bp *Bam*HI-*Pst*I fragment. B, *Bam*HI; E, *Eco*RI; H, *Hind*III; P, *Pst*I.

experiments of metacyclic-derived trypanosomes. Results showed the putative promoter region can drive efficient expression of the CAT reporter gene in day 5 metacyclic-derived trypanosomes, as expected, at a level 75% of that of a bloodstream VSG gene expression site promoter which is not stage-regulated in transient transfection experiments and was used as a positive control (Graham and Barry, 1995). The data from transient transfection of metacyclic-derived trypanosomes supports the nuclear run-on experiments and suggests the 426 bp fragment contains signal sequences that initiate transcription of the 1.22 M-VSG gene, i.e. the 'core' promoter. Using RNA from metacyclic-derived trypanosomes transiently transfected with a reporter construct containing the putative 1.22 M-VSG gene promoter, 5' reverse transcriptase PCR identified the precise position of the transcriptional start site of this promoter at 2828 bp 5' of the VSG gene (Graham and Barry, 1995). This transcriptional start site is 167 nucleotides from the 5' end of the 426 bp putative promoter fragment (see Fig. 3.2.7.1).

When the 426 bp putative promoter fragment of the 1.22 M-VSG gene was tested in transient transfection of procyclic trypanosomes it was found to be inactive or minimally active (Graham and Barry, 1995). This is in contrast to the PARP and bloodstream expression site promoters which were highly active when tested in transient transfection of procyclic trypanosomes (Zomerdijk *et al.*, 1991c; Biebinger *et al.*, 1996). Therefore the mechanism of transcription initiation from the putative 1.22 M-VSG gene promoter appears to be distinct from that which initiates transcription from the PARP and bloodstream expression site promoters.

3.1.3 Assaying the 1.22 M-VSG gene promoter

Although transient transfection of day 5 metacyclic-derived trypanosomes with reporter constructs gave high levels of reporter gene expression (75% of the positive control), these trypanosomes require laborious fly transmissions (the method of isolating cloned, metacyclic-derived trypanosomes is outlined in section 1.7) and yield a very limited number of cells (1-3x10^6 trypanosomes per mouse). To proceed with functional analysis of the promoter, an easier method of assay for 1.22 M-VSG gene promoter activity was required.

After injection into the mammalian host, metacyclic trypanosomes readily differentiate to bloodstream trypanosomes (Vickerman, 1985) but continue to express metacyclic VSG genes for several days after differentiation (Barry *et al.*, 1979; Esser *et*

al., 1982). Within this window of up to 7 days post-infection, metacyclic VSG genes are transcribed *in situ* from metacyclic VSG gene promoters (Graham *et al.*, 1990; Graham and Barry, 1995). Transcription factors necessary for transcription from metacyclic VSG gene promoters are therefore present in early bloodstream trypanosomes (metacyclic-derived trypanosomes) and these factors may persist after expression has switched from metacyclic to bloodstream VSG genes. In addition, the PARP promoter and bloodstream expression site promoter, which like the putative 1.22 M-VSG gene promoter are insensitive to α-amanitin, are both active when assayed by transient transfection of bloodstream trypanosomes (Biebinger *et al.*, 1996). Although, the nuclear run-on experiments had already shown that the putative 1.22 M-VSG gene promoter was inactive in bloodstream trypanosomes *in vivo*, previous experiments had shown that trypanosome promoters may be differentially active *in vivo* and in transient transfection (Zomerdijk *et al.*, 1991c; Rudenko *et al.*, 1994; Berberof *et al.*, 1995; Biebinger *et al.*, 1996; see section 3.3.1). Transient transfection assays the transcriptional activity of a promoter sequence on an extrachromosomal plasmid and could uncouple the promoter from negative or positive regulatory effects in the genome. Therefore, I decided to test whether the 1.22 M-VSG gene promoter was active in transient transfection of long-slender bloodstream trypanosomes. If activity of the putative 1.22 M-VSG gene promoter could be detected in bloodstream trypanosomes this would allow the preparation of large numbers of bloodstream trypanosomes in laboratory animals and easy analysis of the promoter and its mutations or deletions.

In this chapter I tested for transcriptional activity of the putative 1.22 M-VSG gene promoter at the bloodstream stage by transient transfection. The results indicated that the putative promoter was active in bloodstream trypanosomes. The 1.22 M-VSG gene was further characterised by assaying deletions of the promoter sequence in transient transfection of bloodstream trypanosomes.

3.2 RESULTS

3.2.1 Sequencing of the 1.22 M-VSG gene promoter region

The 426 bp *Bam*HI-*Pst*I restriction fragment that contains the putative 1.22 M-VSG gene promoter region had previously been cloned into pBluescript and sequenced (Graham and Barry, 1995). However, sequences upstream of the putative promoter region may also be important in regulating transcription initiation from the promoter or

control of the promoter in the trypanosome life cycle, e.g. by acting as an enhancer region. Both the PARP promoter and the ribosomal RNA gene promoter in trypanosomes are flanked upstream by enhancer-like sequences that increase the level of transcription initiation from the 'core promoter' (Zomerdijk *et al.*, 1991c; Brown *et al.*, 1992; Janz and Clayton, 1994). Therefore, I sequenced 1322 bp upstream of the 426 bp promoter region. A 1748 bp *Hind*III-*Pst*I fragment had already been cloned into pBluescript (plasmid pMT1.22HPI; Graham and Barry, 1995) which contains the 426 bp putative promoter region and 1322 bp of sequence immediately upstream of the promoter (see Fig. 3.2.1.2). Sequencing was performed in both directions in a step-wise fashion, first using the T7 primer against the T7 promoter of pBluescript (USB) and in the reverse direction a primer designed against the putative 1.22 M-VSG gene promoter region, and then using primers designed against novel sequence in the ~1.3 kb fragment (the primers used in the sequencing reactions are listed in Appendix 1). The sequencing reactions were performed exactly as described in Materials and Methods (see section 2.5) and the sequence is shown in figure 3.2.1.2.

3.2.2 Reporter plasmids

All reporter constructs used in the following experiments were derived from plasmid pHD52 (Hug *et al.*, 1993). pHD52 contains the 149 bp promoter sequence of the bloodstream VSG gene expression site which contains the 221 VSG gene (Zomerdijk *et al.*, 1990; 1991c). The promoter is upstream of a luciferase reporter gene which is flanked at its 5' end by the actin gene splice acceptor signal and 5' untranslated region (5' UTR), and at its 3' end by the actin gene 3' UTR. In transient transfection experiments of *Trypanosoma brucei,* the origin of the 5' splice acceptor signal and the 3' UTR used to flank the reporter gene can affect the level of reporter gene expression. In particular, the 3' UTR of life cycle stage-regulated genes can affect stage-specific regulation of a reporter gene in transient transfection experiments (Jefferies *et al.*, 1991; Hug *et al.*, 1993; Berberof *et al.*, 1995; see section 1.8). However, the actin gene is not thought to be differentially regulated during the trypanosome life cycle (Amar *et al.*, 1988) and the actin splice acceptor signal and 3' UTR have been shown to allow reasonable levels of reporter gene expression in transient transfection of bloodstream trypanosomes (Hug *et al.*, 1993). For these reasons the actin splice acceptor signal and

*Hind*III
1 **AAGCTT**AAAGCAATCTGATCCCTCAGTAAAATTCGAGTCAGAAATCTATCCTCTGAAAAGAACAAAGTATTCCCATCCCA 80

*Kpn*I
81 ACAAAATATAAAAAGAGACTCGTTTAAACATTAATTGTTGTATATTTTTGTTCGGGGTATTTCATTTTC**GGTACC**TTTCA 160

161 CACTCGTTTTATCTTTTCGAACGGGGCTCCGATCAGTTTTAGGCCAATATTCTCAGTGATAAAACGTGCAATGAGCGGCA 240

241 GTTCCGCATTTACTTCCGCTTCTTCTGGAGACTCAGAATAAGAGGTATTAAAAAATTTGGGATGCCAAATAACAGCGGGA 320

321 ATGACAACAATACCCAGGAGCGTCCTTCAGTATTTGGCATTGTAGAGTTTAGCATCCGTCAAACCTTAAACCTTGCCTGG 400

401 ATATTTCTAATCTCACGGCCAATCTATTTTTACAGCGGTGCGTGTATCCAAGTTTCCGAGCTTATGGGAAGCAATTGTTT 480

481 CTTTCGCCACAACTAGCCGAAATGTTTTCTTTAACAAACAATTTTGATAGCAGAAATATGAACTGTGGACTGAATTATTT 560

561 TACGCAGTTTCAAGTTCAAATCCTCGGCGAACAGGAGTGTTTTGTTAGGAAATTGCCCTTCCCCCTTCGTCCGTGTGTTT 640

641 CTTCCGGTACAGATAAACCTTTCAATGCTATATATGCTTATGCACTTGCCCAATTAAACAACTCAAGAACTGTACGATGA 720

721 CACTAGAAACATGCGGCAGGACATTCACGTGTTTGGTTGCATTCCATGGCACCATGCACTGCCATCGCCTTGAAAAGAAG 800

801 CAGTTATCATCAAAACAGGCGCTTACCACTTCGCTTCCAAGCGTAGCCACGTAAAGGGGGGCTGAAAGCGGCAACTTCTA 880

881 TTTGGGCAACTGCCCACATAAGTCGcGcTCGCCCGTGCAATTCTTACGGAATAGCAAATCTAGCGCCTTCTAGCTGCGTC 960

*Xba*I
961 CCCGATAACTACACAGAAGGAAGAGAGTAATAAATTTCGcGTAGTTTTTCGAAGGTCGATTTCCACATCACC**TCTAGA**TT 1040

1041 AAATTTCTCCCTTTCATGTCAGCAAAAGTTAAAATTACATGCCTTCCTATCTATCAAAATACTGCCGAAATCAATCACAG 1120

1121 GCACAATCCGTCGACACCTGCGCTGAACTTTTTTTTTCGTTTTTGCCCGCTGACACTTCCGCTGGCCACGGAGAAGGGAT 1200

1201 ACTTAAGTGAATTTCGATGGCCTGAGGTAGTCGAGGCCGTGGTCGGCGGAAACGGTTTGGGCCAACGAGCACGTTTTCCT 1280

*Bam*HI
1281 TTTTGTTCAGGTTCGAGTTCAACATCGGGTGCTTGAGCCGAA**GGATCC**CTATAGAGTATCAGAATATCTCAAGACGAGTT 1360

1361 TCCTTGCTTAAAGCCGACATCCGTACCCAAAAAGAATAAGTTGTAGTAAGTGTATTTCTCGCTGTTCTGACTCTTAGGGT 1440

1441 TTTAGATTTCGTGCCGAGGACAGAATGGGAAACCATCGTCTATGGGCTCATCGCCATTTCCGCAGCGACATTCGTCTTGG 1520

1521 CGCTTGAGGTGTAGCTTTGTGAACCCTGGAAGCATTGAAAGTCTACCGTATTTTCCGCCAACTACTCGGAGGCCCGGACG 1600

1601 TGGCAGGCAATTTTCGATGTGAAACCTTGTCAATGCGCTCGCAATACGTGTCTTGTCAGCGTGCGACTCCGCAAGCCTGG 1680

*Pst*I
1681 CCCATGCGACTCGACCGCGGGGGTAGTGGCATTGATAGAGGTGGCGGCGATGCTTTTCTCCG**CTGCAG**

Figure 3.2.1.2. **The sequence of the 1.22 M-VSG gene promoter region**. The sequence of the *Bam*HI-*Pst*I 426 bp fragment has been reported previously (Graham and Barry, 1995). The arrow shows the transcriptional start site of the 426 bp promoter fragment determined in metacyclic-derived trypanosomes (Graham and Barry, 1995). Restriction fragments of 426 bp (*Bam*HI-*Pst*I) and 1.6 kb (*Kpn*I-*Pst*I) were tested in bloodstream trypanosome transient transfection (see sections 3.2.5 and 3.2.6). Only the relevant restriction sites are shown.

actin 3' UTR were used in all reporter constructs for bloodstream trypanosome transient transfection experiments.

3.2.3 Construction of plasmids

pHD52CAT is the equivalent of pHD52 (Hug *et al.*, 1993) except that a chloramphenicol acetyl transferase (CAT) reporter gene replaces the luciferase gene. In this plasmid, the 221 bloodstream VSG gene expression site promoter is positioned upstream of the CAT gene which is flanked by the actin gene derived splice acceptor and 3' UTR. The luciferase gene was removed from pHD52 by digestion with *Hind*III and *Bam*HI and replaced directly by the CAT gene from pJP44 (Sherman *et al.*, 1991) on a *Hind*III-*Bam*HI fragment (Fig. 3.2.3.1A).

For p-HD52CAT, the bloodstream VSG gene expression site promoter was removed completely from pHD52CAT, leaving a promoterless construct: pHD52CAT was digested with *Sac*I and then blunted before *Sma*I digestion and religation (Fig. 3.2.3.1B)

For p1.22sHD52CAT the bloodstream VSG gene promoter of pHD52CAT was replaced completely by the 426 bp putative promoter region from the 1.22 M-VSG gene locus. This is the 5' most 426 bp *Bam*HI-*Pst*I fragment of pMG7.1-1 (Cornelissen *et al.*, 1985; Fig. 3.2.1.1) subcloned into pBluescript (denoted pMT1.22BPs; Fig. 3.2.1.1), this fragment contains the transcription initiation site for the 1.22 M-VSG gene as determined by nuclear run-on, RNase protection and 5' reverse transcriptase PCR experiments in day 5 metacyclic-derived trypanosomes (Graham and Barry, 1995). pMT1.22BPs was digested with *Sac*I and *Eco*RV (which cut within the pBluescript polylinker flanking the insert) and the 426 bp insert ligated into pHD52CAT digested with *Sac*I and *Sma*I to remove the 221 bloodstream VSG gene expression site promoter (Fig. 3.2.3.1C).

For p1.22lHD52CAT the bloodstream VSG gene promoter of pHD52CAT was replaced completely by a 1.6 kb fragment of the 1.22 M-VSG gene locus. This is the 1.6 kb *Kpn*I-*Pst*I fragment derived from pMT1.22HPl, a 1.75 kb *Hind*III-*Pst*I subclone of pMG7.1-1 in pBluescript (Fig. 3.2.1.1). The 1.6 kb *Kpn*I-*Pst*I fragment contains the 426 bp *Bam*HI-*Pst*I fragment of p1.22sHD52CAT at its 3' end together with ~1.2 kb of sequence immediately upstream (Fig. 3.2.1.2). pMT1.22HPl was digested with *Kpn*I

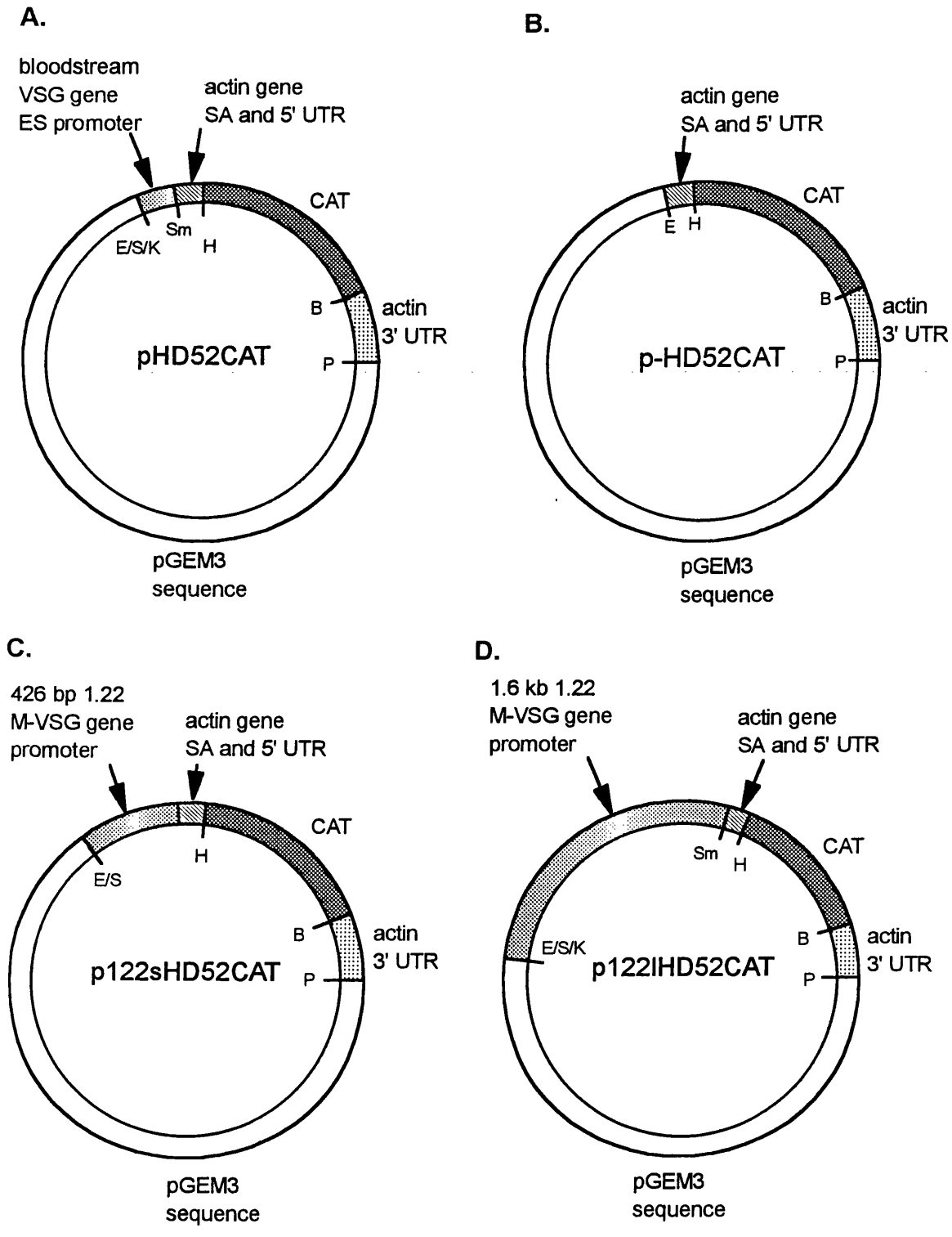


Figure 3.2.3.1. **Schematic representation of the reporter plasmids used in transient transfection of bloodstream trypanosomes.** Each plasmid contains the CAT reporter gene which is flanked at the 3' end by the actin gene 3' untranslated region (3' UTR) and at the 5' end by the actin gene splice acceptor signal (SA) and 5' UTR. pHD52CAT contains a 149 bp promoter fragment from the 221 bloodstream VSG gene expression site (ES). p122sHD52CAT contains a 426 bp *Bam*HI-*Pst*I putative promoter fragment from the 1.22 M-VSG gene expression telomere. p122lHD52CAT contains a larger 1.6 kb *Kpn*I-*Pst*I putative promoter fragment from the 1.22 M-VSG gene expression telomere. p-HD52CAT contains no eukaryotic promoter sequence. Each of the reporter plasmids contains pGEM3 sequence. E, *Eco*RI; S, *Sac*I; K, *Kpn*I; Sm, *Sma*I; H, *Hind*III; B, *Bam*HI; P, *Pst*I.

(which cuts near the 5' end of the insert) and *Sma*I (which cuts within the polylinker at the 3' end of the insert) and the 1.6 kb insert ligated into pHD52CAT digested with *Kpn*I and *Sma*I to remove the bloodstream VSG gene promoter (Fig. 3.2.3.1D).

All plasmids made were checked for correct insertions or deletions upstream of the actin slice acceptor signal by restriction digest analysis and sequencing. Sequencing was performed using the T3actinSA primer (see section 2.5), which anneals to the actin splice acceptor signal and directs sequencing upstream.

3.2.4 Transient transfection of bloodstream trypanosomes

Transient transfection of bloodstream trypanosomes has been performed in several laboratories using reporter plasmids containing either the CAT or luciferase reporter genes. Transient transfection of trypanosomes is achieved by electroporation with supercoiled plasmids. In all cases of bloodstream trypanosome transient transfection, results have been presented as a percentage of a positive control due to the intrinsic variability in the transfection assay (Jefferies *et al.*, 1991; Hug *et al.*, 1993; Berberof *et al.*, 1995; Urmenyi and Van der Ploeg, 1995; Vanhamme *et al.*, 1995a; Biebinger *et al.*, 1996; Pham *et al.*, 1996). The variation in the transfection assay is likely be due to a variation in the transfection efficiency of separate bloodstream trypanosome preparations that may be due to different cell growth rates or different positions in the log phase of growth of separate preparations of trypanosomes. In addition, transient transfection of bloodstream trypanosomes is much less efficient than transient transfection of procyclic trypanosomes. Estimates for transfection efficiency of bloodstream trypanosomes range from 100 to more than 1000 fold lower than procyclic trypanosomes (Hug *et al.*, 1993; Vanhamme *et al.*, 1995a; Pham *et al.*, 1996). Lower transfection efficiency of bloodstream trypanosomes with respect to procyclic trypanosomes is partly due to the fact that bloodstream trypanosomes are much more susceptible to electroporation than procyclic trypanosomes, only ~50% of bloodstream trypanosomes survive electroporation whereas ~90% of procyclic trypanosomes are alive following electroporation. In the experiments presented here, transient transfection of separate preparations of bloodstream trypanosomes with the same plasmid, gave variable levels of CAT gene expression. However, within a single bloodstream trypanosome preparation, the relative level of CAT gene expression directed by different reporter plasmids usually remained constant. The results are therefore presented as a

percentage of the positive control plasmid (pHD52CAT containing the 221 bloodstream expression site promoter) transfected in each preparation of bloodstream trypanosomes.

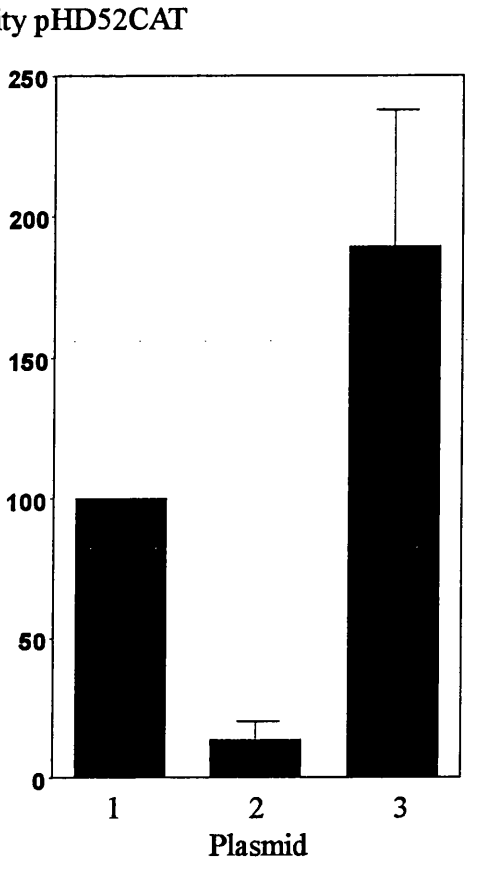
3.2.5 A metacyclic VSG gene promoter active at the bloodstream stage

Bloodstream trypanosomes were transiently transfected with equal amounts of plasmids pHD52CAT as a positive control, p-HD52CAT as a negative control and p1.22sHD52CAT as the test construct exactly as described in Materials and Methods (see section 2.9). Each of the reporter plasmids are identical (i.e. contains the same actin RNA processing signals and plasmid background, pGEM3) except for the promoter region upstream of the actin splice acceptor signal. Therefore, the level of CAT gene expression directed by each transfected plasmid can be directly compared. Transiently transfected trypanosomes were left to recover for 5 hours at 37°C following electroporation before CAT activity was determined by a standard CAT assay using xylene phase extraction (Seed and Sheen, 1988; see section 2.10). An experiment consisted of electroporation of a bloodstream trypanosome preparation with each of the three reporter plasmids, performed in duplicate. The results of a single transfection experiment are shown in table 3.2.5.2 and the data from eight independent experiments are summarised in figure 3.2.5.1.

The positive control, pHD52CAT, contains the promoter of the bloodstream expression site containing the 221 bloodstream VSG gene. The 221 bloodstream VSG gene expression site promoter has been shown to drive high expression of a reporter gene in transient transfection of bloodstream trypanosomes (Hug *et al.*, 1993; Biebinger *et al.*, 1996). Indeed, activity of the 221 bloodstream expression site promoter is greater than the trypanosome ribosomal RNA gene promoter; rRNA gene promoters are usually highly active in eukaryotes (Sollner-Webb and Tower, 1986), when assayed by transient transfection of bloodstream trypanosomes (Biebinger *et al.*, 1996). The 221 bloodstream VSG gene expression site promoter is therefore a good positive control for the putative metacyclic VSG gene promoter in bloodstream transient transfections.

p-HD52CAT contains no eukaryotic promoter sequences upstream of the CAT gene and so specific transcription initiation and transcription of the CAT gene is unlikely to occur in trypanosomes. Spurious transcription initiation from the bacteriophage T7 RNA polymerase promoter, which is upstream of the CAT gene in p-HD52CAT, cannot be detected in trypanosomes in the absence of T7 polymerase (Wirtz *et al.*, 1994).

A.



B.

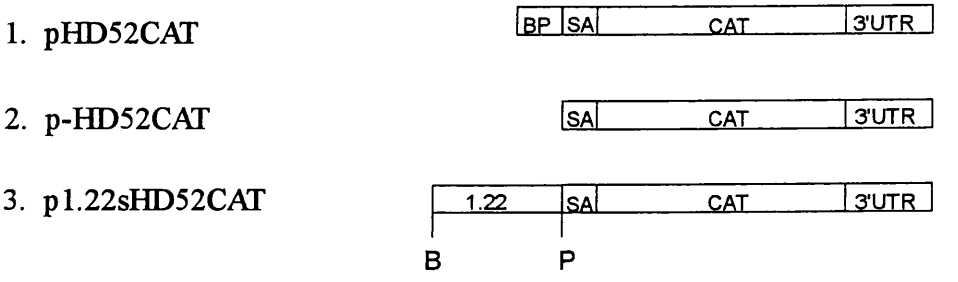


Figure 3.2.5.1. **Functional assay of the putative 1.22 M-VSG gene promoter in transient transfection of bloodstream trypanosomes.** (A) Results of 8 separate experiments, i.e. different preparations of bloodstream trypanosomes electroporated in duplicate with equal amounts of each of the three plasmids, pHD52CAT, p-HD52CAT and p1.22sHD52CAT. The bar chart shows the mean CAT activity and standard deviation for each reporter plasmid represented as a percentage of the positive control pHD52CAT. (B) Schematic diagrams of the three reporter plasmids. CAT, CAT gene coding sequence; SA, actin gene splice acceptor signal and 5' UTR; 3' UTR, 3' untranslated region; BP, promoter of the 221 bloodstream expression site; 1.22, 426 bp *Bam*HI-*Pst*I putative promoter fragment from the 1.22 M-VSG gene expression telomere; B, *Bam*HI; P, *Pst*I.

Plasmid	Promoter	CAT activity (cpm/5x10^7 cells electroporated)	
		duplicate values	*mean*
pHD52CAT	B-VSG	16013 19367	17690
p-HD52CAT	no promoter	2323 2325	2324
p1.22sHD52CAT	426 bp 1.22 M-VSG	39814 38768	39291
p1.22lHD52CAT	1.6 kb 1.22 M-VSG	28830 28219	28525
pBluescript	-	2153 1844	1999

Table 3.2.5.2. **Table showing results of a representative bloodstream trypanosome transient transfection experiment**. Duplicate aliquots of 5 x 10^7 trypanosomes from a single preparation of bloodstream trypanosomes were each electroporated with 10 μg of a reporter plasmid. (see section 2.9). CAT activities were measured in counts per minute (cpm) using a scintillation counter following a standard CAT activity assay (see section 2.10). The table shows the CAT activity of each transfection (duplicate values) and the mean of each duplicate (mean) represented as cpm/5 x 10^7 cells electroporated. B-VSG, 221 bloodstream VSG gene expression site promoter; 426 bp 1.22 M-VSG, *Bam*HI-*Pst*I fragment of the 1.22 M-VSG gene promoter; 1.6 kb 1.22 M-VSG, *Kpn*I-*Pst*I fragment of the 1.22 M-VSG gene promoter. The mean values from this experiment are incorporated into the data shown in figures 3.2.5.1 and 3.2.6.1.

Therefore, as expected, p-HD52CAT directs only low levels of CAT activity in bloodstream trypanosomes (13.8%±6% of pHD52CAT; Fig. 3.2.5.1). This level of CAT activity was equivalent to the pBluescript plasmid (Fig. 3.2.6.1) which contained no CAT reporter gene and confirms that the CAT gene in p-HD52CAT is not transcribed. This level of CAT activity was considered to be the background level.

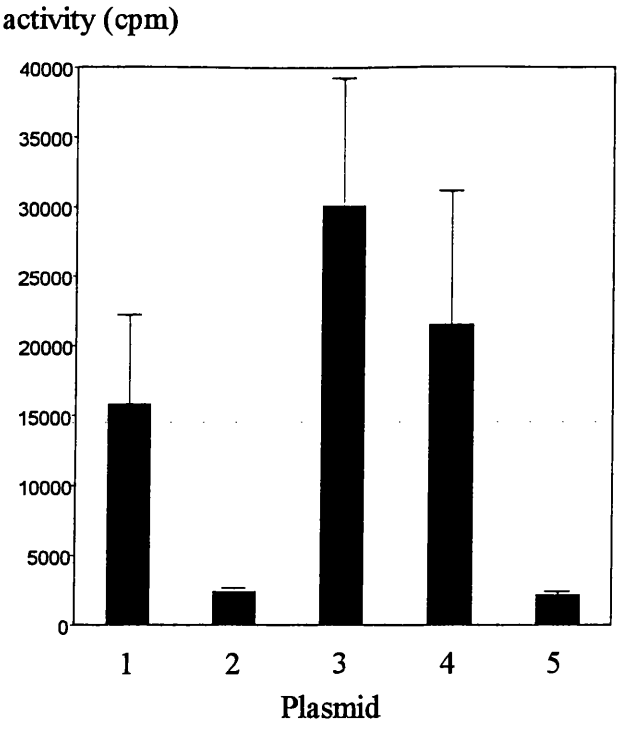
I found that p1.22sHD52CAT directed very high levels of CAT gene expression in bloodstream trypanosomes (189%±48% of pHD52CAT; Fig. 3.2.5.1). This indicates that the 426 bp *Bam*HI-*Pst*I fragment containing the putative 1.22 M-VSG gene promoter is able to efficiently initiate transcription and direct transcription of the CAT gene, not only in metacyclic-derived trypanosomes, but also in bloodstream trypanosomes when assayed by transient transfection. The putative 1.22 M-VSG gene promoter is able to direct CAT gene expression that is approximately 1.5-2 times that directed by the bloodstream expression site promoter in these bloodstream trypanosome transient transfections.

3.2.6 The effect of upstream sequences on the putative promoter of the 1.22 M-VSG gene

Although the putative 1.22 M-VSG gene promoter was able to direct high levels of CAT gene expression in transient transfection of bloodstream trypanosomes, the same promoter region was inactive in bloodstream trypanosomes *in vivo* at the 1.22 M-VSG gene expression telomere as assayed by nuclear run-on analysis (Graham and Barry, 1995). The promoter may be down-regulated in bloodstream trypanosomes at the metacyclic expression telomere by sequences of the telomere that are not included in the 426 bp test fragment. These sequences could be silencer sequence elements that bind repressor molecules and interfere with the mechanism of transcription initiation from the promoter as observed in yeast (Saha *et al.*, 1993; Tjian, 1995). Silencer sequence elements seem to be most efficient when positioned a short distance (within 500 bp) upstream of the binding sites of transcription factors (Saha *et al.*, 1993). Therefore, I tested a more extensive region of sequence immediately upstream of the 1.22 M-VSG gene promoter for modulation of promoter activity in transient transfection of bloodstream trypanosomes.

In three of the experiments quoted in section 3.2.5 two additional plasmids were tested in duplicate, pBluescript as an additional negative control (see section 3.2.6) and

A.

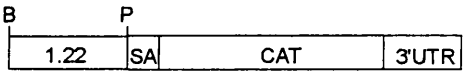


B.

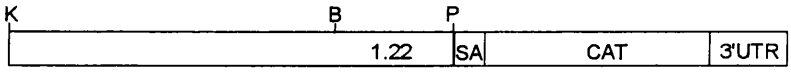
1. pHD52CAT

2. p-HD52CAT

3. p1.22sHD52CAT



4. p1.22lHD52CAT



5. pBluescript

Figure 3.2.6.1. **Functional analysis of the short and long fragments of the putative 1.22 M-VSG gene promoter in transient transfection of bloodstream trypanosomes.** (A) The mean and standard deviation of 3 experiments are represented in the bar chart. Each experiment consisted of different preparations of bloodstream trypanosomes electroporated in duplicate with equal amounts of each of the 4 reporter plasmids and pBluescript. CAT activity is shown in counts per minute (cpm). (B) Schematic representations of each of the 4 reporter plasmids. pHD52CAT, -HD52CAT and p1.22sHD52CAT are as for figure 3.2.4.1. p1.22lHD52CAT contains the same 426 bp *Bam*HI-*Pst*I putative 1.22 M-VSG gene promoter fragment as p1.22s HD52CAT but with an additional 1.2 kb of sequence that is directly upstream at the 1.22 M-VSG gene expression telomere (see Fig. 3.2.1.2). BP, 221 bloodstream expression site promoter; SA, actin gene splice acceptor and 5' UTR; CAT, CAT coding sequence; 3' UTR, actin gene 3' UTR; 1.22, 1.22 M-VSG gene promoter fragment; B, *Bam*HI; P, *Pst*I; K, *Kpn*I.

p1.22lHD52CAT. p1.22lHD52CAT contains a 1.6 kb *Kpn*I-*Pst*I fragment derived from the 1.22 M-VSG gene locus positioned upstream of the CAT gene and actin splice acceptor signal. This includes the *Bam*HI-*Pst*I fragment of plasmid p1.22sHD52CAT containing the putative promoter at its 3' end, plus 1.2 kb of sequence immediately 5' of this at the metacyclic expression telomere (see Fig. 3.2.1.2). Figure 3.2.6.1 summarises the results of 3 experiments giving the mean of absolute counts (cpm), the data from one of these experiments is shown in table 3.2.5.2. pBluescript gave only background levels of CAT activity. Transfections with p1.22lHD52CAT yielded CAT activity at a level approximately 70% of that for p1.22sHD52CAT. The difference in the levels of CAT activity directed by plasmids p1.22lHD52CAT and p1.22sHD52CAT can be accounted for in the actual number of copies of plasmid transfected. p1.22sHD52CAT is 4.4 kb and p1.22lHD52CAT is 5.6 kb in size, consequently when transfecting 10 μg of plasmid DNA, I transfected 30% more copies of p1.22sHD52CAT than p1.22lHD52CAT. Therefore, the 1.2 kb of sequence directly upstream of 426 bp promoter fragment does not significantly alter the level of CAT gene expression directed by the putative 1.22 M-VSG gene promoter in transient transfection of bloodstream trypanosomes.

3.2.7 5' to 3' deletion analysis of the 1.22 M-VSG gene promoter

To determine the 5' limit of the putative 1.22 M-VSG gene promoter, reporter plasmids were prepared containing deleted mutations of the 426 bp promoter fragment. If a deletion removed sequences of the putative promoter that were essential to direct transcription initiation, then this would be reflected in the level of CAT gene expression directed by the promoter fragment in transient transfection of bloodstream trypanosomes.

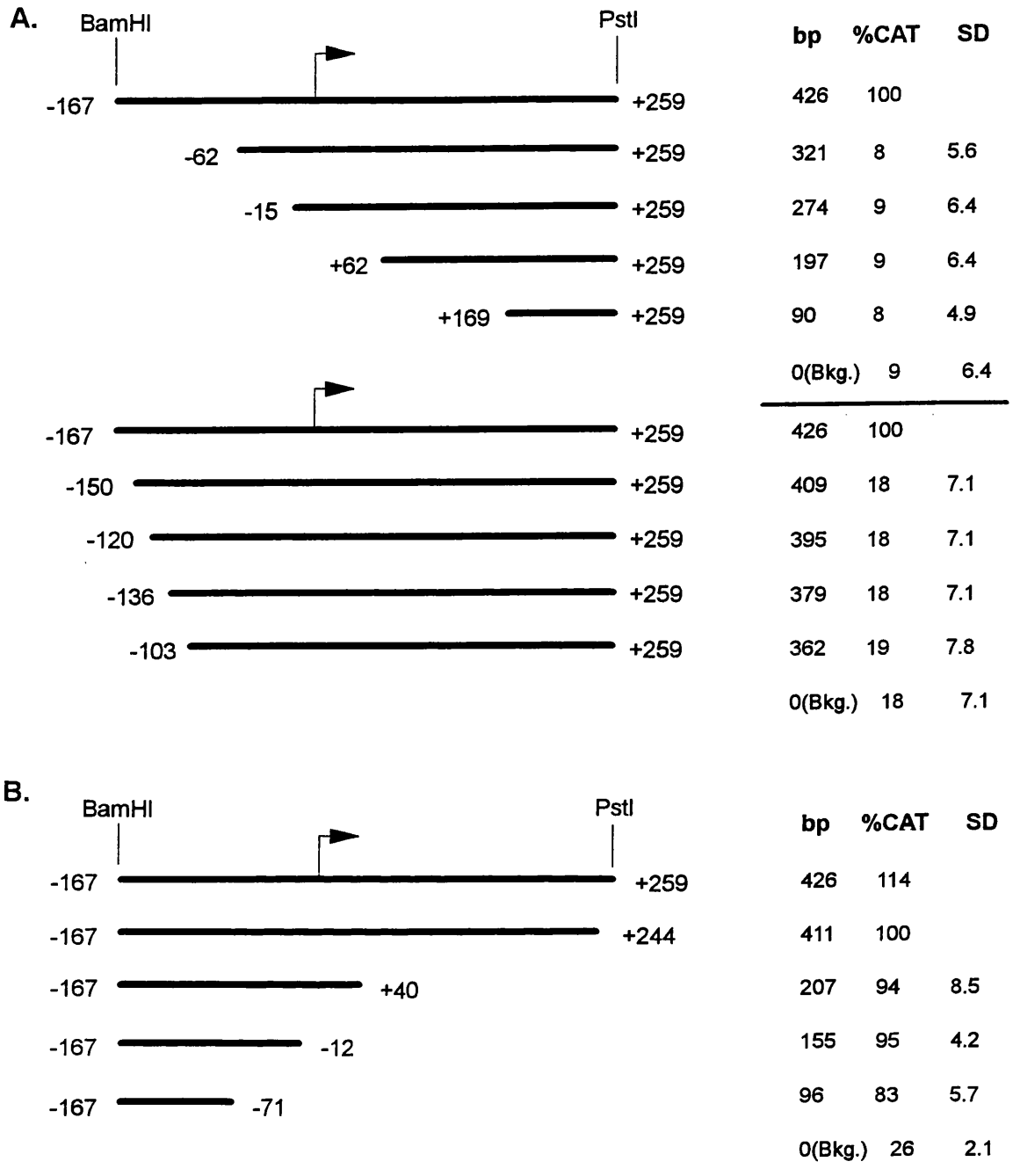
Firstly, reporter plasmids were prepared containing a series of deletions through the putative 1.22 M-VSG gene promoter region in a 5' to 3' direction. The p1.22sHD52CAT plasmid, containing the 426 bp *Bam*HI-*Pst*I fragment, was digested with *Spe*I and *Sac*I and deleted with Exo III and S1 nuclease (see section 2.4). A number of time points were taken after initiating Exo III digestion to generate a series of directional deletions spanning the entire promoter region. Religated clones were checked for deletion by restriction analysis and the precise size of the remaining fragment determined by sequencing. As all deletion plasmids were derived from

p1.22sHD52CAT, each plasmid was identical except for the promoter region upstream of the actin splice acceptor signal.

Figure 3.2.7.1 summarises the results of all 5' to 3' deletion constructs assayed by transient transfection of bloodstream trypanosomes. The CAT activity (measured in cpms) of trypanosomes transiently transfected with each plasmid was variable between bloodstream trypanosome preparations as described previously. For example, two preparations of bloodstream trypanosomes gave CAT activities of 11,000 and 38,000 cpms for p1.22sHD52CAT, and respectively 1370 and 3080 cpms for p-HD52CAT (background). In an experiment (a single preparation of bloodstream trypanosomes) each plasmid was transfected in duplicate and a mean CAT activity determined, the results were then calculated as a percentage of the undeleted promoter construct (p1.22sHD52CAT). Each deleted promoter construct was tested in at least two independent experiments and the mean results are presented in figure 3.2.7.1.

Initially, four deleted promoter plasmid constructs were selected and tested. These plasmids contained deletions of 105 bp, 152 bp, 229 bp and 336 bp from the *Bam*HI restriction site at the 5' end of the 426 bp promoter fragment (Fig. 3.2.7.1A). When tested all four plasmid constructs gave background levels of CAT activity, equivalent to the plasmid p-HD52CAT (~1300 cpm). The shortest deletion of 105 bp leaves a promoter fragment of 321 bp but only 62 bp upstream of the transcriptional start site determined previously in metacyclic-derived trypanosomes (Graham and Barry, 1995). 62 bp of sequence upstream of the transcriptional start site is therefore not sufficient for minimal promoter activity in transient transfection of bloodstream trypanosomes presumably due to removal of motifs in the deleted region of 105 bp that are essential for promoter activity.

To try and dissect this 105 bp region of the metacyclic VSG gene promoter four further deletion constructs were prepared containing deletions of only 17 bp, 31 bp, 47 bp, and 64 bp from the *Bam*HI site at the 5' end of the 426 bp promoter fragment (Fig. 3.2.7.1A). Respectively, these deletions produced constructs containing promoter fragments extending 150 bp, 136 bp, 120 bp and 103 bp upstream of the transcriptional start site. These four deletion promoter constructs also gave background levels of CAT activity (Fig. 3.2.7.1A). This implies that there are sequence motifs between 150 bp and 167 bp upstream of the transcriptional start site (within 17 bp 3' of the *Bam*HI site) that are essential for transcriptional activity of the 1.22 M-VSG gene promoter in transient



bp	%CAT	SD
426	100	
321	8	5.6
274	9	6.4
197	9	6.4
90	8	4.9
0(Bkg.)	9	6.4
426	100	
409	18	7.1
395	18	7.1
379	18	7.1
362	19	7.8
0(Bkg.)	18	7.1

bp	%CAT	SD
426	114	
411	100	
207	94	8.5
155	95	4.2
96	83	5.7
0(Bkg.)	26	2.1

Figure 3.2.7.1. **Deletion analysis of the 426 bp *Bam*HI-*Pst*I putative 1.22 M-VSG gene promoter fragment in transient transfection of bloodstream trypanosomes.** (A) 5' to 3' deletions with the positions at each end of the deleted promoter fragment numbered with respect to the transcriptional start site (black arrow) determined in metacyclic-derived trypanosomes. The chart on the right shows the size of each deleted construct (bp), the CAT activity of each deleted construct as a percentage of p1.22sHD52CAT containing the undeleted promoter (%CAT), and the standard deviation from 2 experiments performed in duplicate (SD). Two sets of experiments were carried out with the gross deletions at the top, and finer deletions of the region closer to the *Bam*HI restriction fragment at the bottom. The background level of CAT activity (Bkg.) at the bottom of each of the sets of data is CAT activity derived from trypanosomes transfected with p-HD52CAT. (B) 3' to 5' deletions of putative 1.22 M-VSG gene promoter. Numbering of the promoter fragments is as above. The CAT activity of trypanosomes transfected with the deleted fragments are represented as a percentage of the deletion closest to the 426 bp fragment. Background (Bkg.) is of a promoterless construct in pBluescript. Standard deviations (SD) are of two experiments carried out in duplicate.

transfection of bloodstream trypanosomes. No shorter deletions have been assayed and so the 5' extent of the putative 1.22 M-VSG gene promoter can be approximated to this *Bam*HI site 167 bp upstream of the transcriptional start site.

3.2.8 3' to 5' deletion analysis of the 1.22 M-VSG gene promoter

To determine the 3' limit of the putative 1.22 M-VSG gene promoter, reporter plasmids were prepared containing a series of sequential deletions through promoter region in a 3' to 5' direction. Plasmid pMT1.22BPs does not contain the appropriate restriction sites flanking the insert to allow easy cloning of deletions into a reporter plasmid and therefore plasmid pMT1.22BE was used to generate 3' to 5' deletions of the 426 bp putative promoter fragment. Plasmid pMT1.22BE contains a 1 kb *Bam*HI-*Eco*RI fragment from the 1.22 M-VSG gene locus that includes the 426 bp *Bam*HI-*Pst*I fragment containing the putative 1.22 M-VSG gene promoter at the 5' end (Fig. 3.2.1.1). pMT1.22BE was digested with *Eco*RI and *Apa*I and deleted in a 3' to 5' direction with ExoIII and S1 nuclease (see section 2.4). Deletions were checked by restriction analysis and appropriate sized fragments sequenced for the precise size of the deletion. In the plasmids pHD52CAT and p-HD52CAT there are a very limited number of restriction sites upstream of the actin splice acceptor signal that are available to clone the deleted fragments of the putative promoter. Therefore, the 3' to 5' deleted promoter fragments were cloned into another plasmid p-52CAT. In p-52CAT the actin splice acceptor, 5' UTR, CAT gene and actin 3' UTR have been completely removed from p-HD52CAT by digestion with *Sma*I and *Pst*I and subcloned into pBluescript (SK-) to provide the appropriate restriction sites upstream of the actin splice acceptor and 3' UTR.. The plasmids containing the 3' to 5' deletions were digested with *Kpn*I, blunted and then digested with *Sac*I to remove the insert and ligated into plasmid p-52CAT that had been digested with *Sma*I and *Sac*I. The nature of the plasmid background (i.e. pGem3 or pBluescript) used in transient transfection experiments does not significantly effect the level of reporter gene expression (S. V. Graham, unpublished observations).

Four deleted promoter constructs were selected and tested by transient transfection of bloodstream trypanosomes. The promoter fragments tested extending 3' from the 5' *Bam*HI site were 411 bp, 207 bp, 155 bp and 96 bp in size. In relation to the described transcriptional start site of the 1.22 M-VSG gene promoter (Graham and Barry, 1995) these positions are +244 bp, +40 bp, -12 bp and -71 bp (fig. 3.2.7.1B).

The 411 bp fragment is nearly the same as the full 426 bp fragment in p1.22sHD52CAT. The remaining 3 deletions include the 207 bp fragment containing the transcriptional start site, the 155 bp fragment with the start site deleted, and the 96 bp fragment with the start site deleted and 70 bp immediately upstream. (see Fig. 3.2.7.1B).

All four deleted promoter fragments gave levels of promoter activity equivalent to the undeleted promoter when assayed in transient transfection of bloodstream trypanosomes. This suggests that there are no motifs essential for transcription initiation from the 1.22 M-VSG gene promoter, within 330 bp upstream of *Pst*I site in the 426 bp *Bam*HI to the *Pst*I promoter fragment. Maximum transcription from the putative 1.22 M-VSG gene promoter is achieved by assaying just 96 bp of sequence 3' of the *Bam*HI site. Surprisingly, this sequence does not include the transcriptional start site and extends from position -71 bp to -167 bp with respect to the start site mapped in metacyclic-derived trypanosomes. Other trypanosome promoters analysed to date all contain sequences essential for transcription initiation within 70 bp immediately upstream of the start site (Zomerdijk *et al.*, 1991c; Brown *et al.*, 1992; Janz and Clayton, 1994; Pham *et al.*, 1996).

3.3 DISCUSSION

In this chapter, transient transfection of bloodstream trypanosomes with a reporter plasmid construct that includes a 426 bp fragment containing the putative 1.22 M-VSG gene promoter, indicates that this promoter is able to initiate transcription at this life cycle stage when assayed on a plasmid episomal vector. A larger 1.6 kb fragment of the 1.22 M-VSG gene promoter region was also tested in bloodstream trypanosome transient transfection; this fragment includes the 426 bp promoter fragment at the 3' end and upstream, 1.2 kb of sequence immediately upstream of the promoter at the 1.22 M-VSG gene expression telomere. Inclusion of the upstream sequence had no significant effect on the level of reporter gene expression directed by the 1.22 M-VSG gene promoter. In addition, transient transfection of bloodstream trypanosomes with a series of reporter plasmids containing sequential deletions through the putative 1.22 M-VSG gene promoter fragment in a 5' to 3' and 3' to 5' direction revealed a minimal promoter sequence of no more than 96 bp.

3.3.1 Life cycle stage-specific control of the 1.22 M-VSG gene promoter

The 426 bp *Bam*HI-*Pst*I restriction fragment that was identified in nuclear run-on reactions as the region of transcription initiation of the 1.22 M-VSG gene in metacyclic-derived trypanosomes, was also able to direct expression of a reporter gene in transient transfection of metacyclic-derived trypanosomes (Graham and Barry, 1995). In transient transfection of bloodstream trypanosomes this promoter region was able to direct levels of reporter gene expression that were greater than those directed by the positive control promoter, the 221 bloodstream expression site promoter. This positive control promoter directs transcription of the bloodstream expression site containing a single copy of the 221 VSG gene (Zomerdijk *et al.*, 1990). Transcription of this single copy gene at the 221 bloodstream expression site is sufficiently high to encode the entire VSG surface coat of approximately 10^7 VSG molecules (Cross, 1975; 1990): at the bloodstream stage the transcripts of a single VSG gene can account for as much as 10% of the polyadenylated RNA in trypanosomes (Pays *et al.*, 1994). Therefore, it is not surprising that the bloodstream expression site promoter is able to direct high levels of reporter gene expression in transient transfection of bloodstream trypanosomes (Hug *et al.*, 1993; Biebinger *et al.*, 1996). In comparison with the bloodstream expression site promoter, the 1.22 M-VSG gene promoter is able to direct high levels of reporter gene expression in transient transfection of bloodstream trypanosomes. This suggests that the 1.22 M-VSG gene promoter can be highly active at the bloodstream stage and the transcriptional machinery that initiates transcription from the 1.22 M-VSG gene promoter region in metacyclic trypanosomes may also be present in bloodstream trypanosomes.

However, though the putative 1.22 M-VSG gene promoter is highly active in transient transfection of bloodstream trypanosomes, it is not active at the bloodstream stage *in vivo* (Graham and Barry, 1995). The inactivity of the 1.22 M-VSG promoter in bloodstream trypanosomes *in vivo* may be due to the environment of the promoter in the genome. For example, the chromosomal context of the promoter in bloodstream trypanosomes may exclude components of transcriptional machinery, or sequences at the metacyclic expression telomere may bind molecules that interfere with transcription initiation (see section 5.3.3). Comparable results have been obtained with other promoters of *Trypanosoma brucei* that are regulated in the life cycle *in vivo* (see section 1.10). For instance, the bloodstream expression site promoter is highly active in

transient transfection of procyclic trypanosomes (Zomerdijk *et al.*, 1991c) though the promoter is active at only a low level in procyclic trypanosomes *in vivo* (Pays *et al.*, 1990; Rudenko *et al.*, 1994). Similarly, the PARP promoter is highly active in transient transfection of bloodstream trypanosomes but down-regulated 5-10 fold in bloodstream trypanosomes *in vivo* (Berberof *et al.*, 1995; Biebinger *et al.*, 1996). The issue of down-regulation of the 1.22 M-VSG gene promoter at the 1.22 M-VSG gene expression telomere in bloodstream trypanosomes is considered more detail in Chapter 5.

At the procyclic stage, control of the 1.22 M-VSG gene is very different. As with bloodstream trypanosomes, transcription could not be detected from the putative 1.22 M-VSG gene promoter in procyclic trypanosomes by nuclear run-on experiments (Graham and Barry, 1995). However, the 426 bp fragment which contains the putative 1.22 M-VSG gene promoter and directs high levels of reporter gene expression in transient transfection of bloodstream and metacyclic-derived trypanosomes, directed only very low levels of reporter gene expression in transient transfection of procyclic trypanosomes (Graham and Barry, 1995). This suggests that the complement of transcription factors that initiate transcription from the 1.22 M-VSG gene promoter region in bloodstream and metacyclic-derived trypanosomes, are not all present in procyclic trypanosomes. Alternatively, there may be sequences in the 426 bp fragment that interfere with transcription initiation specifically at the procyclic stage. In either case, the mechanism for controlling transcription from the 1.22 M-VSG gene promoter region at the procyclic stage is very different from the control of the promoter in bloodstream trypanosomes. Interestingly, the 1.22 M-VSG gene promoter is the first promoter of *Trypanosoma brucei* that has been identified which is regulated during the life cycle when assayed by transient transfection. The trypanosome promoters that have been identified, the PARP gene promoter, the bloodstream expression site promoter, and the ribosomal RNA gene promoter, are all highly active in transient transfection of bloodstream and procyclic trypanosomes (Zomerdijk *et al.*, 1991c; Biebinger *et al.*, 1996; see section 1.9).

3.3.2 1.2 kb of upstream sequence does not affect transcription from the 1.22 M-VSG gene promoter

In addition to the reporter plasmid that contained the 426 bp 1.22 M-VSG gene promoter fragment, a longer 1.6 kb promoter fragment was tested in transient transfection of bloodstream trypanosomes. This fragment contains the 426 bp promoter sequence at the 3' end and a further 1.2 kb of sequence immediately upstream of the 426 bp promoter sequence at the 1.22 M-VSG gene expression telomere (see Fig. 3.2.6.1 and Fig. 3.2.1.2). The 1.2 kb of sequence upstream of the 1.22 M-VSG gene promoter at the metacyclic expression telomere did not significantly affect the level of reporter gene expression directed by the 1.22 M-VSG gene promoter in transient transfection of bloodstream trypanosomes. This result indicates that 1) there are no sequences in the 1.2 kb fragment that increase transcription initiation from the 1.22 M-VSG gene promoter at the bloodstream stage in a transient transfection assay, and 2) there are no sequences in this fragment that can repress transcription initiation from the putative promoter. Sequences that increase the level of transcription from the 1.22 M-VSG gene promoter may be either additional sequence elements of the promoter or enhancer elements. Enhancers may act at many kilobases from the promoter (Tjian, 1995), and therefore possible effects of enhancers on the 1.22 M-VSG gene promoter when in its endogenous telomere location cannot be ruled out. However, it is likely that any additional promoter elements would be located directly upstream of the *Bam*HI site of the 426 bp promoter fragment and contained in the 1.2 kb fragment that was included in this reporter plasmid. In particular, there is no evidence for an upstream control element which is observed upstream of the 'core' PARP and rRNA gene promoters in trypanosomes (Zomerdijk *et al.*, 1991c; Brown *et al.*, 1992; see section 1.9). Transcription of the 1.22 M-VSG gene in metacyclic-derived trypanosomes is insensitive to α-amanitin (Graham and Barry, 1991), and is therefore probably transcribed by RNA polymerase I. Promoters that direct transcription by RNA polymerase I in trypanosomes, and other eukaryotes, often include an upstream control element that is 100-200 bp upstream of the transcriptional start site and increases transcription from the core promoter which is directly upstream of the transcriptional start site (Sollner-Webb and Tower, 1986; Jacob, 1995; Vanhamme and Pays, 1995). The upstream control element is distinct from an enhancer element because it is entirely dependent on its spacing from the core promoter (Jacob, 1995). Assuming that the mechanism of

transcription initiation from the 1.22 M-VSG gene promoter is the same *in vivo* at the metacyclic stage and in transient transfection of bloodstream trypanosomes, then I conclude that the entire 1.22 M-VSG gene promoter is contained on the 426 bp *Bam*HI-*Pst*I restriction fragment tested.

The 1.2 kb of sequence directly upstream of the 1.22 M-VSG gene promoter also does not appear to contain any sequences that reduce transcription initiation from the 1.22 M-VSG gene promoter region at the bloodstream stage. As the 1.22 M-VSG gene promoter is active in transient transfection of bloodstream trypanosomes but inactive *in vivo*, the promoter may be repressed at the 1.22 M-VSG gene expression telomere. This may be effected by silencer sequences at the metacyclic expression telomere that bind repressor molecules and interfere with transcription initiation from the 1.22 M-VSG gene promoter region. Like enhancer elements, it is possible that these silencer elements can act at considerable distances from the transcriptional start site, but evidence suggests that these elements are more effective within 500 bp of the promoter elements (Saha *et al.*, 1993). Therefore, it is unlikely that silencer sequences within the 1.2 kb *Kpn*I-*Bam*HI fragment are responsible for down-regulating of the 1.22 M-VSG gene promoter *in vivo* at the bloodstream stage.

Enhancer sequences, and possibly silencer sequences, can act at distances of many kilobases from the site of transcription initiation. The upstream regulatory elements and the promoter can be brought into close physical proximity by looping-out the intervening sequences (Tjian, 1995) or packaging the intervening sequence into nucleosomal arrays (Wolffe, 1994). However, when considering the large regions of sequence that must be cloned and tested, it is clear that the transient transfection assay is an inappropriate method of screening the genome for sequences that may regulate transcription initiation from the 1.22 M-VSG gene promoter. In addition, the binding of a repressor to a silencer element or an activator to an enhancer element may require features of the metacyclic expression telomere other than DNA sequence. For instance, the plasmids used in these transient transfections are likely to differ from the metacyclic expression telomere in chromatin structure, DNA modifications and degree of DNA supercoiling that may be critical for protein-DNA interactions. The potential repression of the 1.22 M-VSG gene promoter region in bloodstream trypanosomes at the 1.22 M-VSG gene expression telomere may be related to transcriptional repression of promoters

close to telomeres in other organisms. Transcriptional silencing at telomeres is considered in more detail in section 5.3.3.

3.3.3 The minimal 1.22 M-VSG gene promoter sequence

5' to 3' deletion analysis of the *Bam*HI-*Pst*I 1.22 M-VSG gene promoter fragment showed that even the smallest deletion of 17 bp was sufficient to reduce reporter gene expression, directed by the promoter, to background levels in transient transfection of bloodstream trypanosomes. Sequences within this 17 bp are therefore essential for transcription initiation from the 1.22 M-VSG gene promoter fragment at the bloodstream stage and the *Bam*HI site defines the 5' limit of the promoter region. The 5' limit of the 1.22 M-VSG gene promoter at -167 bp in relation to the transcription start site, indicates that the 'core' promoter may be as much as 167 bp in size. Other core promoters that have been identified in trypanosomes are no more than 70 bp in size and positioned directly upstream of the transcriptional start site (Vanhamme and Pays, 1995; see section 1.9). However, subsequent deletion analysis of the 1.22 M-VSG gene promoter in a 3' to 5' direction revealed that the 3' limit of the promoter in bloodstream trypanosomes appeared to be well upstream of the transcriptional start site identified in metacyclic-derived trypanosomes. Sequence could be removed up to and including the site of transcription initiation with no effect on the high level of reporter gene expression in bloodstream trypanosomes. The largest 3' to 5' deletion tested, removed sequence up to -71 bp with respect to the transcriptional start site and left only 96 bp of sequence between positions -71 and -167 bp. Surprisingly, this fragment was still able to direct reporter gene expression at a similar level to the undeleted promoter fragment. Because the 96 bp deleted promoter fragment is directly adjacent to the actin splice acceptor signal and 5' UTR in the reporter construct, initiation of transcription at a position that approximates to the start site identified in metacyclic-derived trypanosomes, i.e. ~70 bp downstream, would mean that most of the splice acceptor signal and 5' UTR would be omitted from the nascent transcript. It has previously been reported that the presence a splice acceptor signal is necessary for efficient reporter gene expression in transient transfection of trypanosomes (Huang and Van der Ploeg, 1991). Therefore, it is very unlikely that the minimal promoter sequence of the 1.22 M-VSG gene promoter identified in bloodstream trypanosomes, initiates transcription at the start site identified in metacyclic-derived trypanosomes.

The 96 bp deletion sequence of the 1.22 M-VSG gene promoter, which is transcriptionally active in bloodstream trypanosome transient transfections, shows some limited homology to a bloodstream VSG gene expression site promoter (the bloodstream expression site promoters identified to date are highly homologous). In particular, there is homology to a sequence of 8 bp located at -60 to -67 bp in the bloodstream expression site promoter (Fig. 3.3.3.1). In the upstream region of the 1.22 M-VSG gene promoter, the 8 bp sequence is directly adjacent to the *Bam*HI site and would be removed by the smallest 5' to 3' deletion of 17 bp that reduced promoter activity to background levels. In the bloodstream expression site promoter the 8 bp sequence was essential for activity of the promoter in transient transfection of both bloodstream and procyclic trypanosomes. Deletion of this region reduces promoter activity to about 3% of the wild-type promoter (Vanhamme *et al.*, 1995b; Pham *et al.*, 1996) whilst single nucleotide or dinucleotide mutations within the -60 to -67 bp box can reduce reporter gene expression to less than 10% of the wild-type promoter (Vanhamme *et al.*, 1995b). Regulatory elements at equivalent positions with respect to the transcriptional start site, also exist in the PARP gene promoter and ribosomal RNA gene promoter, suggesting conservation of a regulatory element at this position in trypanosome promoters that are insensitive to α-amanitin (Sherman *et al.*, 1991; Brown *et al.*, 1992; Janz *et al.*, 1994). Moreover, DNA binding experiments have shown that this regulatory element of the bloodstream expression site, PARP gene and ribosomal RNA gene promoters can bind specifically to proteins in nuclear extracts of bloodstream and procyclic trypanosomes (Vanhamme *et al.*, 1995b) although these proteins are single-stranded DNA binding proteins and as yet no single-stranded binding proteins have been shown to be transcription factors. In addition to the 8 bp sequence homology, there is also similarity between the upstream region of the 1.22 M-VSG gene promoter and the sequence at the transcriptional start site of the bloodstream expression site promoter (Fig. 3.3.3.1). Again, this region of the bloodstream expression site has been shown to be functional in promoter activity by mutational analysis (Vanhamme *et al.*, 1995b). However, at another element of the bloodstream expression site promoter that was shown to be functional, there is no sequence similarity with the upstream region of the 1.22 M-VSG gene promoter. The deletion analysis of the 1.22 M-VSG gene promoter, and the comparison of the sequence with other trypanosome promoters, suggests that transcription may be initiated in bloodstream trypanosome transient transfection by a cryptic promoter resembling the bloodstream expression site promoter.

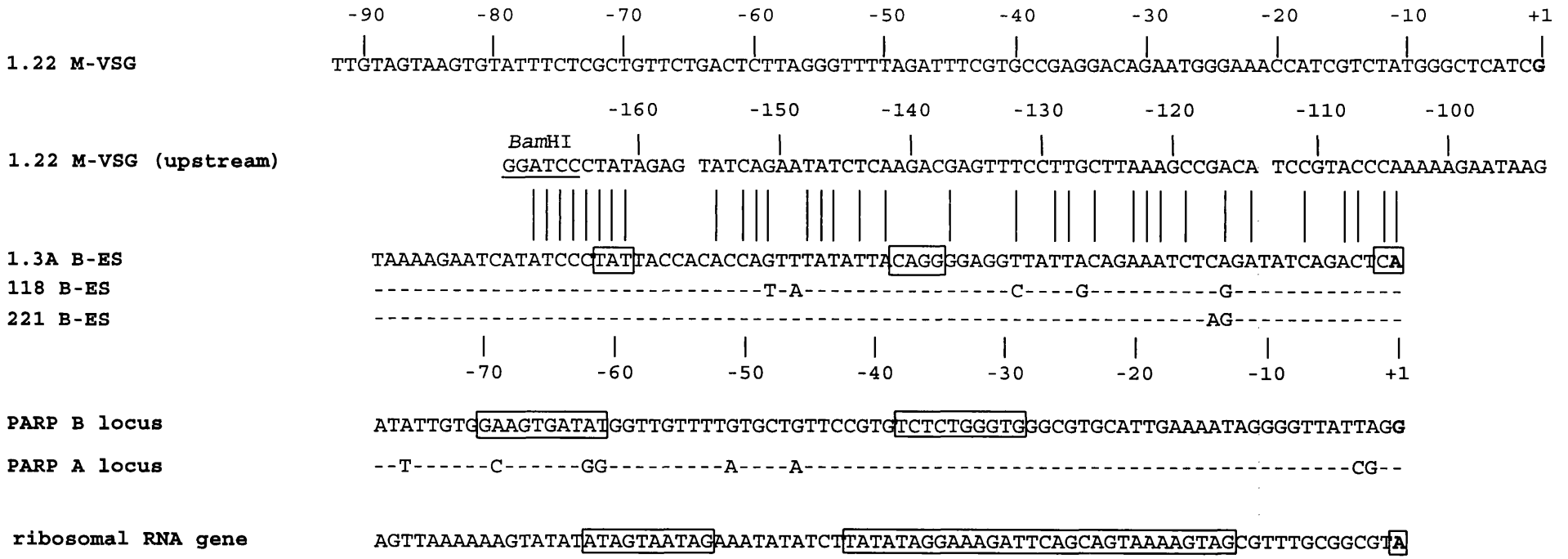


Figure 3.3.3.1. Sequence homology of the upstream region of the 1.22 M-VSG gene promoter with the bloodstream VSG gene expression site promoters. The promoter sequence of the 1.22 M-VSG gene putative promoter is shown from the *Bam*HI restriction site to the putative start site determined in metacyclic-derived trypanosomes. Numbering is in relation to the putative start site. Regions of homology to the 1.3A bloodstream expression site promoter are shown as vertical lines. The sequence of 3 bloodstream expression site promoters is shown with identical sequence to the 1.3A promoter representented as dashes. Numbering is from the transcriptional start site. Also shown for comparison are the PARP B locus and PARP A locus promoters and the ribosomal RNA gene promoter. The transcriptional start sites of each of the promoters is shown in bold and sequences that were shown experimentally to be necessary for transcription initiation are boxed. Analysis of the 1.3A promoter (Vanhamme *et al.*, 1995a), the PARP B locus promoter (Brown *et al.*, 1992) and the ribosomal promoter (Janz and Clayton, 1994).

This cryptic promoter may be inactive in bloodstream trypanosomes *in vivo* due its chromosomal context at the 1.22 M-VSG gene expression telomere. The activity of the 1.22 M-VSG gene promoter region in bloodstream trypanosomes at the 1.22 M-VSG gene expression telomere can be tested by inserting reporter constructs to the telomere in bloodstream form trypanosomes. The axenic culture of bloodstream trypanosomes required for these experiments is described in Chapter 4.

Chapter 4

Characterisation of Bloodstream Trypanosomes in Axenic Culture

4.1 INTRODUCTION

Nuclear run-on analysis showed that the 1.22 M-VSG gene promoter was inactive in bloodstream trypanosomes *in vivo* (Graham and Barry, 1995), whilst transient transfection analysis of bloodstream trypanosomes suggested the same promoter was highly active when assayed on an episomal element (3.2.4). It was proposed that the chromosomal context of the 1.22 M-VSG gene promoter at the 1.22 M-VSG gene expression telomere may affect the transcriptional activity of the promoter *in vivo* (3.3.1). In order to test whether the chromosomal context does in fact affect transcription initiation from the 1.22 M-VSG gene promoter, I decided to integrate copies of the promoter to different chromosomal positions in bloodstream form trypanosomes and assess their transcriptional activity. An expressible copy or copies of prokaryotic antibiotic resistance genes have been successfully integrated into procyclic trypanosomes by homologous recombination at various positions in the genome (see section 5.2.1), conveying antibiotic resistance to a cell upon expression (Lee and Van der Ploeg, 1990a; ten Asbroek *et al.*, 1990; 1993 ; Eid and Sollner-Webb, 1991; Zomerdijk *et al.*, 1991a). Trypanosomes that have been stably transformed by integration of the antibiotic resistance gene into the genome, may then be selected by addition of antibiotic to the procyclic trypanosome medium. Bloodstream trypanosome stable transformants have also been obtained by integrating an antibiotic resistance gene into the genome of procyclic trypanosomes and selection for antibiotic resistance bloodstream trypanosomes in mice following the aberrant differentiation of procyclic trypanosomes directly to the bloodstream form (Murphy *et al.*, 1993). However, the uptake or removal of antibiotic from the murine bloodstream *in vivo* means that the level of antibiotic in the bloodstream, required for selection of transformed trypanosomes, cannot be accurately controlled. Another approach to produce transformed bloodstream trypanosomes involves the stable transformation of procyclic trypanosomes, which are selected with antibiotic in culture, and then transmitted through tsetse flies to develop a metacyclic infection in the salivary glands. The salivary gland metacyclic trypanosomes can then be injected into mice where they differentiate into transformed bloodstream trypanosomes (Jefferies *et al.*, 1993). Unfortunately, this method requires the transmission of trypanosomes through tsetse flies which can be technically difficult and time-consuming. In order to routinely transform and select bloodstream trypanosomes directly, it would be most advantageous to establish bloodstream form trypanosomes in axenic culture, i.e. in the absence of feeder cell layers.

The maintenance of African trypanosome bloodstream forms in axenic culture has only recently become possible with the development of new media and culture techniques. Initially bloodstream trypanosomes could only be maintained in culture with a feeder cell layer of mammalian macrophages or embryonic fibroblasts (Hirumi *et al.*, 1977; Brun *et al.*, 1981) but later experiments allowed axenic culture of bloodstream trypanosomes following a period of initiation and adaptation with a feeder cell layer (Baltz *et al.*, 1985; Duszenko *et al.*, 1985). Thereafter it was possible to adapt several species of bloodstream trypanosomes to axenic culture conditions using a variety of mediums and maintain them indefinitely in the absence of a feeder cell layer (Hirumi and Hirumi, 1994). These media and techniques were used to maintain some highly laboratory-adapted lines of bloodstream *Trypanosoma brucei* in axenic culture which could be grown as clonal colonies on agarose plates containing the bloodstream trypanosome medium (Carruthers and Cross, 1992). These trypanosomes were then successfully stably transformed by integration of a selectable marker gene to the tubulin locus and clones isolated by the selection of transformed trypanosomes on agarose plates containing antibiotic (Carruthers *et al.*, 1993). Subsequent experiments have integrated selectable markers to the bloodstream expression sites in highly laboratory-adapted lines of bloodstream trypanosomes and transformed cells selected on agarose plates containing antibiotic (Horn and Cross, 1995; Rudenko *et al.*, 1995).

To perform stable transformation experiments that examine transcription initiation from the 1.22 M-VSG gene promoter in bloodstream form *Trypanosoma brucei* of our non-laboratory-adapted stock EATRO 795, bloodstream trypanosomes of this stock have successfully been adapted to axenic culture conditions. This allows electroporation and subsequent selection of stably transformed bloodstream trypanosomes in the absence of feeder cell layers that would either be killed during selection with antibiotic or transformed themselves confusing the experimental results. In this chapter I describe the adaptation to axenic culture of bloodstream form stock EATRO 795, a non-laboratory adapted strain which maintains fly transmissibility. To try to establish whether the phenotype of the strain has been altered during adaptation, the axenic culture has been characterised by light microscopy, electron microscopy, immunofluorescence of the surface coat, differentiation to procyclic trypanosomes *in vitro* and transmission through tsetse flies.

4.2 RESULTS

4.2.1 Adaptation to axenic culture of bloodstream form *Trypanosoma brucei* stock EATRO 795

Bloodstream trypanosomes of stock EATRO 795 expressing the ILTat 1.2 VSG were recovered from a rat by cardiac puncture and adapted to culture as described in Materials and Methods (see section 2.11). Briefly, the whole blood was centrifuged at a low speed and a small number of trypanosomes recovered from the buffy coat layer and placed in HMI-9 medium (Hirumi and Hirumi, 1994). The cells were counted and the concentration adjusted to 2 x 10^5 cells/ml in 5 ml of HMI-9 medium in a 25 ml flask that was then incubated at 37°C in the absence of CO_2. Each day 2.5 ml of the culture was removed and replaced with fresh medium irrespective of the concentration of trypanosomes. Daily passaging provided fresh medium and serum and ensured the concentration of the bloodstream trypanosomes was kept low (initially these trypanosomes divide rapidly, doubling every 6-10 hours). After 8 days the culture was below 2.5 x 10^3 cells/ml at which point the trypanosomes were no longer passaged and left at 37°C. 10 days after passaging was stopped, a trypanosome population grew up to approximately 1 x 10^6 cells/ml. These trypanosomes were then passaged every 2 days by removing all the medium from a flask and replacing with fresh medium. The doubling time of the culture was 8-10 hours when in the exponential phase of growth which is equivalent to the doubling time of bloodstream trypanosomes *in vivo* (Turner and Barry, 1989). The maximum cell density was generally 1-2x10^6 when passaged every 2 days but higher concentrations of trypanosomes could be obtained by more rapid passaging. This maximum cell density is very low in relation to *in vivo* bloodstream infections that can reach cell densities above 1x10^9 cells/ml of blood and approximately 10 fold lower than procyclic trypanosomes *in vitro*. Cell density of bloodstream trypanosomes in culture may be limited by growth factors and nutrients in the medium that are either used quickly or are degraded, or by toxic products of cell growth that are removed from the bloodstream *in vivo*. Once the maximum cell density of the bloodstream trypanosome culture was reached the trypanosomes would die very rapidly, with typically >90% dead within 24 hours.

To begin assessing the phenotype of the culture-adapted bloodstream trypanosomes, 4x10^6 bloodstream trypanosomes that had been continuously passaged in culture for 5 months, were injected into 2 mice that had been immunosuppressed by treatment with cyclophosphamide (at 250 mg/kg body weight) 24 hours previously

(Turner and Barry, 1989). Both mice developed high parasitaemias (> $1x10^8$ cells/ml of blood) within 3 days of infection with a similar proliferation profile to bloodstream trypanosomes of stock EATRO 795 model monomorphic line that have not been adapted to culture. The bloodstream trypanosomes were harvested from the mouse and re-introduced to HMI-9 medium. These trypanosomes proliferated in HMI-9 medium without the lag phase associated with the initial adaptation to culture and therefore adaptation to axenic culture may be a stable characteristic of these bloodstream form *Trypanosoma brucei* that does not affect growth of the trypanosomes *in vivo*. Also, because trypanosomes lacking a surface coat are neutralised by complement mediated lysis in the mammalian bloodstream, this experiment showed that the bloodstream trypanosomes in axenic culture must retain expression of the VSG surface coat.

Since the cloning of individual trypanosomes would be an essential step in establishing stably transformed bloodstream trypanosome lines, and cloning of bloodstream trypanosomes *in vitro* has only been described for the laboratory-adapted strain 427 of *Trypanosoma brucei* (Carruthers and Cross, 1992; Carruthers *et al.*, 1993; Horn and Cross, 1995; Rudenko *et al*, 1995; Li and Gottesdiener, 1996), I attempted to clone the axenic culture of stock EATRO 795. Clones of the bloodstream trypanosome culture can be grown from single cells without the use of conditioned medium as is necessary for procyclic trypanosomes (Carruthers and Cross, 1992). I tried cloning the culture-adapted bloodstream trypanosomes by plating trypanosomes on agarose plates containing HMI-9 medium and by serial dilution in HMI-9 medium in 96 well plates (see section 2.16). Cloning these trypanosomes by spreading on agarose plates was successful on several occasions (see 5.2.5) and small, opaque colonies of parasites could be seen on the plates with the unaided eye after about 5 days. Under the inverted microscope the trypanosomes were seen as tight colonies that remained highly motile on the semi-solid plates and few or no trypanosomes were observed migrating away from the colonies. However, cloning efficiency was very low and less than 1% of trypanosomes developed into trypanosome colonies. This is probably because the growth of bloodstream trypanosomes as colonies on agarose plates was very sensitive to the level of humidity in the incubator and moisture on the surface of the agarose plates, and optimal conditions were not achieved. Syringe-passaged bloodstream form *Trypanosoma brucei* of strain 427, that had been adapted to axenic culture, could be cloned on agarose plates with approximately 70% cloning efficiency (Carruthers and

Cross, 1992). The cloning of the axenic culture by serial dilution was much more efficient with greater than 50% cloning efficiency on most occasions.

4.2.2 The gross morphology of bloodstream form trypanosomes in axenic culture

In the early stages of a mammalian infection, a pleomorphic strain of *Trypanosoma brucei* is predominantly long slender in morphology but later in the infection the rapidly dividing long slender trypanosomes can differentiate to non-dividing short stumpy forms. Short stumpy forms are thought to be partially adapted to the tsetse fly midgut environment (Vickerman, 1985) and readily differentiate to procyclic trypanosomes *in vitro* (Rolin *et al.*, 1990; Ziegelbauer *et al.*, 1990; Pays *et al.*, 1993; Matthews and Gull, 1994) and probably *in vivo* (Ashcroft, 1960; Wijers and Willett, 1960). In a monomorphic strain of *Trypanosoma brucei*, short stumpy forms do not normally develop and consequently monomorphic trypanosomes do not easily differentiate to procyclic trypanosomes *in vitro* (Czichos *et al.*, 1986; Overath *et al.*, 1986) or *in vivo* (Overath *et al.*, 1986). The bloodstream trypanosome infection that initiated the bloodstream trypanosome culture was a 'model' line of EATRO 795 expressing ILTat 1.2 as its surface antigen type. These trypanosomes are monomorphic in that they remain long slender during a mouse infection but unusually can still differentiate to procyclic trypanosomes *in vitro* with the addition of citrate and *cis*-aconitate, and can be transmitted through tsetse flies, i.e. they develop a mature metacyclic trypanosome infection in the salivary glands of tsetse flies (Turner and Barry, 1989; Graham *et al.*, 1990). To study the morphology of the EATRO 795 bloodstream trypanosomes in the axenic culture, smears of trypanosomes were Giemsa stained and examined under the light microscope. All the bloodstream trypanosomes in axenic culture appeared to be long slender in morphology (>1000 trypanosomes examined) and evidence of their undulating membrane attached to the flagellum can be seen (Figure 4.2.2.1). The Giemsa stain highlights the nucleus and the kinetoplast of the trypanosomes, the kinetoplast is situated close to the posterior end whilst the nucleus in situated centrally between anterior and posterior ends (Figure 4.2.2.1). This means that the distance between the kinetoplast and the nucleus is quite large. For short stumpy forms and procyclic forms the distance between the kinetoplast and nucleus is relatively small (Vickerman, 1985). I did not observe short stumpy forms in the bloodstream trypanosome culture by phase-contrast light microscopy of the live trypanosomes or light microscopy of stained slides at stationary or exponential phases of culture growth

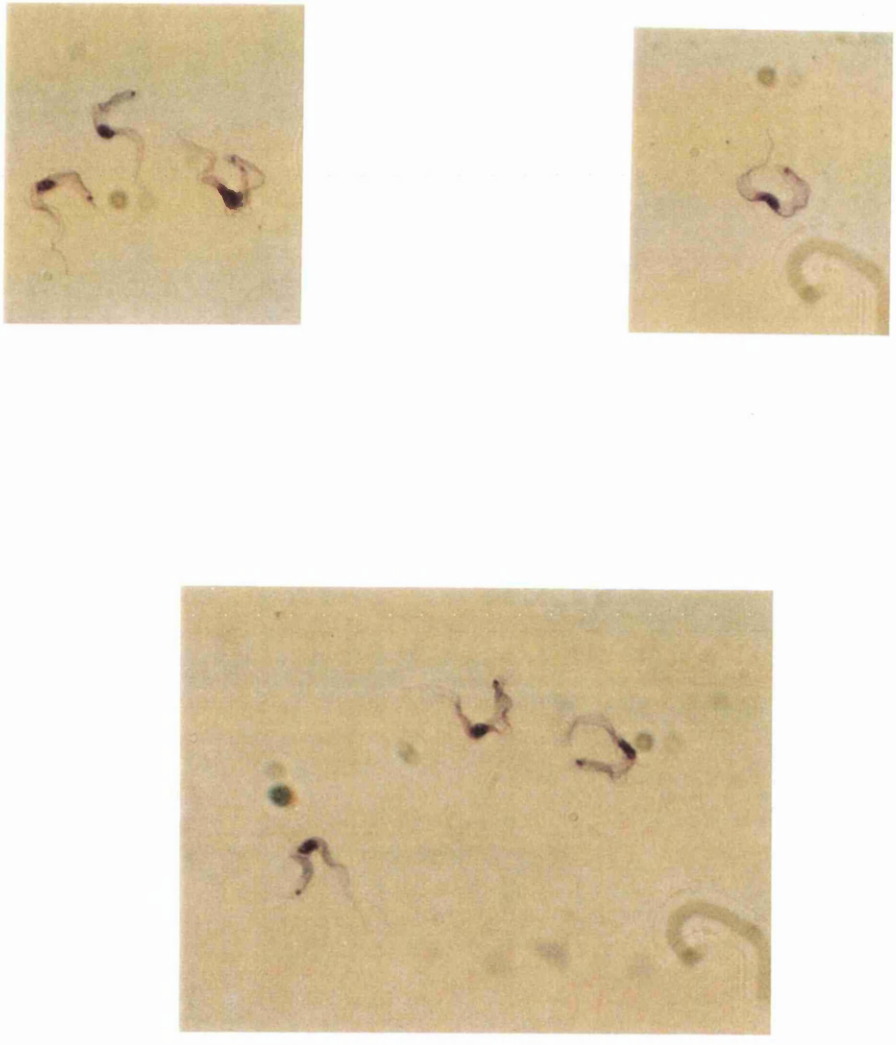


Figure 4.2.2.1. **Bloodstream form *Trypansoma brucei* in axenic culture stained with Giemsa.** Bloodstream trypanosomes that had been maintained in axenic culture (HMI-9 medium) were smeared onto glass slides, fixed and Giemsa stained. The figure shows the small, densely stained kinetoplast near the posterior end of each trypanosome and the larger and centrally positioned nucleus. All bloodstream trypanosomes in axenic culture observed following Giemsa staining and live cultured bloodstream trypanosomes observed under an inverted microscope were long, slender in morphology. Magnification x100.

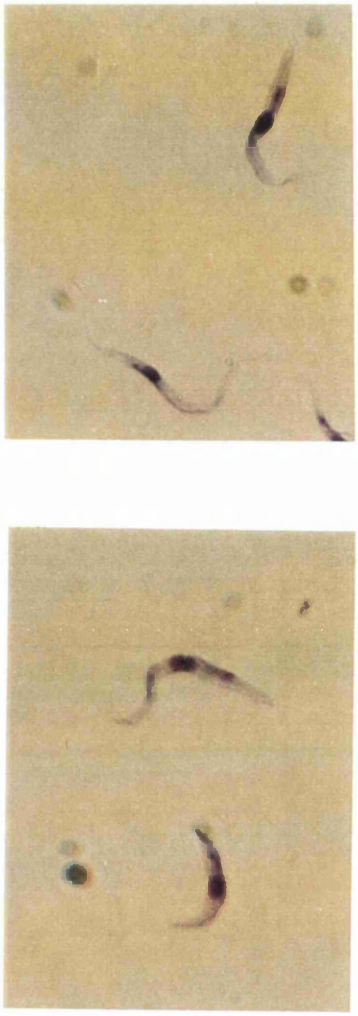


Figure 4.2.2.2. **Procyclic form *Trypanosoma brucei* stained with Giemsa**. Established procyclic trypanosomes maintained in SDM-79 medium were smeared onto glass slides, fixed and Giemsa stained. The figure shows that the procyclic trypanosomes are slightly larger and more elongated than the bloodstream trypanosomes in figure 4.2.2.1. The small, densely staining kinetplast is also positioned closer to the nucleus in procyclic trypanosomes. Magnification x100.

(>1000 trypanosomes examined). Therefore the gross morphology of the cultured bloodstream trypanosomes is very similar to that of long slender bloodstream trypanosomes *in vivo* (Vickerman, 1985).

At the same time, procyclic *Trypanosoma brucei* which had previously been differentiated from the model bloodstream trypanosome line (expressing ILTat 1.2) and maintained in SDM-79 medium, were stained with Giemsa stain and examined under the light microscope. These procyclic trypanosomes are clearly distinct from the bloodstream trypanosomes (Figure 4.2.2.2). The cells are larger and without the obvious undulating membrane, the kinetoplast is situated further from the posterior end of the trypanosomes and is relatively close to the nucleus (Vickerman, 1985)

4.2.3 Ultrastructural features of bloodstream form trypanosomes in axenic culture

Under a light microscope the cultured trypanosomes in HMI-9 medium were identified as bloodstream form on the basis of their shape, small size with respect to procyclic trypanosomes, the posterior position of the kinetoplast, the distal position of the kinetoplast in relation to the nucleus. In addition, the motility of live trypanosomes in medium was similar to that of trypanosomes from mice infections when placed in medium. The use of transmission electron microscopy to study the organisation and structure of organelles in the cultured bloodstream trypanosomes can confirm their similarity to bloodstream trypanosomes *in vivo*. Some features of trypanosome ultrastructure are characteristic of particular life cycle stages, and correspond to aspects of trypanosome biochemistry that are stage-specific (see Vickerman, 1985). At the procyclic stage the trypanosome derives energy from the oxidation of amino acids, mostly proline, in the mitochondrion and therefore the mitochondrion is well developed (occupying as much as 25% of the cell volume), branched and contains discoid cristae. At the bloodstream stage glucose is oxidised instead of amino acids in specialised organelles called glycosomes containing all the enzymes necessary for glycolysis. The active glycosomes are spherical and very prominent in bloodstream trypanosomes but are less obvious in procyclic trypanosomes where they are inactive and become bacilliform in shape. Pyruvate resulting from glycolysis in the glycosomes does not enter the mitochondrion where further oxidation could occur, but is removed from the cell into the bloodstream. Consequently, the mitochondrion is inactive in bloodstream trypanosomes and occupies only about 5% of the cell volume with few branches or cristae. The main characteristic of bloodstream form trypanosomes is the dense surface coat that is

composed of VSG molecules and covers the entire cell including the flagellum. This is readily visible under the electron microscope as a layer 12-15 nm thick overlying the cell membrane.

Bloodstream trypanosomes from axenic culture were prepared for transmission electron microscopy by fixation in osmium tetroxide and glutaraldehyde and then stained with uranyl acetate before embedding in resin and sectioning (see section 2.12). Electron micrographs were prepared with assistance from Dr. L. Tetley. Figure 4.2.3.1 shows a longitudinal section through the flagellar pocket (FP) and the axoneme (Ax) of the flagellum. On the surface of the flagellum and the internal surface of the flagellar pocket can be seen the densely staining surface coat (SC) which is between 12 and 15 nm in thickness and lies above the surface membrane, a 7 nm thick lipid bilayer. Figure 4.2.3.2 is a longitudinal section of a bloodstream trypanosome at much lower magnification showing the organisation of organelles in the cell. At the centre is the nucleus (N) showing some regions of more densely staining heterochromatin associated with the nuclear envelope, as well as the densely staining nucleolus (Nuc). Along the upper edge of the cell is the mitochondrion (Mit) contained in a double lipid bilayer, though the distinctive kinetoplast of trypanosome mitochondria cannot be seen in this micrograph. The mitochondrion is not branched, represents only a small proportion of the overall cell volume and contains no obvious cristae. Several prominent, spherical glycosomes (G) can be seen and the flagellum (F) is visible in cross section. Figure 4.2.3.3 is a cross section of the flagellum towards the anterior end of the trypanosome and the adjacent region of the cell body at high magnification. Again the micrograph shows the trypanosome surface coat (SC) that covers the whole cell including the flagellum. The axoneme (Ax) of the flagellum can be clearly seen and is composed of 9 outer microtubule doublets centred around 2 inner microtubules. Adjacent to the axoneme and along the length of the flagellum, is the paraxial rod (PR). The main body of the trypanosome is 'caged' within a dense array of longitudinal pellicular microtubules (PM) (Vickerman, 1969). The flagellum is attached to the main body of the trypanosome by means of the macula adherens (MA) that may be considered functionally equivalent to desmosomes in higher organisms but are structurally quite different (Vickerman, 1969). A glycosome (G) is marked at the bottom of the figure. The prominent, spherical glycosomes, degenerate mitochondrion and dense surface coat are ultrastructural features of bloodstream form EATRO 795 trypanosomes in axenic culture that indicate these cells are very similar to bloodstream trypanosomes *in vivo*. In addition, the repressed mitochondrion and prominent glycosomes

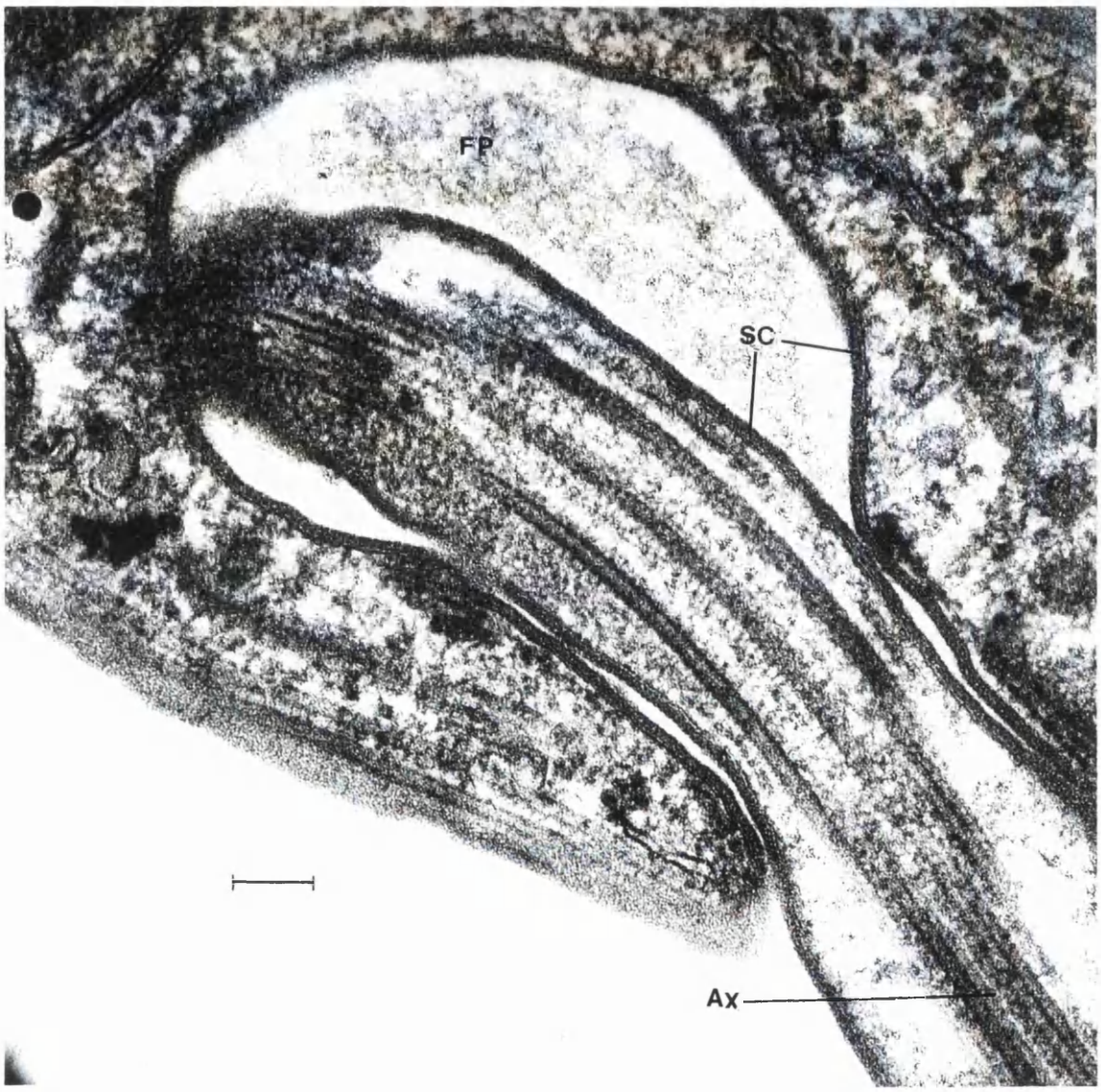


Figure 4.2.3.1. **Electron micrograph showing a longitudinal section through the flagellar pocket of *T. brucei* cultured bloodstream form**. The section shows the flagellum containing the axoneme (Ax) microtubule structure which runs along the length of the flagellum. The flagellum is closely associated with the cell body where the flagellum emerges from the flagellar pocket (FP). The dense staining surface coat (SC) can be clearly seen on the flagellum and the internal surface membrane of the flagellar pocket.

x 100,000. Scale bar = 0.1 μm.

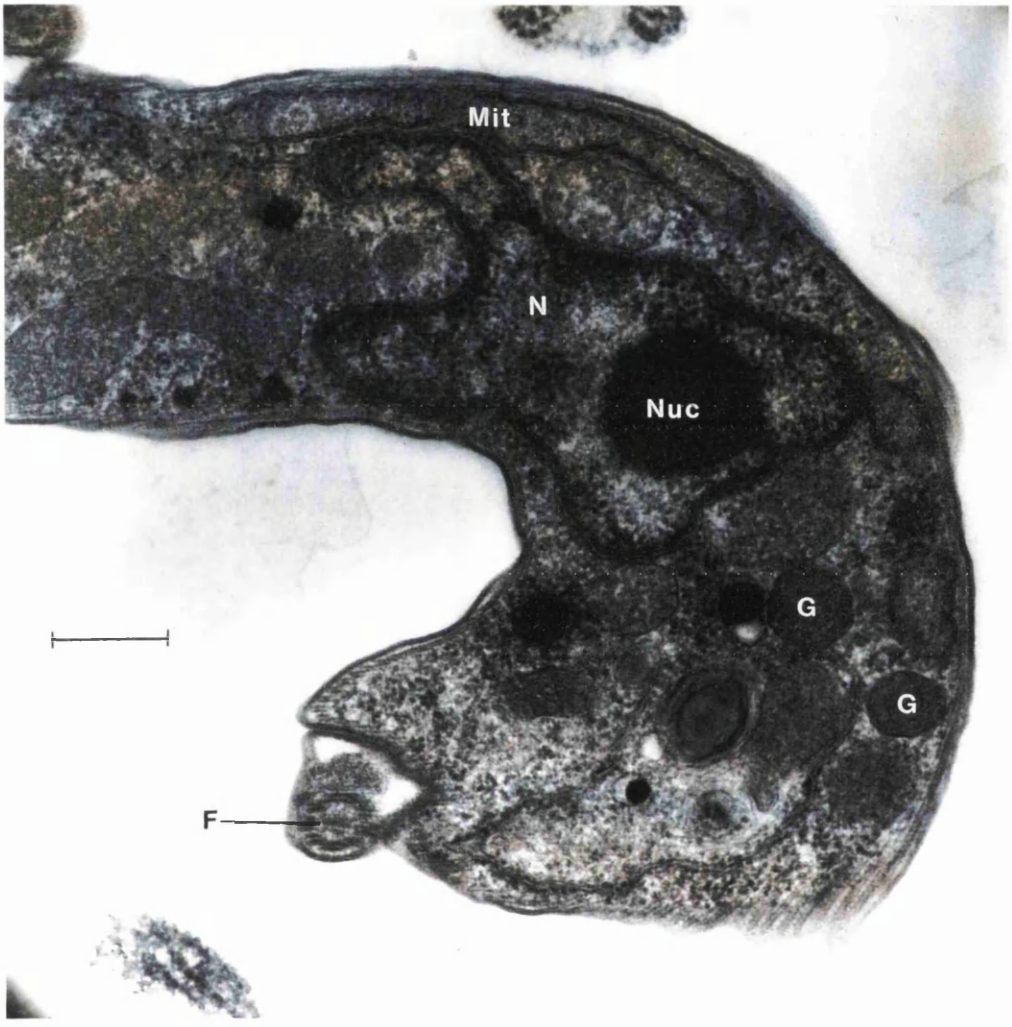


Figure 4.2.3.2. **Electron micrograph showing longintudinal section of *T. brucei* cultured bloodstream form**. The section shows the dense packing of organelles in the bloodstream trypanosome. At the top is the mitochondrion (Mit) which is contained in a double lipid bilayer. The mitochondrion contains no observable cristae and (from this and other micrographs) represents only a small proportion of the cell volume relative to the mitochondrion in procyclic trypanosomes. The nucleus (N) contains densely staining heterochromatin that is largely associated with the nuclear envelope and the densely staining nucleolus (Nuc). Several spherical glycosomes (G) can be seen and the flagellum (F) is shown in cross section.

x 40,000. Scale bar = 5 μm.

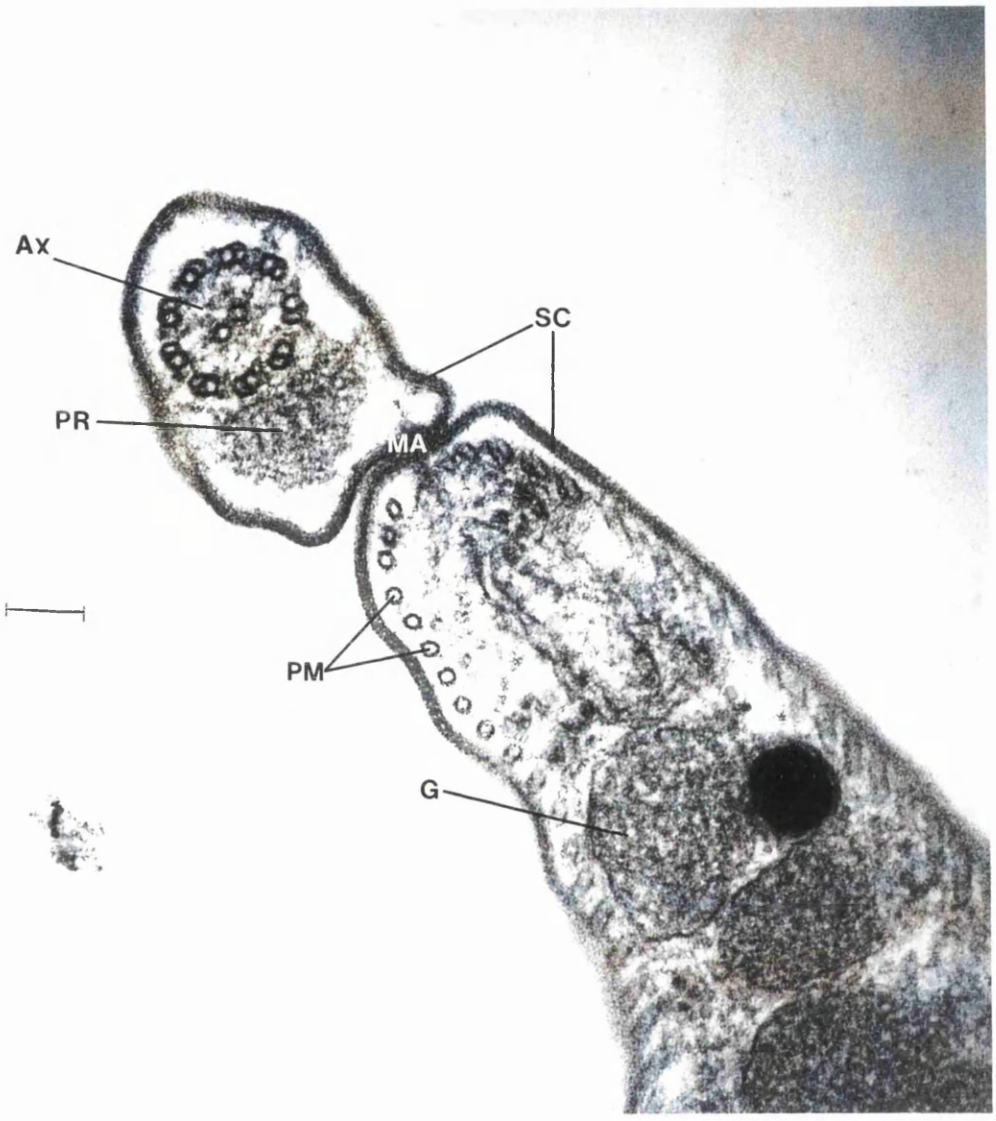


Figure 4.2.3.3. **Electron micrograph showing cross section of flagellum and associated region of the cell body of cultured bloodstream *T. brucei*.** The axoneme (Ax) of the flagellum shows a structure of 2 inner microtubules surrounded by a ring of 9 microtubule doublets. Adjacent to the axoneme and running along the length of the flagellum is the paraxial rod (PR). The flagellum is attached to the cell body by a macula adherens junction (MA) where there is a break in the pellicular microtubules (PM) of the cell body. The surface of the trypanosome flagellum and cell body is covered by the surface coat (SC). Also shown is a glycosome (G).

x 100,000. Scale bar = 0.1 μm.

suggest bloodstream forms in axenic culture oxidise glucose rather than amino acids and are therefore, in at least this respect, biochemically equivalent to bloodstream forms *in vivo*.

4.2.4 Immunodetection of the variant antigen type expressed *in vitro*

VSG molecules of one variant antigen type go to make up the dense surface coat of bloodstream *Trypanosoma brucei* apart from exceptional circumstances where two variant antigen types can be detected on the surface of a single trypanosome (Esser and Schoenbeckler, 1984). Such double expressors are probably an intermediate stage where a trypanosome is switching from one antigen type to another. Each variant antigen type corresponds to the expression of a single VSG gene and within a bloodstream trypanosome population *in vivo*, expression of one VSG gene predominates. Bloodstream trypanosomes of line 427 that were adapted to growth in laboratory animals and then maintained *in vitro* in HMI-9 medium were also predominantly of one antigen type (Horn and Cross, 1995; Rudenko *et al.*, 1995). Switching did occur in these bloodstream trypanosomes, though because switching events in these experiments were selected in mice pre-immunised against the predominant VSG, it is not clear if switching occurred *in vitro* or *in vivo*.

The antigen type expressed by the EATRO 795 bloodstream trypanosomes in culture was analysed by indirect immunofluorescence in collaboration with Kathleen Milligan (Department of Biochemical Parasitology, Glasgow University) using 6 monoclonal antibodies and 21 polyclonal rabbit antisera that each react against a distinct antigen type of the EATRO 795 serodeme. Of the 27 monoclonal antibodies and antisera screened, none reacted against the surface coat of the bloodstream trypanosome culture (see Table 4.2.4.1 for a list of antibodies used and the VSGs that they react against). The bloodstream trypanosomes that initiated the axenic culture were expressing the ILTat (ILRAD Trypanozoon antigen type) 1.2 VSG on their surface. However, ILTat 1.2 was not detected by indirect immunofluorescence and therefore this VSG was not expressed by the bloodstream trypanosomes in culture, though the cultured bloodstream trypanosomes still express a surface coat as visualised in transmission electron microscopy (4.2.3) and as demonstrated by their infectivity in mice (4.2.1). These trypanosomes have presumably switched the VSG expressed from ILTat 1.2 to another VSG whilst in axenic culture, thus indicating that bloodstream trypanosomes in culture may undergo antigenic variation in the absence of cues from the animal host.

VAT	IsoVAT	Antibody	Dilution
GUTat 7.1	ETat 1.2, ILTat 1.22	GUPM 27.1	neat supernatant
7.2		GUPM 18.7	neat supernatant
7.3		Rabbit antisera	400
7.4		"	200
7.5		"	300
7.6		"	400
7.8		"	150
7.9		"	200
7.10		"	100
7.11		"	100
7.12		"	200
7.13		GUPM 17.1	ascites 500
ETat 1.1		Rabbit antisera	300
1.3		"	100
1.4		"	200
1.5		"	100
1.6		"	100
1.7		"	200
1.9	ILTat 1.21	"	100
1.10	GUTat 7.17	"	100
1.11	GUTat 7.7	"	100
1.12	ILTat 1.2	"	100
ILTat 1.3	ETat 1.8, GUTat 7.14	"	200
1.4		"	200
1.61	GUTat 7.15	GUPM 23.1	300
M-VAT		GUPM 10.1	500
M-VAT		GUPM 11.7	500

Table 4.2.4.1. **The antibodies and antisera used to detect the variant antigen type or types expressed by EATRO 795 bloodstream trypanosomes in culture.** The table shows the variable antigen type (VAT) and IsoVATs detected by each monoclonal antibody (GUPM for Glasgow University Protozoology Monoclonal antibody) or VAT specific antisera (rabbit antisera). Dilution shows the dilution of each monoclonal or antisera. GUTat, Glasgow University Trypanozoon antigen type; ETat, Edinburgh Trypanozoon antigen type; ILTat, ILRAD Trypanozoon antigen type; M-VAT, metacyclic VAT.

Antigenic variation of bloodstream trypanosomes in culture has been demonstrated previously (Baltz *et al.*, 1985) but again it is not clear whether switching occurred in the axenic culture or during the initiation or adaptation phases of the culture both of which required macrophage feeder cell layers. Furthermore, bloodstream trypanosomes expressing the ILTat 1.2 VSG readily switch *in vivo* to VSGs detected by the antibodies and antisera used in this screening (J. B. Barry, personal communication). As none of the antibodies or antisera reacted with the cultured bloodstream trypanosomes, these trypanosomes may have switched several times *in vitro* or switched to a VSG not usually expressed *in vivo*.

4.2.5 Differentiation of cultured bloodstream trypanosomes to procyclic trypanosomes *in vitro*

The 'model' trypanosome line of EATRO 795 expressing ILTat 1.2 that was used to initiate the bloodstream trypanosome culture, is capable of differentiation to procyclic trypanosomes *in vivo* and *in vitro*, although this does not occur readily *in vitro* and requires the citric acid cycle intermediates citrate and *cis*-aconitate (Turner and Barry, 1989; Graham and Barry, 1995). To determine if these bloodstream trypanosomes maintain this feature after adaptation to axenic culture, attempts were made to differentiate the cultured bloodstream trypanosomes to procyclic trypanosomes *in vitro*. In this laboratory procyclic trypanosomes are routinely cultured at 27°C in SDM-79 medium supplemented with 10 % foetal calf serum and haemin at 7.5 μg/ml (Brun and Schonenberger, 1979). Several attempts were made to differentiate cultured bloodstream trypanosomes to procyclic trypanosomes by transferring bloodstream forms to this procyclic culture medium but all attempts were unsuccessful even in the presence of conditioned procyclic SDM-79 medium or the citric acid cycle intermediates, citrate and *cis*-aconitate, which are known to stimulate differentiation to procyclic trypanosomes *in vitro* (Brun and Schonenberger, 1981; Hunt *et al.*, 1994). However, using differentiation trypanosome medium (DTM), which is essentially bloodstream trypanosome medium but without glucose (Overath *et al.*, 1986), bloodstream trypanosomes in axenic culture were successfully differentiated to procyclic trypanosomes. $1x10^7$ bloodstream trypanosomes were centrifuged and resuspended in 5 ml of DTM medium supplemented with 3 mM citrate and 3 mM *cis*-aconitate and incubated at 27°C. The bloodstream trypanosomes differentiated rapidly and under a light microscope using phase contrast >80% of the trypanosomes were morphologically

procyclic forms within 24 hours. After 48 hours no trypanosomes resembling bloodstream forms were present. Monomorphic and pleomorphic bloodstream trypanosomes from mouse blood differentiate to procyclic forms at a very similar rate *in vitro* (Overath *et al.*, 1986; Ziegelbauer et al., 1990; Bass and Wang, 1991). To encourage division of the cells, the culture was passaged 1 in 5 every 2 days with fresh DTM medium but without citric acid cycle intermediates. Once the procyclic culture was established, passaging 1 in 2 every day with fresh DTM medium was found to be optimal for cell division. The trypanosomes divided slowly, doubling every 20-30 hours (rather than 10-12 hours in SDM-79) with maximum density of approximately $5x10^6$ ($2x10^7$ in SDM-79). The successful differentiation of bloodstream trypanosomes to procyclic trypanosomes in DTM medium and not in SDM-79 medium is presumably due to DTM medium being optimised for growth of bloodstream trypanosomes whereas SDM-79 medium is optimised for the growth of procyclic trypanosomes. These trypanosomes could not be transferred to SDM-79 medium even in the presence of 20% serum which implies these trypanosomes are distinct from procyclic EATRO 795 trypanosomes cultured routinely in this laboratory.

Indirect immunofluorescence of these 'procyclic-like' trypanosomes differentiated from cultured bloodstream trypanosomes was carried out using a monoclonal antibody (#418) that reacts against the PARP molecule which is expressed abundantly on the surface of procyclic trypanosomes (Roditi *et al.*, 1989). At the same time, and with the same concentration of primary and secondary antibodies, cultured bloodstream trypanosomes from which the procyclic-like trypanosomes were derived were analysed for the expression of PARP on the trypanosome surface. Figure 4.2.5.1A shows the aberrant procyclic forms after staining with DAPI illustrating that the trypanosomes are large with nuclei and kinetoplasts that are indistinct in some cells. After indirect immunofluorescence with the PARP antibody (Figure 4.2.5.1 B) each of the trypanosomes is clearly labelled with the FITC conjugated secondary antibody indicating that these aberrant procyclic trypanosomes do express PARP on their surface. Figure 4.2.5.2A shows cultured bloodstream trypanosomes stained with DAPI which confirms the posterior position of the kinetoplast and central position of the nucleus seen when the trypanosomes were stained with Giemsa stain. When these trypanosomes were reacted with the PARP monoclonal antibody, little reactivity was detected (Figure 4.2.5.2B) indicating the cultured bloodstream trypanosomes do not express PARP on their surface. Therefore, the aberrant procyclic forms derived from cultured bloodstream

A.

B.

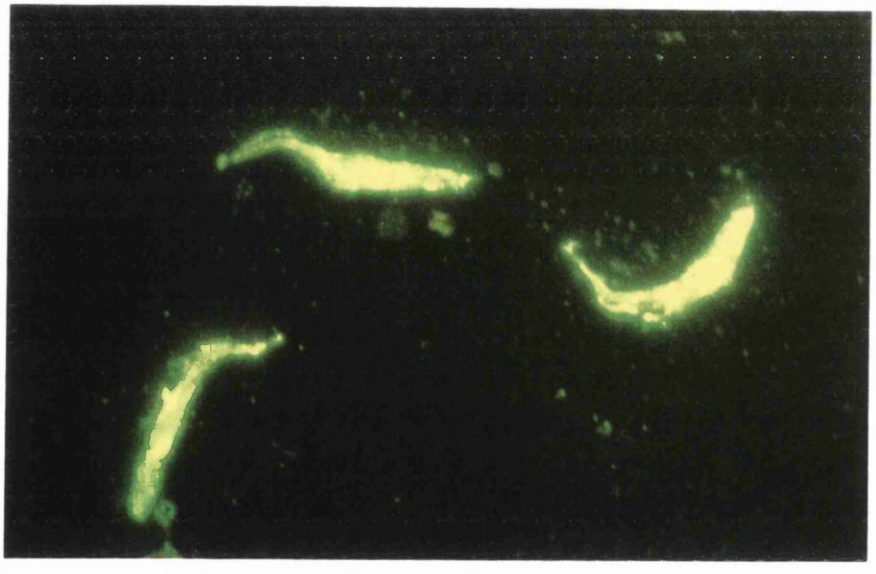
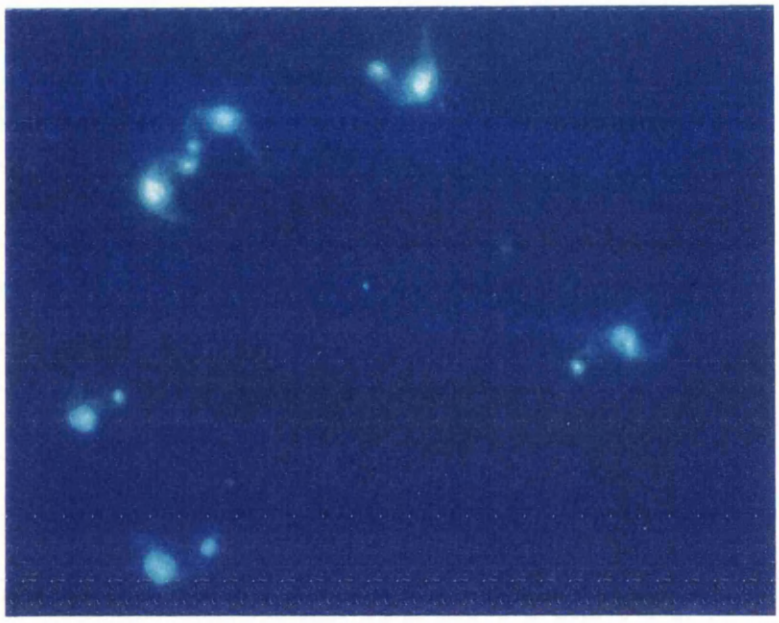


Figure 4.2.5.1. **Detection of PARP on the surface of aberrant procyclic trypanosomes**. Cultured bloodstream form *Trypanosoma brucei* were differentiated to procyclic forms *in vitro* in DTM medium. The resulting culture of 'procyclic-like' trypanosomes were larger and less motile than procyclic trypanosomes in SDM-79 medium, doubled at a slower rate and would not divide in SDM-79 medium. These aberrant procyclic trypanosomes were tested for the expression of the PARP surface molecule by immunofluorescence with a monoclonal antibody that reacts against PARP. (A) DAPI-stained aberrant procyclic trypanosomes. (B) The same trypanosomes stained with the monoclonal antibody against PARP and a FITC-conjugated secondary antibody. Magnification x200.

A.



B.

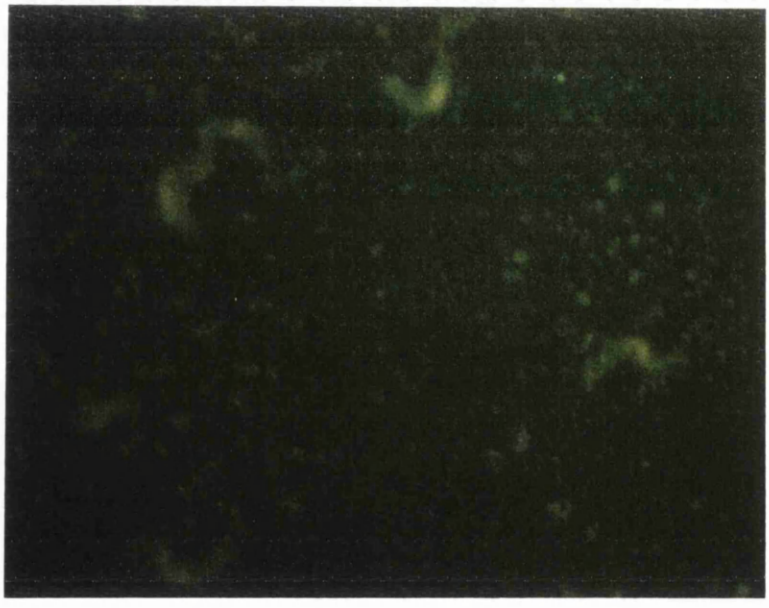


Figure 4.2.5.2. **Immunofluorescence of cultured bloodstream form *Trypanosoma brucei* using a monoclonal antibody that reacts against PARP**. (A) Bloodstream form *Trypanosoma brucei* in axenic culture stained with DAPI. (B) The same trypanosomes stained with a monoclonal antibody that reacts against PARP and a FITC-conjugated secondary antibody. Magnification x200.

trypanosomes do express a major molecular marker of the procyclic stage, PARP, even though they are abnormal in terms of morphology, growth rate and medium requirements.

4.2.6 Transmission of cultured bloodstream trypanosomes through tsetse flies

The *in vitro* differentiation of cultured EATRO 795 bloodstream trypanosomes to procyclic-like trypanosomes suggested axenicly cultured bloodstream trypanosomes may be able to differentiate to procyclic forms *in vivo* and thereby initiate a mature infection in the midgut of tsetse flies. The procyclic trypanosomes in the midgut could then go on to develop a mature metacyclic infection in the tsetse fly salivary glands and complete the life cycle of bloodstream trypanosomes established in culture. This would be a significant technological step forward since most lines of cultured bloodstream trypanosomes established to date, are monomorphic strains and cannot be transmitted through tsetse flies. Cultured bloodstream trypanosomes in HMI-9 medium were mixed with sheep's blood or horse's blood and fed to teneral tsetse flies as described in Materials and Methods (see section 2.14). Approximately 50 flies were fed on cultured bloodstream trypanosomes on at least 5 separate occasions and the trypanosomes subsequently maintained on uninfected blood meals. From day 21 after the infected fly feed, the saliva of the flies was probed onto a microscope slide and analysed for the presence of metacyclic trypanosomes under a light microscope (a metacyclic infection can develop 21-35 days after a fly feed depending on the trypanosome strain). Metacyclic trypanosomes were not detected in the tsetse fly saliva of any flies that survived beyond day 21. However, of 250 flies fed on the cultured bloodstream trypanosomes, less than 5% survived to day 21. This is a very high fatality rate but was the same as other experiments, conducted at the same time, infecting flies with bloodstream or procyclic trypanosomes. Therefore, the high fatality rate was probably not a consequence of toxic factors in the HMI-9 medium but the result of poor health of the flies. From these experiments it is not possible to determine whether the cultured bloodstream trypanosomes are likely to transmit through tsetse flies and develop a metacyclic infection in the salivary glands as the sample size for day 21 flies was too low. Further experiments could be performed perhaps using a tsetse fly line that is selected for competence in transmission of trypanosomes (Maudlin and Dukes, 1985).

4.3 DISCUSSION

Bloodstream trypanosomes of stock EATRO 795 expressing ILTat 1.2 were adapted to axenic culture in HMI-9 medium. Adaptation of bloodstream trypanosomes was characterised by a lag phase in cell growth of approximately 5-6 days during which time most trypanosomes died. This was followed by an exponential growth phase where trypanosomes doubled at approximately the same rate as bloodstream trypanosomes *in vivo*. The maximum cell density of trypanosomes in axenic culture was much lower than trypanosomes *in vivo* but long slender bloodstream trypanosome morphology was maintained at exponential and lag phases of population growth. The parasites displayed several key ultrastructural features that are characteristic of long slender bloodstream forms; they expressed a dense surface coat, many prominent spherical glycosomes were observed, and the mitochondrion was in a repressed state. The bloodstream trypanosomes also switched the VSG expressed on their surface in axenic culture and remained infective to mice for prolonged periods in culture. However, in contrast to bloodstream trypanosomes *in vivo*, those maintained in axenic culture did not differentiate fully to procyclic trypanosomes *in vitro* (though they could be induced to differentiate to procyclic-like trypanosomes under particular conditions).

4.3.1 The adaptation phase of bloodstream trypanosomes to axenic culture

Bloodstream trypanosomes do not readily proliferate in HMI-9 medium but instead go through a lag phase of growth before establishment in culture. As there are no discernible differences between trypanosomes before and after the adaptation phase of growth, it is not clear what occurs during this phase. During the lag phase of growth, trypanosomes may make subtle adjustments to their requirements for nutrients and growth factors, the availability of which is likely to be different in the HMI-9 medium from the situation in the bloodstream. Because, during the adaptation phase of growth the bloodstream trypanosome population is reduced to a very low cell density, only a few trypanosomes make the transition to bloodstream trypanosomes that are adapted to growth in axenic culture. It is possible that the cultured bloodstream population is derived from a few cells or just a single cell. Once bloodstream trypanosomes have adapted to growth *in vitro*, they can also grow readily *in vivo* without any lag phase and therefore, probably without adaptation or differentiation. When re-introduced to HMI-9 medium these bloodstream trypanosomes grow *in vitro* without a lag phase indicating the adaptation to culture is a stable trait. The nature of the adaptation phase of

bloodstream trypanosome growth in culture may imply adaptation involves a specific mutation of one or a few genes rather than minor adjustments to gene regulation and function. However, Hesse *et al.* (1995) have recently shown that bloodstream *T. brucei* (at least strain 427) may be grown in cell-free medium without a lag phase of growth characteristic of adaptation to culture, implying that gene mutation is not necessary for bloodstream trypanosomes to grow in axenic culture unless, of course, these trypanosomes have already undergone a change in phenotype or genotype that allows them to grow readily in culture.

4.3.2 The low maximum cell density may be due to a soluble inhibitory factor

The maximum cell density achieved with these bloodstream trypanosomes in axenic culture was approximately $3x10^6$ cells/ml. This is consistent with other studies of bloodstream trypanosomes *in vitro* (Baltz *et al.*, 1985; Carruthers and Cross, 1992; Hirumi and Hirumi, 1994) but is several orders of magnitude lower than bloodstream trypanosomes *in vivo* that can grow to densities higher than $1x10^9$. Hesse *et al.* (1995) studied bloodstream trypanosomes in axenic culture but adopted a slightly different culturing technique to the one used here. Instead of diluting trypanosomes every 2 days in fresh medium, they centrifuged trypanosomes gently at different time intervals (6-48 hours) and resuspended the bloodstream trypanosomes in an equal volume of fresh medium such that the cell density remained the same but nutrients and growth factors were replenished. Several interesting features of this bloodstream trypanosome population were observed. By completely replacing the trypanosome medium every 24 hours, cell densities of $2x10^7$ cells/ml could be obtained at a doubling rate of about 6 hours but each peak of cell density was followed by the appearance of short stumpy forms and a precipitous fall in the cell density followed by lag phase and then another peak of cell density. The oscillation in trypanosome density resembles that of a trypanosome population in a chronic infection of a host suggesting these waves of parasitaemia may not be due to host-parasite interactions, such as clearance of an antigenic type by an immune response, but rather are the result of the trypanosomes controlling their own population numbers. By adding either 10% serum or 10% medium to trypanosomes that had been centrifuged and resuspended in 90% of their conditioned medium at intervals of 2 hours or 6 hours, the trypanosome maximum cell density was halved with respect to the control where all the medium was replaced with fresh medium. The authors proposed therefore, that the maximum cell density was not limited by

nutrient or growth factor depletion, but by a soluble factor secreted by the trypanosomes (similar to the cytokines in mammalian systems) that may also control differentiation of long slender forms to stumpy forms. The soluble factor should have a short half life such that as the population increases, so does the concentration of the secreted factor which at high concentrations restricts cell division and induces differentiation. However, the trypanosome density may be limited inadvertently by a toxic product of trypanosome proliferation that restricts division both in culture and in the bloodstream. In the bloodstream, much higher cell densities may be reached possibly due to clearance of this soluble factor from the bloodstream. The cell density of cultured EATRO 795 bloodstream trypanosomes may also be limited by secretion of a trypanosome factor, but in this case, long slender forms do not differentiate to short stumpy forms at higher cell densities suggesting that restricted cell density and the control of differentiation to short stumpy forms are not associated phenomena.

4.3.3 Cultured bloodstream trypanosomes do not readily differentiate to procyclic trypanosomes *in vitro*

The EATRO 795 bloodstream forms in axenic culture do not easily differentiate to procyclic trypanosomes *in vitro*. This appears to be a feature of *Trypanosoma brucei* bloodstream forms maintained in axenic culture. *Trypanosoma brucei* strain 427 bloodstream forms differentiate to procyclic forms *in vitro* when harvested directly after propagation in mice or after growth of bloodstream trypanosomes in culture supported with a feeder cell layer (Roditi *et al.*, 1989; Ziegelbauer *et al.*, 1990; Czichos *et al.*, 1986). However, when this same monomorphic strain of bloodstream trypanosomes was maintained in axenic culture (HMI-10 medium) they could not differentiate to dividing procyclic forms *in vitro* (Mutomba and Wang, 1995). The cultured bloodstream trypanosomes could begin the differentiation process when shifted from 37°C to 26°C by shedding the VSG coat but the onset of PARP synthesis was much delayed. The authors suggested that this was due to selection of a variant bloodstream form trypanosome that was adapted to growth in HMI-10 medium but deficient in the ability to differentiate to procyclic forms. Experiments with the same bloodstream trypanosome line required DTM medium for successful differentiation of cultured bloodstream trypanosomes to procyclic trypanosomes *in vitro* (Horn and Cross, 1995). DTM medium was required for the differentiation of cultured EATRO 795 bloodstream trypanosomes to procyclic trypanosomes (4.2.5) but probably resulted in procyclic-like trypanosomes that are

dissimilar to procyclic trypanosomes cultured in SDM-79 medium. DTM medium is derived from TM medium, a medium optimised for growth of bloodstream trypanosomes, and is therefore likely to favour the growth of bloodstream trypanosomes or bloodstream-like trypanosomes rather than procyclic trypanosomes. The inability of cultured bloodstream form EATRO 795 to differentiate to procyclic forms *in vitro* may well correlate to the situation *in vivo* in the fly midgut. Cultured bloodstream form EATRO 795 ingested by the fly and taken into the midgut may be unable to differentiate to procyclic trypanosomes and therefore would not establish a mature procyclic infection in the midgut or metacyclic infection in the salivary glands of the tsetse fly.

Chapter 5

Down-Regulation of the 1.22 M-VSG Gene Promoter in the Genome of Bloodstream Trypanosomes

5.1 INTRODUCTION

The 1.22 M-VSG gene is a single copy gene in *Trypanosoma brucei* stock EATRO 795 and is located at a position close to a chromosome end as identified by its sensitivity to the exonuclease *Bal*31 (Cornelissen *et al.*, 1985). Recent data has shown that the 5' end of the 1.22 M-VSG gene is ~5 kb from the chromosome end (S.V. Graham, personal communication). This implies that the 1.22 M-VSG gene is situated close to the telomere repeats that are characteristic of chromosome ends in *Trypanosoma brucei* (Blackburn and Challoner, 1984; Van der Ploeg *et al.*, 1984a; see section 1.4), as well as higher eukaryotes (Blackburn, 1991; Shippen, 1993; Palladino and Gasser, 1994). All other metacyclic VSG genes, that have been studied, are also located close to the telomeres of chromosomes that migrate with the largest set of trypanosome chromosomes when analysed by pulsed field gel electrophoresis (Cornelissen *et al.*, 1985; Lenardo *et al.*, 1986). In addition, transcription of metacyclic VSG genes initiates from a promoter positioned only a short distance upstream (within 3 kb) and the transcription unit contains no other genes (Alarcon *et al.*, 1994; Graham and Barry, 1995). Though very common in higher eukaryotes, such monocistronic transcription units are highly unusual in trypanosomes as all other known trypanosome genes are transcribed as polycistronic transcription units (Imboden *et al.*, 1987; Kooter *et al.*, 1987; Mowatt and Clayton, 1987; Amar *et al.*, 1988; Gibson *et al.*, 1988; Lee and Van der Ploeg, 1990b). The telomeric positioning and monocistronic structure of metacyclic VSG gene expression loci may be features essential for the tight control of metacyclic VSG gene expression in the trypanosome life cycle.

Previously, the 1.22 M-VSG gene promoter was shown to be transcriptionally active only at the metacyclic stage; it is inactive at the bloodstream and procyclic stages when assayed *in vivo* by nuclear run-on analysis (Graham and Barry, 1995). However, results of bloodstream trypanosome transient transfection experiments (see section 3.2.5) suggested that the promoter region could be active in bloodstream trypanosomes when assayed outwith its metacyclic VSG gene expression telomere, i.e. activity of the 1.22 M-VSG gene promoter region was very different when assayed on an episomal vector, than when assayed in the genome. To analyse the transcriptional activity of the 1.22 M-VSG gene promoter region in the genome in more detail, I stably transformed bloodstream forms of *Trypanosoma brucei* with reporter plasmids designed to integrate into the genome by homologous recombination. Each plasmid contained the 1.22 M-VSG gene promoter region directing expression of a CAT reporter gene and,

downstream, a selectable marker gene (*ble*) under control of the constitutively active PARP promoter to allow selection of transformants (Fig. 5.2.2.1). First I wished to determine whether inserting the reporter plasmid construct back into the metacyclic expression telomere would alter the activity of the M-VSG gene promoter directing CAT expression from this vector and secondly, if it was the telomeric location that was important or if another chromosomal context would exert an effect on the M-VSG gene promoter. Therefore, similar constructs were targeted to the 1.22 M-VSG gene expression telomere (Fig. 5.2.2.1) and to a chromosomal-internal position, the non-transcribed spacer region of ribosomal DNA (Fig. 5.2.4.1). Analysis of CAT expression in these stably transformed lines showed that the 1.22 M-VSG gene promoter region was active at the chromosomal-internal position but only minimally active at the 1.22 M-VSG gene expression telomere.

5.2 RESULTS

5.2.1 Stable transformation of trypanosomes

Procyclic *Trypanosoma brucei* have previously been stably transformed with a range of prokaryotic antibiotic resistance genes that allow selection of trypanosome transformants by addition of the appropriate antibiotic to the procyclic trypanosome medium (ten Asbroek *et al.*, 1990; Eid and Sollner-Webb, 1991; Zomerdijk *et al.*, 1991a). Antibiotic resistance genes may be introduced into trypanosomes by electroporation with plasmids that contain the antibiotic resistance gene, which may be maintained as episomes in the cell or integrated into the trypanosome genome (Patnaik *et al.*, 1993; ten Asbroek *et al.*, 1993; Lee, 1995). In order for plasmids containing the antibiotic resistance gene to be maintained episomally in the trypanosome, the plasmid must contain an active trypanosome promoter directing expression of the antibiotic resistance gene and the appropriate RNA processing signals flanking the gene (Patnaik *et al.*, 1993; ten Asbroek *et al.*, 1993). Also, a trypanosome replication origin must be included in the plasmid to support replication of the plasmid in the trypanosome and possibly mitotic segregation of the plasmid copies (Patnaik *et al.*, 1994). In the absence of selection, episomal plasmid copies are lost as the trypanosome population divides, presumably because of insufficient episomal replication or unequal segregation of the episomes during mitosis (Patnaik *et al.*, 1993; Lee, 1995). However, when a plasmid or other exogenous DNA containing an antibiotic resistance gene, is integrated into the trypanosome genome it is usually maintained indefinitely even in the absence of selection.

When integrated into the genome, transcription of the antibiotic gene may be initiated from a promoter sequence within the integrated exogenous DNA or from an upstream promoter sequence in the adjacent chromosomal DNA (ten Asbroek *et al.*, 1990; Eid and Sollner-Webb, 1991).

Introduction of exogenous DNA into chromosomal DNA can occur by two mechanisms, 1) illegitimate integration or 2) homologous recombination. Illegitimate integration events occur when random double-stranded DNA breaks in chromosomal DNA ligate to the double-stranded DNA ends of exogenous DNA. Homologous recombination involves alignment of homologous sequences in the chromosomal (target) DNA and exogenous DNA followed by cross-over events between the aligned sequences. Alignment may be the due to the invasion of a double helix by homologous single-stranded DNA (produced by exonuclease activity on double-stranded DNA ends) to form a transient triple helix which can resolve to generate the cross-over event (Sun *et al.*, 1991; Camerini-Otero and Hsieh, 1993). The result of homologous recombination is a targeted integration of the exogenous DNA to a specific chromosomal site. Illegitimate integration of exogenous DNA to non-specific chromosomal sites, occurs in a high proportion of transformed mammalian cells (>99% of integrants; Thomas and Cappecchi, 1987; Sedivy and Sharp, 1989; Jasin *et al.*, 1990), whilst in yeast this mechanism is rarely observed (Manivasakam *et al.*, 1995) and no illegitimate integration events have been reported in *Trypanosoma brucei*. Instead, integration of exogenous DNA into the genome of *Trypanosoma brucei* occurs exclusively by homologous recombination (Lee and Van der Ploeg, 1990a; Eid and Sollner-Webb, 1991; ten Asbroek *et al.*, 1993; Blundell *et al.*, 1996). In yeast a majority of integration events are also by homologous recombination (Manivaskam *et al.*, 1995) but in mammalian cells integration of exogenous DNA into genomic DNA by homologous recombination is rare (Sedivy and Sharp, 1989). In trypanosomes the efficiency of homologous recombination is greatly increased when the exogenous DNA is linear and the sequences homologous to the target DNA are situated at each end of the molecule (Eid and Sollner-Webb, 1991; ten Asbroek *et al.*, 1993), presumably because these ends will readily form the single-stranded DNA ends homologous to the target DNA and thereby facilitate triplex formation. Also, homologous recombination in trypanosomes seems to require a very high degree of homology between the exogenous DNA and target DNA sequences in the genome (Blundell *et al.*, 1996). Antibiotic resistance genes have been targeted efficiently to several loci in procyclic trypanosomes including the tubulin gene array (ten Asbroek *et*

al., 1990), the calmodulin gene locus (Eid and Sollner-Webb, 1991), the RNA polymerase II large subunit gene locus (Chung *et al.*, 1993), the PARP gene locus (Wirtz and Clayton, 1995), bloodstream VSG gene expression sites (Rudenko *et al.*, 1994; 1995; Horn and Cross, 1995) and the non-transcribed spacer region of the ribosomal gene array (Zomerdijk *et al.*, 1991a).

By transmitting procyclic trypanosome stable transformants through tsetse flies and injecting the contents of the infected salivary glands into mice, it is possible to obtain bloodstream trypanosome transformants (Jefferies *et al.*, 1993). However, this method of generating bloodstream trypanosome stable transformants is time-consuming and may be very difficult as the efficiency of fly transmission is often low. Recently, improved bloodstream trypanosome culture and transfection techniques have permitted the stable transformation of bloodstream *Trypanosoma brucei* directly, and allowed bloodstream trypanosomes to be transfected and selected in culture. In addition, culture-adapted bloodstream trypanosomes grow well as colonies on agarose plates, which can greatly facilitate their selection and cloning (Carruthers and Cross, 1992; Carruthers *et al.* 1993). In this chapter, I have used these transfection techniques to integrate reporter plasmids into the trypanosome genome by homologous recombination, and transform bloodstream trypanosomes that have been adapted to axenic culture (see section 4.2.1).

5.2.2 Stable integration of plasmid pt1.22BC into the 1.22 M-VSG gene expression telomere

Plasmid pt1.22BC was constructed previously in our laboratory (S.V. Graham, unpublished) for stable transformation of procyclic trypanosomes but it is also suitable for transformation of bloodstream trypanosomes. pt1.22BC was designed to integrate to the 1.22 M-VSG gene expression telomere by homologous recombination, whilst maintaining an unaltered copy of the putative 1.22 M-VSG gene promoter region upstream of the metacyclic VSG gene. Figure 5.2.2.1 shows pt1.22BC and the site to which the plasmid is targeted in the 1.22 M-VSG gene expression telomere. pt1.22BC contains two 'cassettes', a reporter gene cassette and a selectable marker gene cassette. In the reporter gene cassette, upstream of the CAT gene is the same 1.6 kb *Kpn*I-*Pst*I fragment of the 1.22 M-VSG gene promoter region that directed high levels of CAT gene expression in the transient transfection of bloodstream trypanosomes (see section

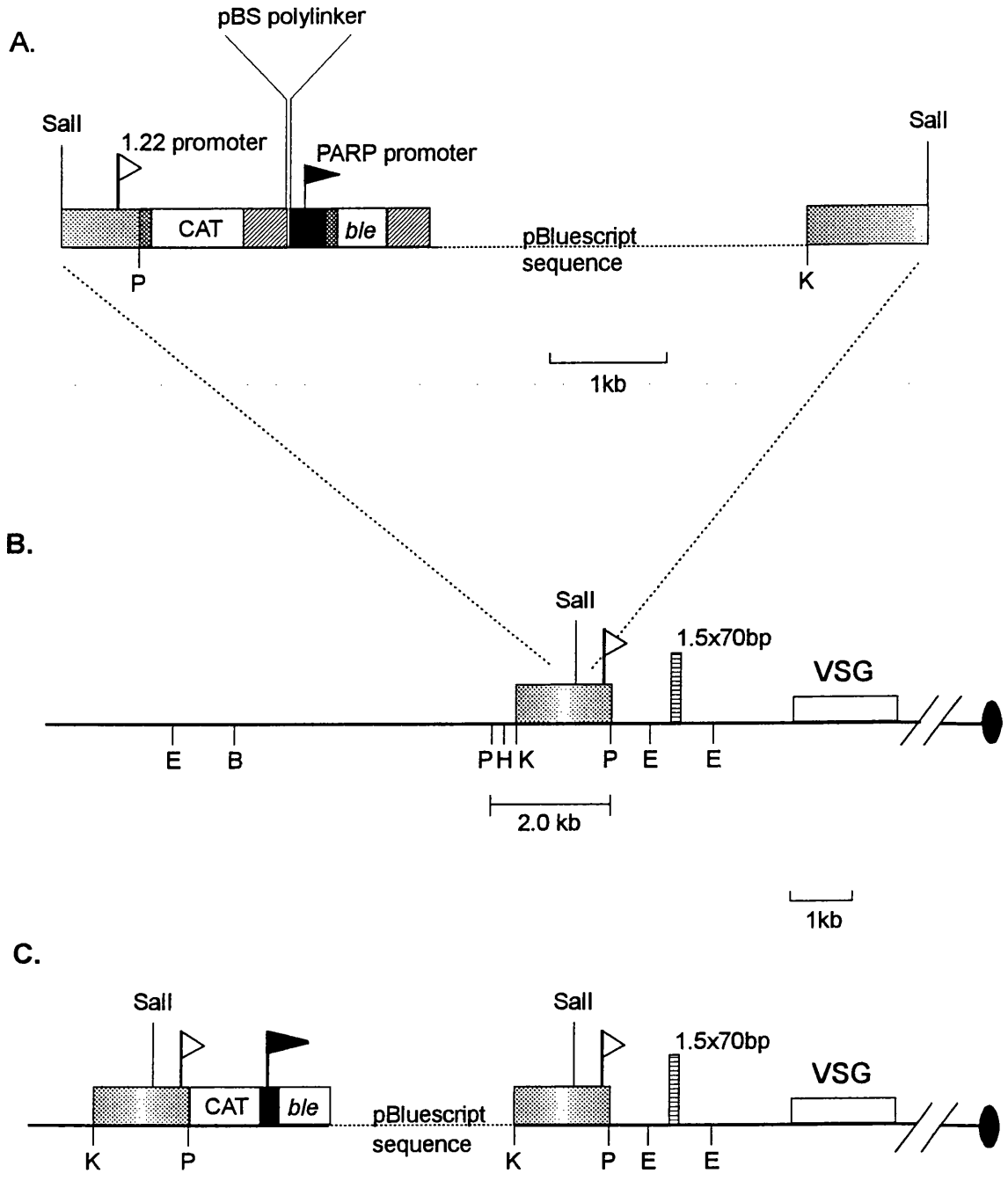


Figure 5.2.2.1. **Schematic representation of pt1.22BC and its integration to the 1.22 M-VSG gene expression telomere.** (A) Plasmid pt1.22BC contains a CAT reporter gene cassette and a *ble* gene cassette. The CAT gene is downstream of the 1.6 kb *Kpn*I-*Pst*I fragment of the putative 1.22 M-VSG gene promoter (white flag). The *ble* gene is downstream of the PARP promoter (black flag). Both the CAT gene and *ble* gene are flanked by the PARP 5' UTR and splice acceptor signal (dark grey box) and by the PARP 3' UTR (diagonal lined box). Between the CAT gene cassette and *ble* gene cassette is a region of pBluescript polylinker that is ~100 bp. Linearisation of pt1.22BC at a unique site in the 1.22 M-VSG gene promoter fragment allows insertion of the plasmid by homologous recombination to the single copy 1.22 M-VSG gene promoter region at the 1.22 M-VSG gene expression telomere. (B) The 1.22 M-VSG gene expression telomere showing site of plasmid insertion and the chromosome end (black oval). (C) The 1.22 M-VSG gene expression telomere following insertion of a single copy of pt1.22BC. pBS, pBluescript; 70bp, 70 bp repeats; VSG, 1.22 M-VSG gene.

3.2.5). PARP RNA processing signals flank the CAT gene and were derived from pJP44 (Sherman *et al.*, 1991). The PARP 3' UTR alone is not sufficient to direct accurate polyadenylation of CAT transcripts since there are sequences in the PARP intergenic region which are essential for processing of RNA from upstream and downstream genes (Schurch *et al.*, 1994; Hug *et al.*, 1994). A reliance on sequences in intergenic regions for accurate processing of RNAs from flanking genes is not unique to PARP gene expression but is probably a common feature of gene expression in Kinetoplastida (Lebowitz *et al.*, 1993; Matthews *et al.*, 1994) (see section 1.8). However, CAT gene transcripts from constructs containing only a PARP 5' splice acceptor region and a 3' UTR (and no intergenic region) are still polyadenylated albeit at a site ~100 bp 5' of the wild type site of poly (A) addition (Graham *et al.*, 1996). The PARP gene 5' splice acceptor region and 3' UTR probably contain cryptic signal sequences that can direct *trans*-splicing and polyadenylation respectively, of CAT gene transcripts to yield functional, mature CAT mRNAs (Hug *et al.*, 1993).

pt1.22BC was used for stable transformation of bloodstream trypanosomes although it had been designed for use in procyclic trypanosome stable transformation (S. V. Graham, unpublished results) and included PARP RNA processing signals which are known to be used inefficiently in bloodstream trypanosomes (Hug *et al.*, 1993). Use of a new construct where the PARP RNA processing signals were replaced with actin signals (known to be equally efficiently used in bloodstream and procyclic trypanosomes) would have been preferable, however, reconstruction of the complex plasmid pt1.22BC may have taken some time and therefore I decided to pursue the investigation with the existing construct.

The selectable marker gene cassette is located downstream of the reporter gene cassette in pt1.22BC. It contains the *ble* gene encoding a prokaryotic polypeptide that binds the antibiotic phleomycin and prevents it from being activated by ferrous ions and oxygen and breaking-down DNA (Drocourt *et al.*, 1990). The *ble* gene is also flanked by the same PARP RNA processing signals as the CAT gene in the reporter gene cassette, and is under control of the PARP B locus promoter which is constitutively active during the trypanosome life cycle (Sherman *et al.*, 1991). Although this promoter is active throughout the trypanosome life cycle, it is down-regulated 5-10 fold at the bloodstream stage (Pays *et al.*, 1990; Vanhamme *et al.*, 1995a; Biebinger *et al.*, 1996). This means that expression of *ble* from pt1.22BC, when stably integrated into the genome of bloodstream trypanosomes, is likely to be low. In stable transformation

experiments of procyclic trypanosomes with pt1.22BC 10 μg/ml phleomycin was used to select for transformed procyclic trypanosomes expressing the *ble* gene product although some cloned lines were resistant to up to 100 μg/ml phleomycin (S. V. Graham, unpublished results). The lower efficiency of the PARP gene promoter and the effects of the PARP gene RNA processing signals, that control expression of the *ble* gene in pt1.22BC, were expected to reduce expression of the *ble* gene in bloodstream trypanosomes in relation to procyclic trypanosomes (see section 1.8). Therefore, a lower final concentration of 5μg/ml phleomycin was used initially for selection of bloodstream trypanosomes transformed with pt1.22BC.

EATRO 795 bloodstream trypanosomes that had been adapted to axenic culture (see section 4.2.1), were electroporated as described in Materials and Methods (see section 2.15). Digestion of pt1.22BC with *Sal*I cuts the plasmid at a unique site in the 1.22 M-VSG gene promoter region which linearises the plasmid and facilitates integration to the 1.22 M-VSG gene expression telomere by homologous recombination (Fig. 5.2.2.1). In the first transformation experiment, 1 x 10^7 bloodstream trypanosomes were electroporated with 30 μg of linear, *Sal*I-digested pt1.22BC. After recovery for 24 hours in 10 mls of HMI-9 medium, transformed trypanosomes were selected by addition of phleomycin to a final concentration of 5μg/ml. At the same time wild-type bloodstream trypanosomes at 1x10^5 cells/ml were treated with final concentrations of 0.1, 0.5 and 1μg/ml phleomycin: within 48 hours no motile cells were observed at any of these phleomycin concentrations. Initially, isolation of transformed trypanosome clones by plating on agarose proved difficult and the uncloned transformed population was passaged continually under selection for 12 weeks prior to successful isolation of clones. Clones were finally isolated by plating on agarose plates containing either no antibiotic or phleomycin at 1 μg/ml, which allows trypanosome clones to grow in the absence of selection or at a low level of selection (see section 2.16). Colonies of transformed trypanosomes were visible on the plates without antibiotic after 5 days, whilst on plates containing antibiotic colonies were visible after 7 days. All colonies obtained in the presence or absence of selection, and placed in liquid medium, maintained resistance to phleomycin at 5μg/ml, however, the efficiency of plating was very low and less than 1% of the trypanosomes plated developed into colonies. Three clones, tA, tB and tC, obtained on plates containing phleomycin at 1μg/ml, were picked and placed in liquid medium under selection to grow sufficient cells for isolation of genomic DNA. Total genomic DNA was digested with *Pvu*II, separated on an agarose gel, Southern blotted

and hybridised with a ^{32}P-labelled probe homologous to the 1.6 kb *Kpn*I-*Pst*I fragment of the 1.22 M-VSG gene promoter (Fig. 3.2.1.1). The restriction enzyme *Pvu*II was selected to digest total genomic DNA to screen for correct plasmid insertions as this enzyme does not cut within the 1.22 M-VSG gene promoter sequence from which the probe was derived. Thus in wild-type trypanosomes the probe should hybridise to a single *Pvu*II fragment of 10.4 kb (previous restriction map analysis of the 1.22 M-VSG gene expression telomere; Paul Shiels, thesis, 1990; Fig. 5.2.3.1B). In trypanosomes transformed with a single copy of ptl.22BC at the 1.22 M-VSG gene expression telomere, the probe should hybridise to two *Pvu*II fragments of 9.6 kb and 2.5 kb since *Pvu*II cuts within ptl.22BC (Fig. 5.2.3.1B). The 9.6 kb *Pvu*II fragment contains most of the sequence of the 10.4 kb fragment in wild-type cells including the upstream integrated copy of the 1.22 M-VSG gene promoter but is limited at the 3' end by the *Pvu*II site in the integrated CAT gene (Fig 5.2.3.1B). The 2.5 kb *Pvu*II fragment extends from a *Pvu*II site in the pBluescript sequence of ptl.22BC at the 5' end, to a *Pvu*II site just upstream of the VSG gene at the 3' end, and includes the downstream endogenous copy of the 1.22 M-VSG gene promoter (Fig. 5.2.3.1B). Figure 5.2.2.2 shows that the probe hybridises to a 2.0 kb fragment in track 1 that contains plasmid DNA of ptl.22BC. This fragment includes the 1.22 M-VSG gene promoter fragment and the 5' end of the CAT gene. Track 2 contains genomic DNA from wild-type trypanosomes digested with *Pvu*II and as expected the probe hybridises to a 10.4 kb *Pvu*II fragment in this DNA (Fig. 5.2.2.2B, top line). For clones tB, tC and the uncloned population (tracks 3, 4 and 5 respectively), *Pvu*II fragments of 9.6 kb and 2.5 kb hybridised as expected. However, an additional *Pvu*II fragment of 2 kb was detected in the DNA of tB, tC and the uncloned population. This fragment was identical in size to the band which hybridised in track 1 i.e. the 1.22 M-VSG gene promoter region fragment from ptl.22BC plasmid DNA. Therefore the best explanation of this extra fragment would be an integration event involving multiple insertions of the plasmid in a tandem array (Fig. 5.2.2.2B, bottom line). This would generate 2 kb *Pvu*II fragments that each contain a copy of the 1.22 M-VSG gene promoter from the integrated plasmid (Fig. 5.2.2.2B). From the comparative intensity of the 2 kb band in relation to the 9.5 kb and 2.5 kb bands in each track, it seemed likely that only a few copies of ptl.22BC were inserted at the 1.22 M-VSG gene telomeric locus (Fig. 5.2.2.2). Selection of transformed trypanosomes over an extended period of time at a high concentration of antibiotic may have selected for a single

Figure 5.2.2.2 **Southern analysis of a triple insertion of pt1.22BC to the 1.22 M-VSG gene expression telomere**. (A) Genomic and plasmid DNA was digested with PvuII and run on an agarose gel before Southern blotting and probing with the 1.6 kb *Kpn*I-*Pst*I fragment of the 1.22 M-VSG gene promoter. The DNA in each track was plasmid pt1.22BC (track 1), genomic DNA from wild-type trypanosomes (track 2), genomic DNA from clone tB (track 3), genomic DNA from clone tC (track 4) and genomic DNA from the uncloned population (track 5). (B) Schematic representation of three copies of pt1.22BC integrated to the 1.22 M-VSG gene expression telomere and the *Pvu*II fragments that hybridise to the probe indicated.

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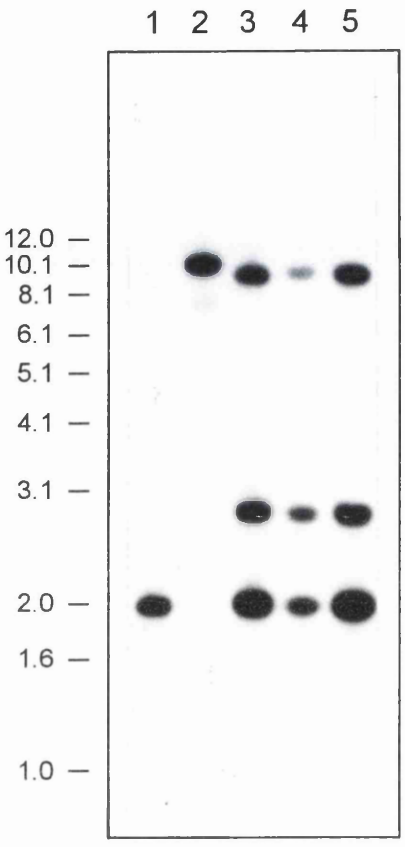
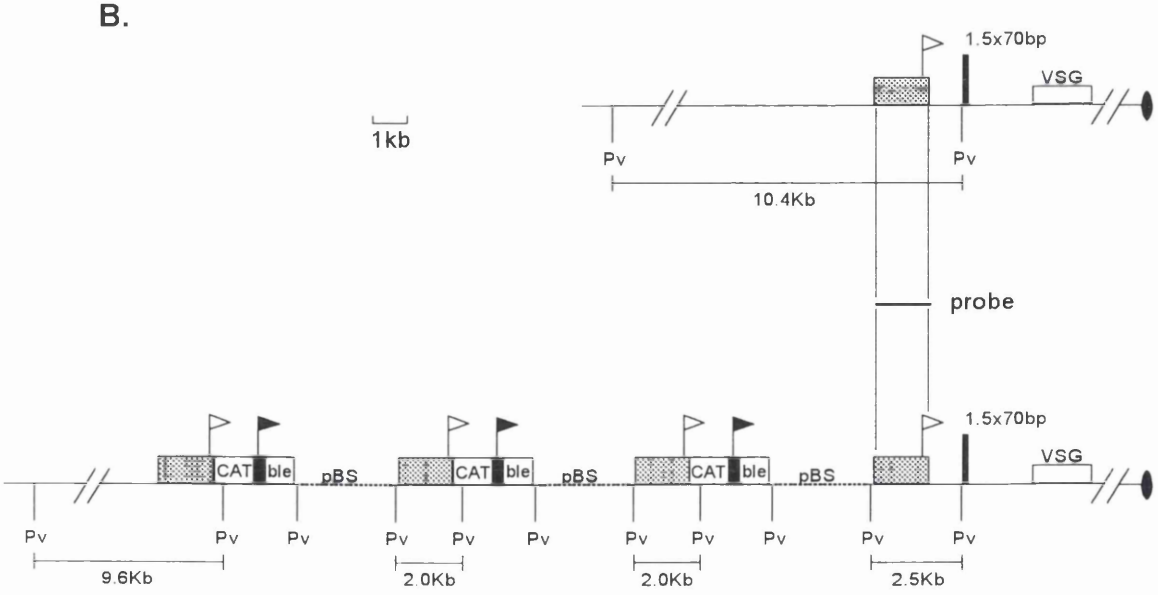
A.


1 2 3 4 5
12.0
10.1
8.1
6.1
5.1
4.1
3.1
2.0
1.6
1.0

B.


1kb
1.5x70bp
VSG
Pv
Pv
10.4Kb
probe
1.5x70bp
VSG
CAT
ble
pBS
CAT
ble
pBS
CAT
ble
pBS
Pv
Pv
Pv
Pv
Pv
Pv
Pv
Pv
Pv
Pv
Pv
9.6Kb
2.0Kb
2.0Kb
2.5Kb

transformant that contains several copies of pt1.22BC and resulted in the uncloned population being largely or completely clonal. This may explain why the probe hybridises the same *Pvu*II fragments in the DNA of cloned and uncloned populations.

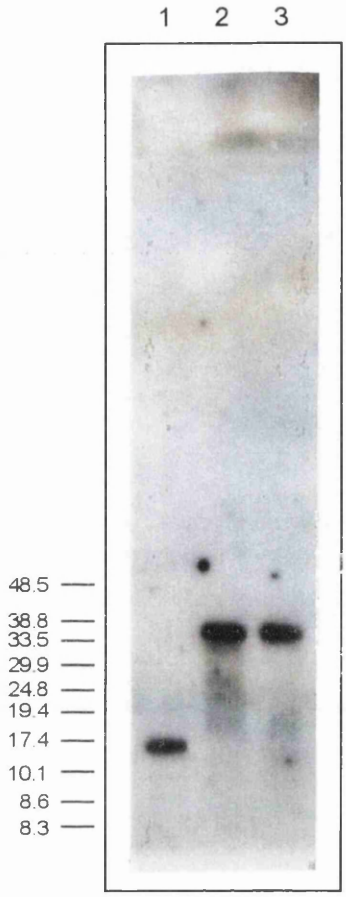
To confirm the integration event was at the 1.22 M-VSG gene expression telomere, and to determine the number of integrated copies of pt1.22BC, the shift in size of a larger *Sac*I fragment was analysed, which contains a large region of the 1.22 M-VSG gene expression telomere including the integration site. Genomic DNAs prepared from wild-type trypanosomes, clone tA (a clone isolated at the same time as tB and tC) and the uncloned population were digested with *Sac*I and separated on an agarose gel by Field Inversion Gel Electrophoresis (FIGE). The DNA was blotted to nylon membrane by Southern transfer and the blot probed with the same ^{32}P-labelled probe homologous to the 1.22 M-VSG gene promoter region used in the first Southern analysis. In DNA prepared from wild-type trypanosomes a band of 16 kb was detected (Fig. 5.2.2.3, track 1) that extends from a *Sac*I site just 3' of the promoter region to a *Sac*I site approximately 14 kb 5' of the promoter region (Fig. 5.2.2.3.B). pt1.22BC integrates within this 16 kb *Sac*I fragment but is not cut by *Sac*I and therefore in transformed trypanosomes the 16 kb band is shifted according to the size of the plasmid insertion. In clone tA and the uncloned transformed population a *Sac*I fragment of approximately 37 kb hybridised (Fig. 5.2.2.3 tracks 2 and 3). This is consistent with the integration of 3 copies of pt1.22BC (each 7 kb in length) in a tandem array directly adjacent to the endogenous copy of the 1.22 M-VSG gene promoter (Fig. 5.2.2.3.B). In conclusion, Southern analysis shows that in clones tA, tB and tC, 3 copies of pt1.22BC have been inserted at the predicted position and in the predicted orientation at the 1.22 M-VSG gene expression telomere.

The integration of 3 copies of pt1.22BC to the 1.22 M-VSG gene expression telomere is probably the result of ligation of 3 plasmid copies prior to the integration event. Electroporation of trypanosomes with a large amount of DNA (30μg) probably increased the chance of multiple plasmid insertions whilst a long period of passaging in the presence of phleomycin may have resulted in selection of trypanosomes with greater resistance to phleomycin. Trypanosomes containing multiple copies of pt1.22BC where the *ble* genes are expressed, will have higher levels of resistance to phleomycin and these cells may have out-grown trypanosomes that contain fewer copies of pt1.22BC under selection and with prolonged passaging.

Figure 5.2.2.3 **Southern analysis of triple insertion of pt1.22BC to the 1.22 M-VSG gene expression telomere**. (A) Genomic DNA was digested with *Sac*I and run on a FIGE gel before Southern blotting and probing with the 1.6 kb *Kpn*I-*Pst*I fragment of the 1.22 M-VSG gene promoter region. DNA was of wild-type trypanosomes (track 1), clone tA (track 2) and the uncloned population (track 3). (B) Schematic representation of integration of three copies of pt1.22BC to the 1.22 M-VSG gene expression telomere in tandem and the *Sac*I fragments that hybridise to the probe indicated.

Facing page 112

A.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2
3
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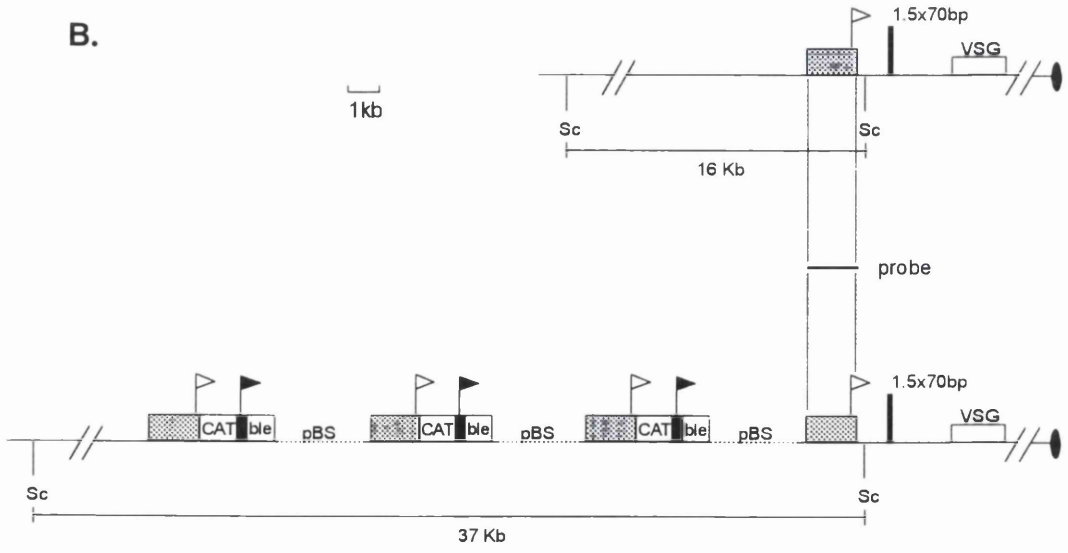
38.8
33.5
29.9
24.8
19.4
17.4
10.1
8.6
8.3

B.


1kb
1.5x70bp
VSG
Sc
Sc
16 Kb
probe
CAT
ble
pBS
CAT
ble
pBS
CAT
ble
pBS
1.5x70bp
VSG
Sc
Sc
37 Kb

5.2.3 Integration of a single copy of pt1.22BC to the 1.22 M-VSG gene expression telomere

Insertion of multiple copies of pt1.22BC to the telomeric locus in a tandem array means that any CAT gene transcription detected may be attributed to one of several copies of the CAT gene. Also, in a tandem array of plasmid copies, transcription of the CAT gene may initiate correctly at the 1.22 M-VSG gene promoter immediately upstream or, for those plasmid copies which are not the 5' most copy in the array, transcription may occur by transcriptional read-through from a copy of the plasmid upstream. In the latter case, an upstream copy of pt1.22BC would contain two promoters, the PARP promoter and the 1.22 M-VSG gene promoter, and transcription may initiate from either one or both of these trypanosome promoters. It is difficult to analyse transcription of a gene from a discrete promoter when multiple copies of the reporter plasmid have been integrated, therefore a second transformation experiment was performed to achieve a single insertion of pt1.22BC. Several strategies were adopted to prevent insertion of multiple copies of pt1.22BC in the second transformation experiment. Firstly, only 3 μg of *Sal*I-linearised pt1.22BC was electroporated into bloodstream trypanosomes (one tenth of that electroporated when a triple insertion was obtained), thereby hopefully reducing the number of copies of pt1.22BC introduced to each trypanosome. Secondly, transformed trypanosomes were selected at a much lower level of phleomycin (0.5μg/ml) so that trypanosomes expressing higher levels of the *ble* gene product due to multiple insertions were less likely to be preferentially selected. Wild-type cultured bloodstream form EATRO 795 were rapidly killed at this concentration of phleomycin and selection of transformed bloodstream trypanosomes at 0.5μg/ml phleomycin has been carried out successfully before (Li and Gottesdiener, 1996). Thirdly, transformed trypanosomes were cloned within 5 weeks of electroporation in order to reduce the likelihood of trypanosomes with multiple insertions out-growing trypanosomes with single insertions due to their increased resistance to phleomycin.

Bloodstream trypanosomes from a single electroporation with *Sal*I-linearised pt1.22BC, were divided equally between 5 flasks before selection with 0.5μg/ml phleomycin. At the same time, wild-type trypanosomes were grown in HMI-9 medium also containing 0.5μg/ml phleomycin and the cell density maintained below $1x10^6$ cells/ml: no motile cells were observed in this flask one week after the antibiotic was added. Phleomycin-resistant trypanosome populations grew-up in all 5 flasks and were

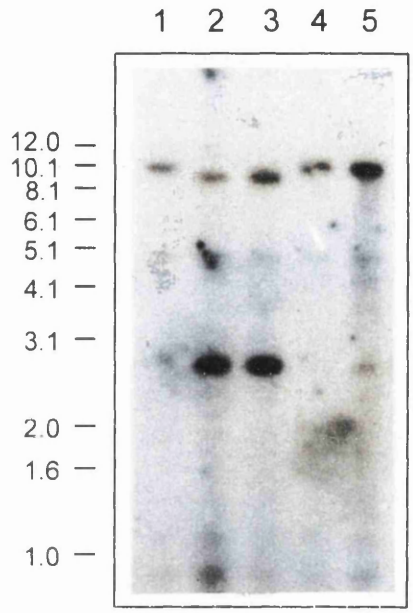
checked for plasmid insertion by Southern analysis. Genomic DNA was prepared from each population and digested with *Pvu*II, separated on an agarose gel and Southern blotted onto nylon membrane. The Southern blot was hybridised with the same ^{32}P-labelled DNA probe homologous to the 1.22 M-VSG gene promoter region that was used in previous hybridisations. Figure 5.2.3.1 shows that the probe hybridises to bands of 9.6 kb and 2.5 kb in only two of the five putative transformed populations (tracks 2 and 3) showing that ptl.22BC had integrated correctly and in single copy to the 1.22 M-VSG gene expression telomere. In the remaining three trypanosome populations, the probe hybridises either with one band of 10.4 kb indicating trypanosomes with no plasmid integration (track 4), or two bands of 10.4 kb and 2.5 kb indicating the plasmid had integrated but not at the 1.22 M-VSG gene expression telomere (tracks 1 and 5). Instead, integration may have occurred at an alternative site in the trypanosome genome with sufficient homology to the target sequence of the 1.22 M-VSG gene promoter to allow integration by homologous recombination. The alternative site appears to contain the same *Pvu*II site 3' of the integration position to give the 2.5 kb band but not the *Pvu*II site 5' of the integration position so the 9.6 kb band is not detected (Fig. 5.2.3.1). Sequences in the genome homologous to the 1.22 M-VSG gene promoter region were not detected by low stringency hybridisation (Paul Shiels; thesis 1990) but it is not yet clear how much homology is required for homologous recombination in *Trypanosoma brucei*. In *E. coli* efficient recombination can occur with as little as 23-27 bp of homologous sequence (Shen and Huang, 1986) whilst in yeast the homology may be only 15 bp (Manivasakam *et al.*, 1995) however, homologous recombination becomes more efficient with increased length of the homologous sequences.

From ptl.22BC transformed population G (Fig. 5.2.3.1; track 2), trypanosomes were cloned by serial dilution in 96 well plates with HMI-9 medium containing 0.5 μg/ml phleomycin. Two trypanosome clones, t8 and t9, were chosen and Southern analysis repeated to confirm integration of ptl.22BC to the 1.22 M-VSG gene expression telomere. DNA was prepared from wild-type and stably transformed trypanosome clones t8 and t9, digested with *Pvu*II and separated on an agarose gel before Southern transfer to nylon membrane. The blot was probed with the same ^{32}P-labelled probe homologous to the 1.22 M-VSG gene promoter region as used before. In figure 5.2.3.2A the probe hybridises to a 2.0 kb *Pvu*II fragment in ptl.22BC plasmid DNA (track 1). The probe hybridised with a 10.4 kb band in wild-type DNA (track 2) and two

Figure 5.2.3.1 **Southern analysis of uncloned bloodstream trypanosomes transformed with pt1.22BC.** (A) Genomic DNA from 5 uncloned trypanosome populations transfected with pt1.22BC (populations F-J in tracks 1-5) digested with *Pvu*II, run on an agarose gel before Southern blotting and probing with the 1.6 kb *Kpn*I-*Pst*I fragment of the 1.22 M-VSG gene promoter region. (B) Schematic representation of a copy of pt1.22BC integrated to the 1.22 M-VSG gene expression telomere and *Pvu*II fragments that hybridise when probed with the probe indicated.

Facing page 115

A.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8.1
6.1
5.1
4.1
3.1
2.0
1.6
1.0

B.

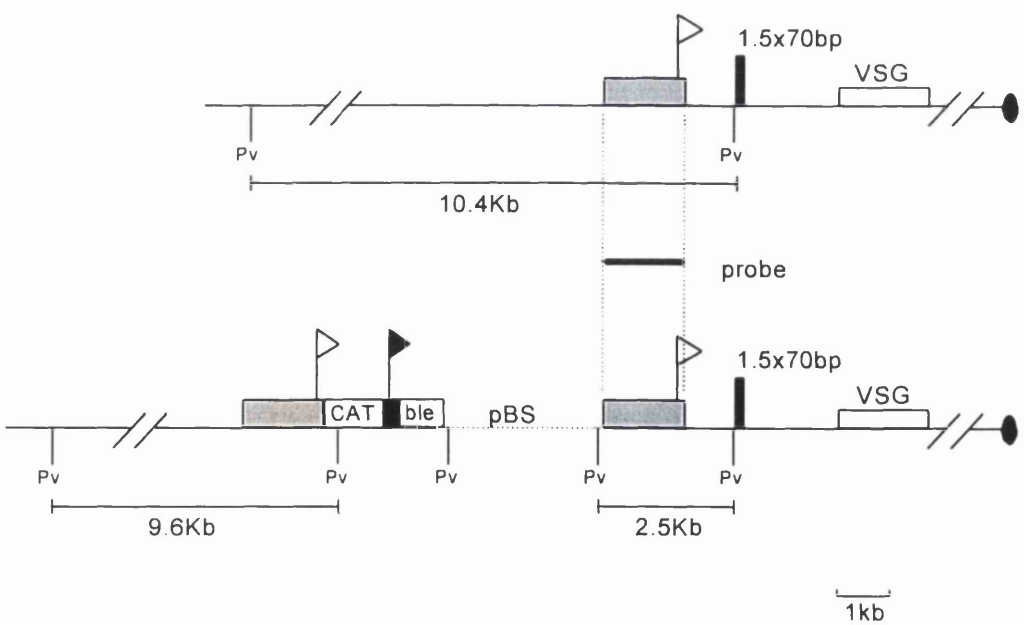
1.5x70bp
VSG
Pv
Pv
10.4Kb
probe
1.5x70bp
VSG
CAT
ble
pBS
Pv
Pv
Pv
Pv
Pv
9.6Kb
2.5Kb
1kb

Figure 5.2.3.2 **Southern analysis of cloned bloodstream trypanosomes transformed with pt1.22BC.** (A) DNA digested with *Pvu*II was separated on an agarose gel, Southern blotted and probed with the 1.6 kb *Kpn*I-*Pst*I fragment of the 1.22 M-VSG gene promoter region indicated in B. DNA digested and probed was plasmid pt1.22BC (track 1), genomic DNA of wild-type trypanosomes (track 2), genomic DNA of clone t9 (track 3) and genomic DNA of clone t8 (track 4). (B) Schematic representation of pt1.22BC integrated to the 1.22 M-VSG gene expression locus and the *Pvu*II fragments that hybridise the probe indicated.

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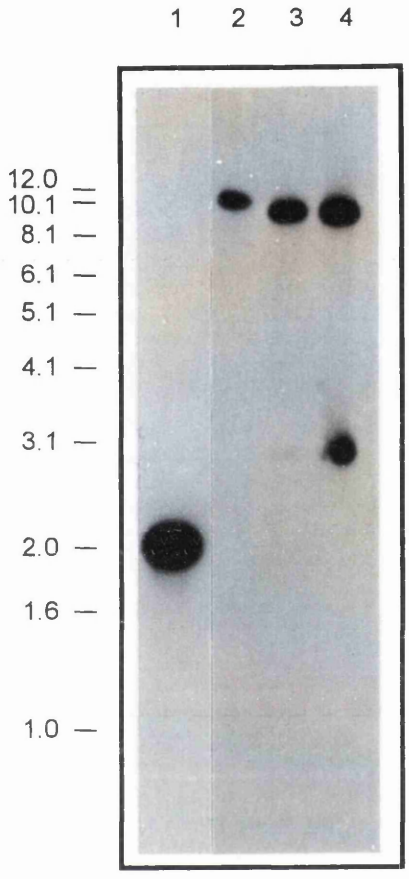
A.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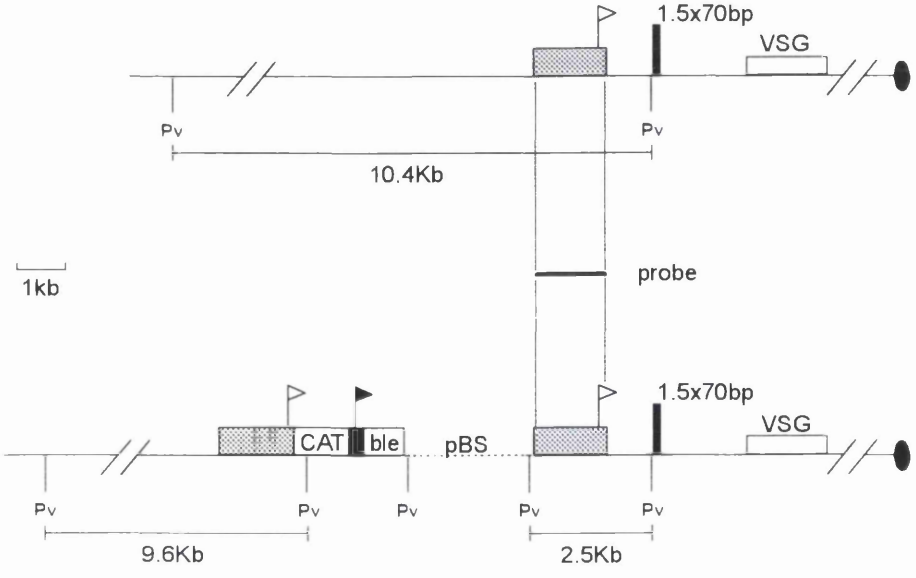
10.1
8.1
6.1
5.1
4.1
3.1
2.0
1.6
1.0

B.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VSG
Pv
Pv
10.4Kb
1kb
probe
1.5x70bp
VSG
CAT
ble
pBS
Pv
Pv
Pv
Pv
Pv
9.6Kb
2.5Kb

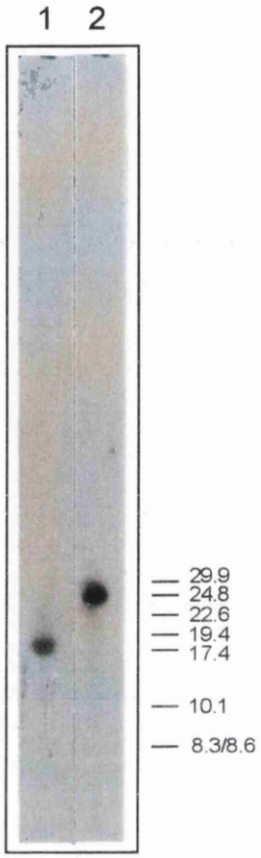
bands of 9.6 kb and 2.5 kb in DNA from clones t8 and t9 (Fig. 5.2.3.2A, tracks 3 and 4). In clone t9 (track 3) the 2.5 kb band is much weaker than the 9.6 kb band in the same lane or the 2.5 kb band in the adjacent track 4 containing DNA from clone t8. This is probably due to incomplete digestion of the *Pvu*II sites closer to the telomere that produce the 2.5 kb *Pvu*II fragment hybridised by the probe. *Pvu*II and *Pst*I restriction sites are subject to base modification at the silent bloodstream VSG gene expression sites of bloodstream trypanosomes (Gommers-Ampt *et al.*, 1991). The base modification does not occur at the single expression site that is transcribed in bloodstream trypanosomes or at any of these expression sites in procyclic trypanosomes. The base modification also occurs with greater frequency closer to the telomeres (F. van Leeuwen, personal communication). These base modifications could also occur at silent metacyclic expression telomeres in bloodstream trypanosomes and hinder *Pvu*II restriction, particularly of those *Pvu*II sites closest to the telomere. Thereby, the two *Pvu*II sites closest to the telomere that produce the 2.5 kb *Pvu*II fragment in clone t9 may not always be cut (Fig. 5.2.3.2B). The incomplete digestion would not necessarily result in a distinct third band as the 2.5 kb *Pvu*II fragment would now be much larger and may include the telomere which can vary greatly in length (Bernards *et al.*, 1983; Van der Ploeg *et al.*, 1984a).

To confirm the integration event was at the 1.22 M-VSG gene expression telomere, genomic DNAs prepared from wild-type trypanosomes and clone t9 were digested with *Sac*I and separated on an agarose gel by Field Inversion Gel Electrophoresis (FIGE). The DNA was blotted to nylon membrane by Southern transfer and the blot probed with the same ^{32}P-labelled probe homologous to the 1.22 M-VSG gene promoter. In DNA prepared from wild-type trypanosomes a band of 16 kb was detected (Fig. 5.2.3.3, track 1). *Sac*I does not cut within pt1.22BC and therefore, in the transformed cell line, the 16 kb band should be shifted according to the size of the inserted plasmid (7 Kb) and a band of approximately 23 kb should be detected. Figure 5.2.3.3, track 2 shows that in clone t9 the probe hybridises to only one *Sac*I fragment of 23 Kb. This confirms insertion of one copy of pt1.22BC to a position directly adjacent to the endogenous 1.22 M-VSG gene promoter and therefore at the 1.22 M-VSG gene expression telomere. In conclusion, Southern analysis shows that in clones t8 and t9 a single copy of pt1.22BC has been inserted at the predicted position and in the predicted orientation at the 1.22 M-VSG gene expression telomere.

Figure 5.2.3.3 **Southern analysis of cloned bloodstream trypanosomes transformed with pt1.22BC.** (A) Genomic DNA digested with *Sac*I and separated by field inversion gel electrophoresis (FIGE) was probed with the 1.6 kb *Kpn*I-*Pst*I fragment of the 1.22 M-VSG gene promoter region indicated in B. DNA on the Southern blot was genomic DNA from wild-type trypanosomes (track 1) and genomic DNA from clone t9 (track 2). (B) Schematic representation of pt1.22BC integrated to the 1.22 M-VSG gene expression telomere and the *Sac*I fragments that hybridise to the probe indicated.

Facing page 118

A.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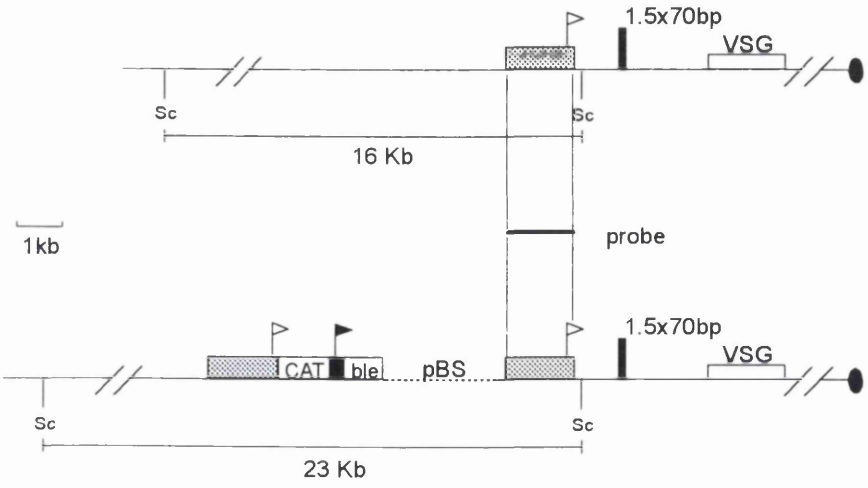
2
29.9
24.8
22.6
19.4
17.4
10.1
8.3/8.6

B.


1.5x70bp
VSG
Sc
Sc
16 Kb
1kb
probe
1.5x70bp
VSG
CAT
ble
pBS
Sc
Sc
23 Kb

5.2.4 Stable insertion of plasmid pr1.22BC into the ribosomal DNA spacer region

To determine the effect on promoter control of placing the M-VSG gene promoter at a chromosomal-internal position, pt1.22BC was adapted for targeting between transcription units of the ribosomal RNA gene tandem repeats. In *Trypanosoma brucei* the ribosomal RNA gene transcription unit contains a promoter driving transcription of the 18S, 5.8S and 28S RNA genes. Transcription is initiated approximately 1.2 kb upstream of the 18S gene and terminates at 250-500 bp downstream of the 28S RNA gene (White *et al.*, 1986). The ribosomal RNA gene transcription units are arranged as tandem repeats clustered on chromosomes that are 2-4 Mb in size (Van der Ploeg *et al.*, 1984b; Zomerdijk *et al.*, 1992) and between each transcription unit there is a ribosomal spacer region of approximately 8 kb (White *et al.*, 1986). Transcription of ribosomal RNA genes occurs exclusively at a discrete nuclear compartment, the nucleolus, in higher eukaryotes (Shaw *et al.*, 1995) and probably in trypanosomes (Rudenko *et al*, 1991; Chung *et al.*, 1992). Ribosomal RNA genes are only transcribed by the α-amanitin insensitive polymerase, RNA polymerase I (Shaw *et al.*, 1995). I decided to integrate the reporter plasmid into this ribosomal spacer region because the M-VSG gene promoter also directs transcription by an α-amanitin insensitive RNA polymerase (Graham and Barry, 1991) that is likely to be RNA polymerase I (see 1.7) and therefore the positioning of the metacyclic VSG gene promoter at the nucleolus may be important. Furthermore, the spacer region is largely untranscribed in *Trypanosoma brucei* (White *et al.*, 1986; Mike Cross, personal communication) and is not positioned close to a telomere. To negate any possible effects of transcriptional read-through from the upstream ribosomal RNA gene transcription unit, the plasmid construct was designed to integrate in a reverse orientation to the direction of transcription of the ribosomal RNA gene tandem repeats. The plasmid pr1.22BC was constructed by insertion of a 700 bp sequence from the ribosomal spacer region upstream of the 1.22 M-VSG gene promoter region in pt1.22BC. The 700 bp fragment of the ribosomal spacer region was derived from plasmid pHD430 (Wirtz and Clayton; 1995) by digestion with *Eco*RI and subcloning the 1 kb fragment into pBluescript (SK-) followed by digestion of the subclone with *Kpn*I and ligation of the 700 bp fragment into *Kpn*I cut pt1.22BC. The new plasmid for targeted integration was checked for correct orientation of the inserted *Kpn*I fragment by restriction enzyme digestion analysis. The 700 bp ribosomal spacer fragment contains a *Not*I site which would be suitable for linearising the plasmid and integration to the ribosomal spacer by

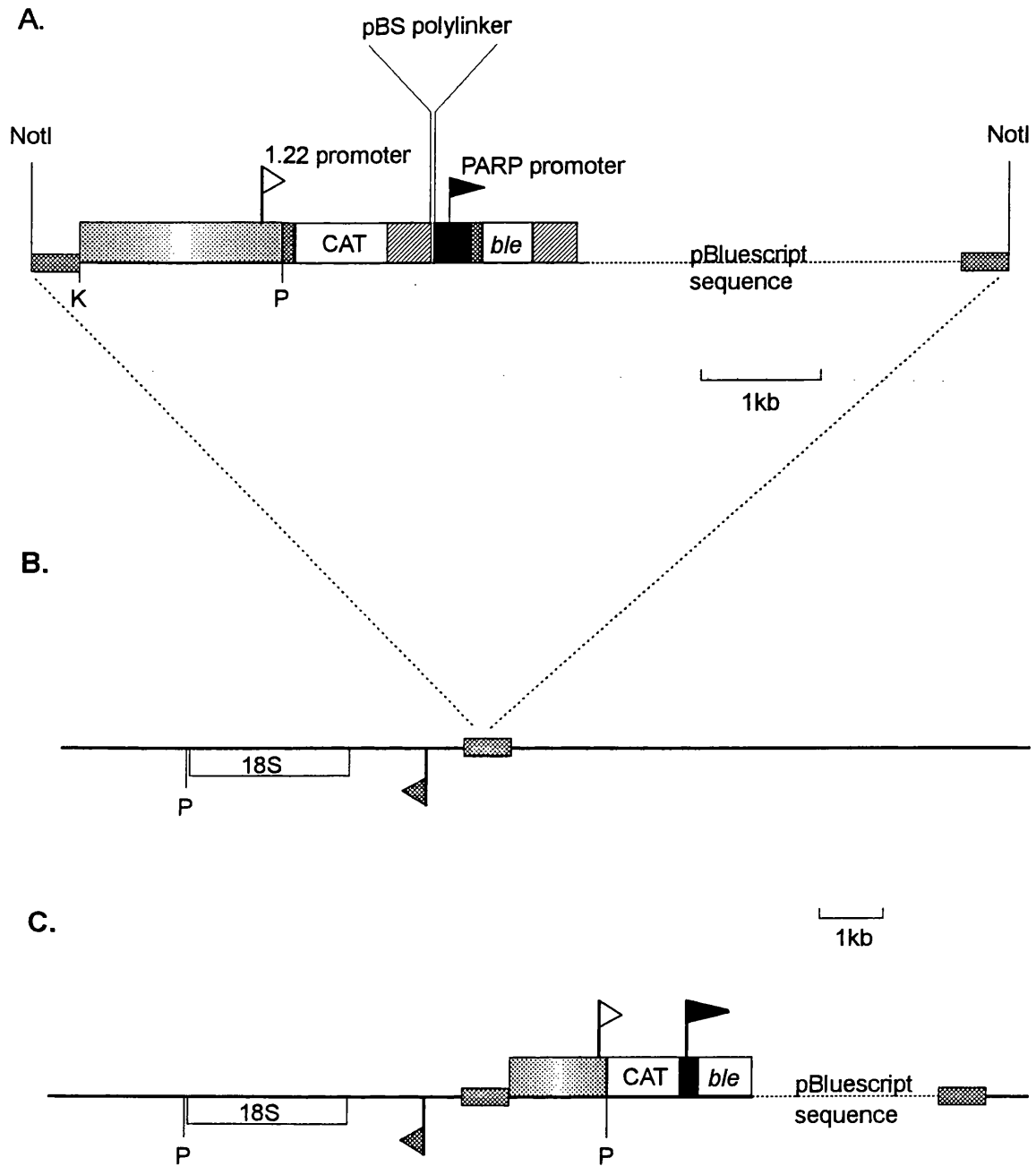


Figure 5.2.4.1. **Integration of plasmid pr1.22BC to the untranscribed spacer of the ribosomal RNA genes.** (A) Schematic representation of pr1.22BC containing the CAT gene and *ble* gene cassettes is the same as for figure 5.2.2.1. except for insertion of a 700 bp sequence that is derived from the untranscribed spacer region of the ribosomal RNA genes (hatched box). This sequence is upstream of the 1.22 M-VSG gene promoter region in pr1.22BC and contains a unique *Not*I restriction site which allows linerisation of the plasmid. (B) Insertion of pr1.22BC linearised with *Not*I by homologous recombination to a site upstream of the ribosomal RNA gene promoter (grey flag) and in the opposite orientation to transcription of the tandemly repeated ribosomal RNA gene transcription units. (C) The untranscribed spacer of the ribosomal RNA gene tandem repeats following insertion of a single copy of pr1.22BC. P, *Pst*I; K, *Kpn*I; white flag, 1.22 M-VSG gene promoter; dark grey box, PARP 5' UTR and splice acceptor signal; CAT, CAT coding sequence; diagonal lined box, PARP 3'UTR; pBS, pBluescript; black flag, PARP promoter; *ble*, phleomycin resistance gene; 18S, 18S ribosomal RNA gene.

homologous recombination. However, there is also a *Not*I site in the pBluescript polylinker between the CAT gene and *ble* gene cassettes of this plasmid. In order to remove this site the plasmid clone was partially digested with *Not*I, such that only one of the two *Not*I sites would cut, then the NotI site was blunt-ended and the plasmid was religated. The resulting colonies were screened for each event by restriction enzyme digestion analysis. The plasmid pr1.22BC was selected wherein the *Not*I site in the pBluescript polylinker had been removed and it could now be linearised to completion with the *Not*I in the 700 bp ribosomal spacer sequence, facilitating insertion into the ribosomal spacer region 1 kb upstream of the ribosomal promoter, in reverse orientation to ribosomal RNA gene transcription (Fig. 5.2.4.1).

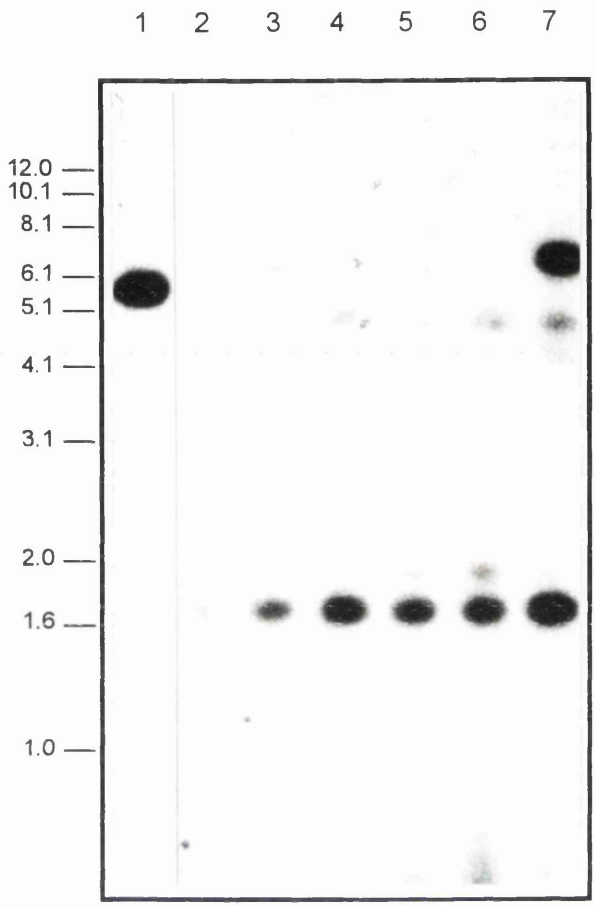
The 700 bp ribosomal spacer sequence to which pr1.22BC would be targeted, is present in multiple copies in the trypanosome genome. To minimise the possibility of plasmid insertions to several ribosomal spacer regions in a single cell, only a small quantity (2μg) of DNA was used in each electroporation. Also, to try to prevent the insertion of tandem arrays of the plasmid pr1.22BC to the same ribosomal spacer region (as observed for pt1.22BC in section 5.2.3), *Not*I digested pr1.22BC was treated with calf intestinal phosphatase (CIP) to prevent ligation of plasmid copies prior to integration. Lastly, trypanosomes containing several copies of pr1.22BC should express higher levels of the *ble* gene product and therefore greater resistance to phleomycin. By selecting for transformants at a low level of antibiotic (0.5 or 1μg/ml), trypanosomes with several copies of pr1.22BC, and therefore greater resistance to phleomycin, would not be preferentially selected in the uncloned population.

Two preparations of bloodstream trypanosomes in axenic culture were each electroporated with 2μg of pr1.22BC that had been linearised with *Not*I and treated with CIP. The two electroporated populations were left to recover for 24 hours then divided equally between 10 flasks before selection with either 0.5μg/ml or 1μg/ml phleomycin (5 flasks each). DNA was prepared for Southern analysis from 6 of the 10 uncloned populations, 5 selected at 0.5μg/ml and one selected at 1μg/ml. Genomic DNA was digested with *Pst*I, separated on an agarose gel, blotted to nylon membrane and probed with the ^{32}P-labelled probe homologous to the 1.22 M-VSG gene promoter region used in previous Southern analysis; the Southern blot is shown in figure 5.2.4.2. In track 1, which contains pr1.22BC plasmid DNA digested with *Pst*I, the probe hybridises to a fragment of 6 kb which contains the 1.22 M-VSG gene promoter region. In the tracks

Figure 5.2.4.2 **Southern analysis of uncloned populations of trypanosomes transformed with pr1.22BC.** (A) Genomic and plasmid DNA was digested with *Pst*I and separated on an agarose gel before Southern blotting and probing with the 1.6 kb *Kpn*I-*Pst*I fragment of the 1.22 M-VSG gene promoter region indicated in B. DNA was plasmid pr1.22BC (track 1), genomic DNA from uncloned populations selected at 0.5μg/ml phleomycin A-E (tracks 2-6) and genomic DNA from one uncloned population selected at 1μg/ml phleomycin (track 7). (B) Schematic representation of the site of integration of pr1.22BC in the ribosomal spacer region and the expected result of integration with the *Pst*I fragment that hybridises with the probe indicated. The 2.0 kb *Pst*I fragment in all the genomic DNA tracks that hybridises to the 1.22 M-VSG gene promoter probe is derived from the 1.22 M-VSG gene expression telomere (see Fig. 5.2.2.1).

Facing page 122

A.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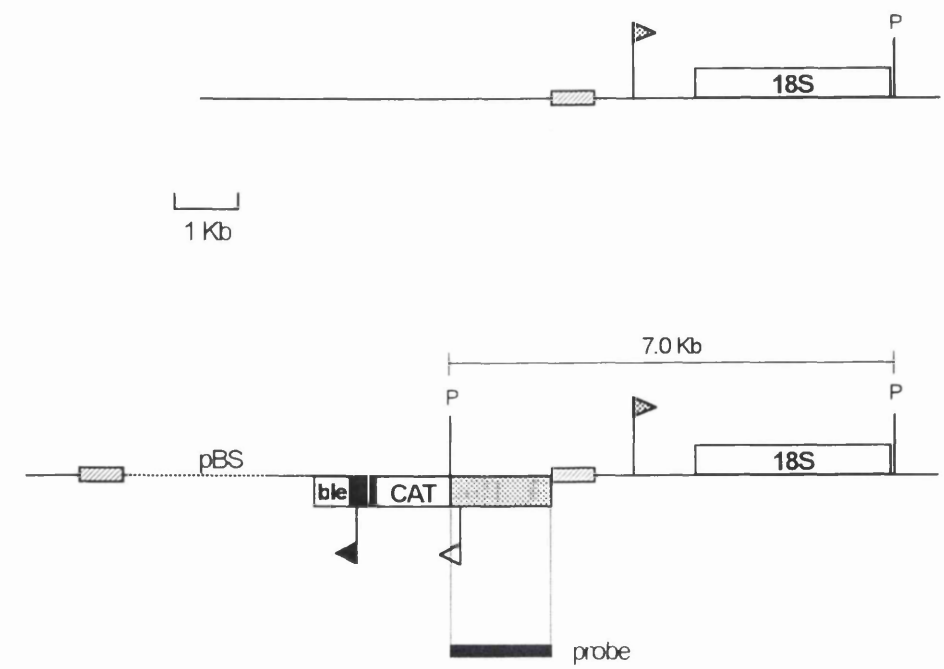
3.1
2.0
1.6
1.0

B.


P
18S
1 Kb
7.0 Kb
P
P
pBS
ble
CAT
18S
probe

containing trypanosome genomic DNA (tracks 2-7) the probe hybridised to a 2 kb *Pst*I fragment. This fragment contains the endogenous copy of the 1.6 kb *Kpn*I-*Pst*I fragment 1.22 M-VSG gene promoter region present at the 1.22 M-VSG gene expression telomere (Fig. 5.2.2.1). In the 5 uncloned populations that were selected at a phleomycin concentration of 0.5μg/ml, the probe detects only this 2 kb fragment indicating that pr1.22BC has not integrated anywhere in the genome (tracks 2-6). These trypanosomes may exhibit slightly greater resistance to phleomycin than wild-type trypanosomes due to transient expression of the *ble* gene product from copies of pr1.22BC in the trypanosome nucleus but not integrated into the genome. In contrast, the uncloned trypanosome population selected at 1μg/ml phleomycin contained two *Pst*I fragments of 2 kb and 7 kb that hybridised to the 1.22 M-VSG gene promoter probe (track 7). While the 2 kb fragment represents the endogenous copy of the 1.22 M-VSG gene promoter at the 1.22 M-VSG gene expression telomere, the 7 kb fragment is the expected size of the PstI fragment containing the 1.22 M-VSG gene promoter that would result from integration of pr1.22BC to the ribosomal spacer (Fig. 5.2.4.2B). The 7 kb band is of very similar intensity to the 2 kb band suggesting there is only one extra copy of the promoter sequence in each trypanosome (the probe is single copy in wild-type trypanosomes) and therefore only one copy of pr1.22BC has integrated per genome. There is some weak hybridisation of the probe to fragments of ~5 kb and ~2 kb in most tracks in the Southern blot. These bands may represent a low level of homology to the 1.22 M-VSG gene promoter region at sites in the genome other than the 1.22 M-VSG gene expression telomere.

Trypanosomes were then cloned from this uncloned population F (track 7, Fig. 5.2.4.2) by serial dilution in HMI-9 medium containing 0.5 μg/ml phleomycin in 96 well plates and clones r8 and r10 were chosen for further analysis. Southern blot analysis was repeated with *Pst*I-digested genomic DNA from wild-type trypanosomes and clone r10, probed with the ^{32}P-labelled probe homologous to the 1.22 M-VSG gene promoter region. Fig 5.2.4.3 shows only one band of 2 kb hybridised in wild-type DNA (track 2) whilst in r10 DNA there are two bands of 7 kb and 2 kb (track 3). The 7 kb band is larger than the 6.5 kb band hybridised by *Pst*I-digested, non-integrated pr1.22BC plasmid (track 1), illustrating the 7 kb band is not derived from plasmid copies maintained in the trypanosomes episomally. Again the hybridisation pattern is consistent with a single copy of pr1.22BC integrated at the ribosomal spacer region in clone r10.

Figure 5.2.4.3 **Southern analysis of cloned trypanosomes transformed with pr1.22BC.** (A) DNA was digested with *Pst*I and run on an agarose gel before Southern transfer to nylon membrane and probing with the 1.6 kb *Kpn*I-*Pst*I fragment of the 1.22 M-VSG gene promoter region. DNA analysed was plasmid pr1.22BC (track 1), genomic DNA from wild-type trypanosomes (track 2) and genomic DNA from clone r10 (track 3). (B) Schematic representation of the site of integration of pr1.22BC in the ribosomal spacer region and the expected result of integration with the *Pst*I fragment that hybridises with the probe indicated. The 2.0 kb *Pst*I fragment that hybridises with the 1.22 M-VSG gene promoter probe in wild-type and transformed trypanosomes is derived from the 1.22 M-VSG gene expression telomere (see Fig. 5.2.2.1).

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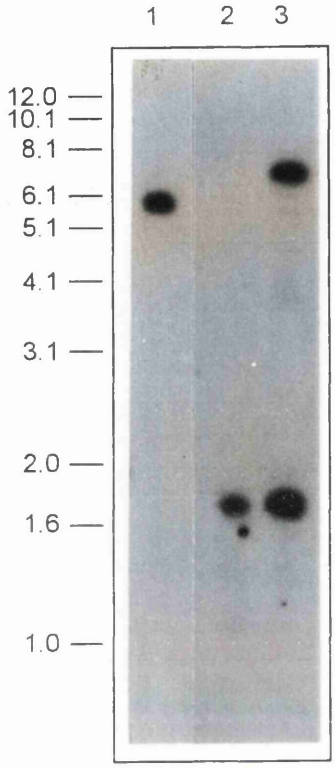
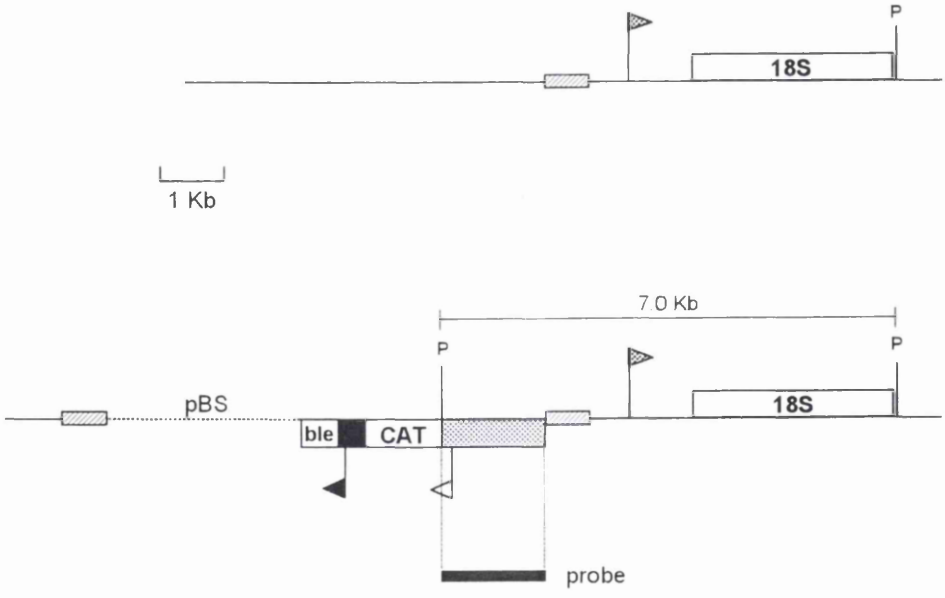
A.


1
2
3
12.0
10.1
8.1
6.1
5.1
4.1
3.1
2.0
1.6
1.0

B.


P
18S
1 Kb
7.0 Kb
P
P
pBS
ble
CAT
18S
probe

To confirm that the integrated copy of pr1.22BC was positioned at the ribosomal spacer region, genomic PCR reactions were performed using two oligonucleotide primers (see section 2.18), one which anneals to a sequence of the 1.22 M-VSG gene promoter region (primer Mprom sequence) and one which anneals to the ribosomal spacer just upstream of the ribosomal promoter region (primer Rprom sequence; Fig 5.2.4.4). Both oligonucleotides anneal to the coding strand of their respective promoter regions and in a PCR reaction these primers would generate a PCR product only when the 1.22 M-VSG gene promoter sequence is positioned close to the ribosomal promoter sequence and in the reverse orientation (Fig. 5.2.4.4). Therefore, if pr1.22BC has integrated upstream of the ribosomal RNA gene promoter (in the ribosomal spacer region) and in the reverse orientation to ribosomal RNA gene transcription, then in PCR reactions a PCR product of approximately 1200 bp should result (calculated from the restriction map of pR4, a plasmid containing a single repeat of the ribosomal RNA gene tandem array, [White *et al.*, 1986]). Figure 5.2.4.4 shows an ethidium bromide stained gel of 5 PCR reactions that were performed each containing the two oligonucleotide primers and uncut genomic DNA from either wild-type trypanosomes (track 2), trypanosomes transformed with pr1.22BC (clones r10 and r8; tracks 3 and 4 respectively) or trypanosomes transformed with pt1.22BC (clones t8 and t9; tracks 5 and 6 respectively). PCR products of approximately 1500 bp were obtained only in reactions containing genomic DNA from trypanosomes transformed with pr1.22BC, clones r10 and r8 (tracks 3 and 4). No PCR products were observed in reactions containing DNA from wild-type trypanosomes (track 2) or trypanosomes containing pt1.22BC integrated to the 1.22 M-VSG gene expression telomere (tracks 5 and 6). The PCR product was slightly larger than the size predicted from restriction map analysis of a cloned trypanosome ribosomal spacer region (pR4, White *et al.*, 1986) but shows that pr1.22BC has integrated to a ribosomal spacer region in both clones r8 and r10. The larger size of PCR product is probably due to heterogeneity between spacer regions in the ribosomal RNA gene arrays (Biebinger *et al.*, 1996) and in clones r8 and r10, pr1.22BC has integrated to a ribosomal spacer region slightly different to that cloned in plasmid pR4. Therefore, Southern blot analysis and genomic PCR analysis confirm that in clones r10 and r8, a single copy of pr1.22BC has integrated at the correct position in the ribosomal spacer region and in the correct orientation.

Figure 5.2.4.4 **Genomic PCR analysis of cloned trypanosomes transformed with pr1.22BC**. (A) Ethidium bromide stained agarose gel of PCR reactions using primers Mprom and Rprom and using uncut genomic DNA from transformed and wild-type trypanosomes as templates. Genomic DNA templates used in each reaction were wild-type (track 2), clones r10 and r8 transformed with pr1.22BC (tracks 3 and 4) and clones t9 and t8 transformed with pt1.22BC (tracks 5 and 6). DNA size standards are in track 1. (B) Schematic representation of the integration of pr1.22BC to the ribosomal spacer region and the relative positions at which the primers Mprom and Rprom anneal. The predicted size of the PCR product is shown below the diagram.

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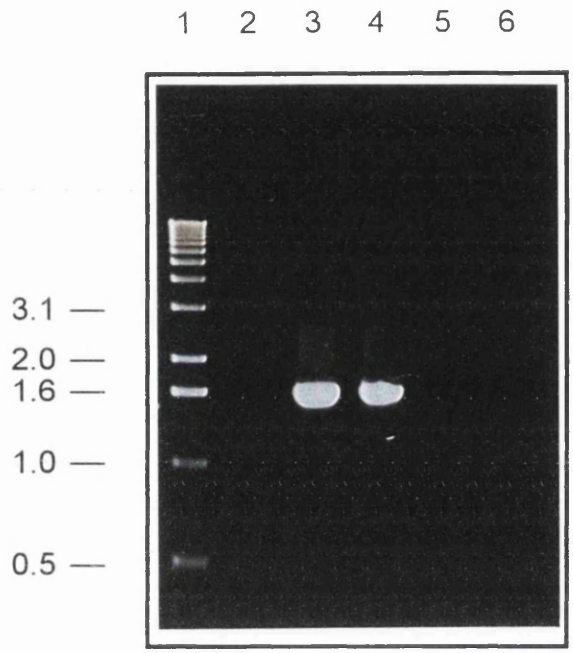
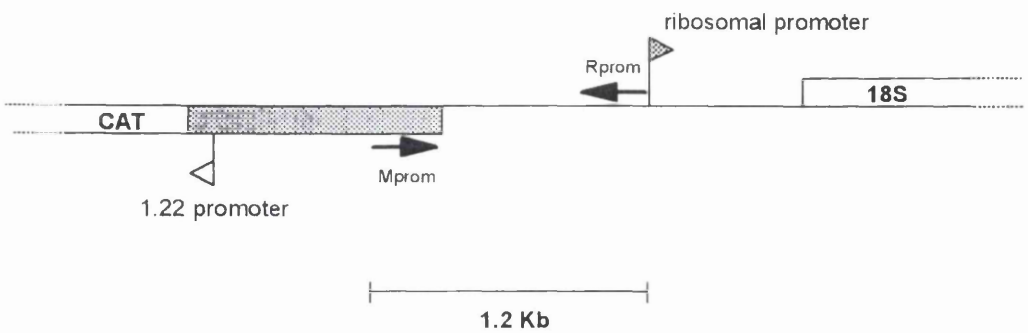
A.


1 2 3 4 5 6
3.1
2.0
1.6
1.0
0.5

B.


ribosomal promoter
Rprom
18S
CAT
Mprom
1.22 promoter
1.2 Kb

5.2.5 CAT activity in the stably transformed cell lines

In both ptl.22BC and prl.22BC, the CAT gene is positioned directly downstream of the 1.22 M-VSG gene promoter region and the PARP gene 5' splice acceptor site. Transcription initiation from the 1.22 M-VSG gene promoter should result in primary transcripts that encode the entire CAT gene and are processed into mature CAT gene mRNAs by *trans*-splicing (at the PARP gene 5' splice acceptor site) and polyadenylation (at a site in the PARP gene 3' UTR). Therefore, CAT gene mRNA abundance and CAT enzyme activity, should be directly proportional to the rate of transcription initiation from the 1.22 M-VSG gene promoter. To measure the level of CAT enzyme activity, crude cell extracts were prepared from wild-type cultured bloodstream trypanosomes and cloned stably transformed trypanosome lines and assayed for CAT enzyme activity exactly as described in Materials and Methods (see section 2.10). Several transformed lines were tested: clones r10 and r8 containing a single insertion of prl.22BC to the ribosomal spacer region, clones t8 and t9 containing a single insertion of ptl.22BC to the 1.22 M-VSG gene expression telomere and clone tA containing a triple insertion of ptl.22BC to the 1.22 M-VSG gene expression telomere. CAT activity of wild-type trypanosomes corresponded very closely to the CAT activity of samples that contained no cell extract (see Table 5.2.5.1). In each experiment, 1 x 10^7 bloodstream trypanosomes, grown in the absence of selection to mid-log phase (4-8 x 10^5 cells/ml), were harvested and assayed in duplicate. As I observed with transient transfection of bloodstream trypanosomes, there was variability in counts between experiments for each stably transformed clone and for the wild-type trypanosomes, but relative CAT activities, between clones in the same experiment, remained consistent. Therefore, the CAT enzyme activity of wild-type trypanosomes was assigned the arbitrary value of one in each of the experiments, and CAT activity of each of the clones was calculated with respect to wild-type trypanosomes in each experiment (results are presented in Table 5.2.5.1). Clone r10 contains a single insertion of prl.22BC to the ribosomal RNA gene repeat untranscribed spacer and gave CAT activity 30-50 fold higher than wild-type trypanosomes. Clone r8 also contains a single insertion of prl.22BC to the ribosomal spacer region but gives a lower level of CAT activity than clone r10, approximately 20 fold higher than wild-type trypanosomes in the single experiment conducted. In bloodstream trypanosome transformation experiments in another laboratory, similar variations in CAT gene expression between clones from a single set of transformation experiments were observed, for a number of different

Table 5.2.5.1A **Table showing the CAT activity of cloned lines of bloodstream trypanosomes transformed with pr1.22BC and pt1.22BC.** For each experiment, 2 aliquots of 1 x 10^7 trypanosomes were harvested from the same flask containing either a cloned line of transformed trypanosomes or wild-type (WT) trypanosomes at mid-log phase. The CAT activity of transformed and wild-type trypanosomes was determined using the same assay as used for transient transfection assays (see section 2.10). The CAT activity of each aliquot of 5 cloned lines of transformed trypanosomes and wild-type trypanosomes are represented in counts per minute (cpm) per 1 x 10^7 trypanosomes. -ve represents the CAT activity of CAT resuspension buffer that contains no cells. Clones r10 and r8 contain a single insertion of pr1.22BC to the untranscribed spacer region of the ribosomal RNA gene repeats. Clones t9 and t8 contain a single insertion of pt1.22BC to the 1.22 M-VSG gene expression telomere. Clone tA contains three copies of pt1.22BC inserted to the 1.22 M-VSG gene expression telomere in tandem. ND, not determined; WT, wild type bloodstream trypanosomes.

Table 5.2.5.1B **Table showing the relative CAT activity of cloned lines of transformed bloodstream trypanosomes.** The CAT activity of wild-type trypanosomes (the mean of each duplicate in table 5.2.5.1A) was assigned the arbitrary figure of one in each experiment (1-4), and the CAT activity of the cloned lines in each experiment (the mean of each duplicate) was calculated relative to the wild-type. Therefore, in experiment 1 the level of CAT activity measured in clone r10 is 45.5 times greater than in wild-type trypanosomes. On the right of the table is shown the mean and standard deviation (SD) of relative CAT activities where sufficient values have been determined. -ve represents the CAT activity of CAT resuspension buffer that contains no cells. The cloned lines of transformed bloodstream trypanosomes are described above in the legend to table 5.2.5.1A. ND, not determined.

A.

CAT activity (cpm/1 x 10^7 trypanosomes)

	Experiment			
	1	*2*	*3*	*4*
WT	3905 4069	2757 2571	4045 3790	5229 5234
-ve	ND	1673 2674	4391 4455	5231 5335
r10	187289 178552	77517 77128	139213 142853	256089 146230
r8	ND	52130 49797	ND	ND
t9	ND	3495 3531	ND	10770 10664
t8	7706 7651	3847 3639	6017 5015	9166 8883
tA	ND	ND	131561 141906	244364 258067

B.

Relative CAT Activity

	Experiment					
	1	*2*	*3*	*4*	*Mean*	*SD*
WT	1	1	1	1	1	-
-ve	ND	1.0	1.1	1.0	1.0	0.06
r10	45.5	29.4	35.7	47.6	39.6	8.5
r8	ND	19.4	ND	ND	-	-
t9	ND	1.3	ND	2.1	1.7	-
t8	1.9	1.4	1.4	1.7	1.6	0.2
tA	ND	ND	34.6	47.6	41.1	-

reporter constructs integrated at the same position in the untranscribed spacer region as pr1.22BC in my experiments. Each reporter construct contained either the PARP gene promoter, VSG gene promoter or ribosomal RNA gene promoter directing expression of the CAT gene and clones transformed with the same construct varied in CAT enzyme activity by as much as 3-fold (Biebinger *et al.*, 1996). The discrepancy in CAT enzyme activity in clones r10 and r8 may be due to independent integration events in each cloned trypanosome line. Since there are as many as 100 ribosomal RNA gene untranscribed spacer regions in a single trypanosome genome (Hasan *et al.*, 1984), in each stably transformed clone pr1.22BC may have inserted to different positions in the same ribosomal RNA gene array or into separate arrays that could be located on different chromosomes. Within or between ribosomal RNA gene arrays there may be variable levels of primary transcript processing or rate of nuclear or cytoplasmic transport of transcripts which may in turn affect the level of CAT gene expression from the reporter construct in the untranscribed spacer region. Alternatively, regions of the ribosomal RNA gene repeat arrays may be more or less competent for transcription.

In contrast to the insertion of the reporter construct to the ribosomal spacer region, integration of a single copy of pt1.22BC at the 1.22 M-VSG gene expression telomere results in very low levels of CAT activity, but these were consistently just higher than the CAT activity of wild-type trypanosomes. Clone t8, containing a single copy of the plasmid pt1.22BC integrated to the 1.22 M-VSG gene expression telomere, gave CAT activity at a mean of 1.6 fold higher than wild-type trypanosomes in four separate experiments. A similar clone, t9, also containing a single copy of pt1.22BC at the 1.22 M-VSG gene expression telomere, gave similar levels of CAT activity in two experiments. However, in clone tA containing three copies of pt1.22BC in a tandem array integrated at the 1.22 M-VSG gene expression telomere (Fig. 5.2.2.2), CAT activity was around 40 fold higher than wild-type trypanosomes, a level of activity equivalent to clone r10. However this result is very difficult to interpret because there are 3 CAT genes in the tandem array and CAT gene expression could be due to transcription of just one CAT gene or the result of transcription of more than one gene. In addition, the tandem arrangement of plasmid copies in the array means that transcription of the CAT genes may be the result of transcription initiation from the 1.22 M-VSG gene promoter copy immediately upstream, or, in the case of the two downstream CAT genes, transcriptional read-through from a 1.22 M-VSG gene

promoter region or PARP promoter in an upstream copy of pt1.22BC (Fig. 5.2.2.2). Transcriptional read-through of a CAT gene from an upstream copy of the PARP promoter would mean that transcription continues through the *ble* gene, the 3 kb of pBluescript vector sequence and the 1.6 kb 1.22 M-VSG gene promoter fragment before transcribing the CAT gene (Fig. 5.2.2.2). This is unlikely because the PARP promoter is minimally active in the genome in bloodstream trypanosomes (Biebinger *et al.*, 1996) and in bloodstream trypanosomes there is no transcriptional read-through of plasmid vector sequence directed by the PARP promoter in a similar reporter construct integrated to the PARP A locus (Vanhamme *et al.*, 1995a). Therefore, the high level of CAT gene expression in clone tA is probably the result of CAT gene transcription which is initiated from one or more of the 1.22 M-VSG gene promoter regions. Unfortunately, it is not possible to determine which of the 1.22 M-VSG gene promoter regions are active in the tandem array but the high level of CAT gene expression in clone tA in relation to clone t8, which contains a single copy of pt1.22BC, suggests that one or more of the copies of the 1.22 M-VSG gene promoter region in the tandem array are more active than in a single insertion of pt1.22BC to the telomere.

If the data from the single plasmid integration events are compared, the results suggest the CAT gene is highly transcribed when pr1.22BC is integrated at the ribosomal spacer region whilst at the 1.22 M-VSG gene expression telomere transcription of the CAT gene from pt1.22BC is down-regulated at least 20-fold. In both cases transcription of the CAT gene is directed by the 1.22 M-VSG gene promoter and therefore reduced CAT gene expression in clones t8 and t9 is probably the result of reduced transcriptional activity of the 1.22 M-VSG gene promoter at the 1.22 M-VSG gene expression telomere.

5.2.6 Northern blot analysis of CAT gene expression in the stably transformed cell lines

Northern blot analysis was performed to confirm that the level of CAT enzyme activity observed in the transformed trypanosome clones reflected the level of CAT steady-state mRNA. Total RNA was prepared from wild-type trypanosomes and clones t8 (telomeric insertion) and r10 (ribosomal locus insertion) and run on a denaturing agarose gel before transfer onto nylon membrane and probing with a ^{32}P-labelled antisense RNA probe for the CAT gene. An antisense RNA probe was used to detect CAT gene transcripts as previous probes prepared by random primer extension of double

stranded DNA were not sufficiently specific to detect CAT gene transcripts in Northern blot analysis. Figure 5.2.6.1 A. shows CAT transcripts cannot be detected in wild-type trypanosomes (track 1) or clone t8 containing a single copy of pt1.22BC integrated at the 1.22 M-VSG gene expression telomere (track 2). In contrast, CAT transcripts can be readily detected in clone r10 (track 3) containing a single copy of pr1.22BC integrated at the ribosomal spacer region. In trypanosomes, primary RNA transcripts are very quickly processed to generate mature mRNAs (Graham, 1995), so the distinct band of approximately 1.5 kb is likely to represent the mature CAT mRNA with a *trans*-spliced mini-exon and poly (A) tail. The CAT mRNA is somewhat larger than would be predicted from the size of the CAT gene (800 bp), the splice leader sequence (39 bp), 5' UTR (30 bp) and 3' UTR (250 bp). From previous Northern blot analysis of a similar construct in procyclic trypanosomes, a transcript of approximately 1.2 kb would have been expected (S. V. Graham, unpublished). This implies that in bloodstream trypanosomes, the CAT primary transcripts were not processed in the same manner as the equivalent transcripts in procyclic trypanosomes. The longer CAT mRNA in bloodstream trypanosomes could be attributable to use of an alternative polyadenylation site downstream of the normal PARP transcript polyadenylation site. Given that the poly (A) tail is likely to be up to 200 bp long (Graham, 1995) the acceptor site for polyadenylation probably lies just upstream of the *ble* gene in the antibiotic resistance cassette portion of pr1.22BC (Fig. 5.2.2.1). The processes of *trans*-splicing and polyadenylation are functionally linked in trypanosomes with the downstream event of *trans*-splicing preceding the polyadenylation event upstream in a polycistronic transcription unit (Ullu *et al.*, 1995). The PARP gene 5' splice acceptor contains a polypyrimidine tract that is functional in signalling polyadenylation upstream (Clayton and Hotz, 1996) and which may signal polyadenylation of the CAT transcript in the plasmid pr1.22BC at a position downstream of the CAT gene and just upstream of the *ble* gene (Fig. 5.2.2.1) and therefore account for the slightly larger size of CAT gene transcript. Northern analysis of CAT transcripts supports the data from the CAT enzyme activity assays and strongly suggests the 1.22 M-VSG gene promoter region is highly active at the ribosomal spacer region whilst at the 1.22 M-VSG gene expression telomere transcription initiation from this promoter is greatly reduced.

The Northern blot that was probed with the CAT gene probe, was stripped and reprobed with a radiolabelled DNA fragment homologous to the *ble* gene (Fig. 5.2.6.1 B.). *ble* transcripts of approximately 800 bp could be detected in both clone t8 (track 2)

Figure 5.2.6.1 **Northern analysis of the trypanosomes transformed with pt1.22BC and pr1.22BC.** Whole RNA was run on an agarose denaturing gel and blotted to nylon membrane before hybridisation with the probes indicated. The RNA loaded was from wild-type bloodstream trypanosomes (track 1), bloodstream trypanosomes transformed with pt1.22BC, clone t8 (track 2), bloodstream trypanosomes transformed with pr1.22BC, clone r10 (track 3). (A) The Northern blot hybridised with a CAT gene probe. (B) The same blot stripped and rehybridised with a *ble* gene probe. (C) the same blot stripped again and reprobed with a trypanosome actin gene probe.

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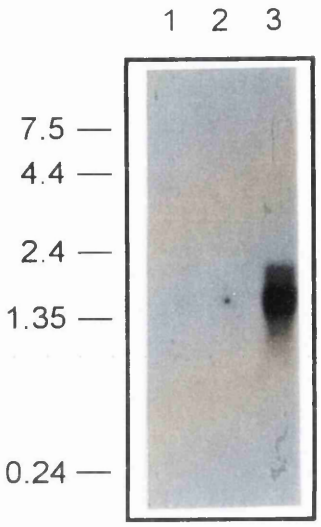
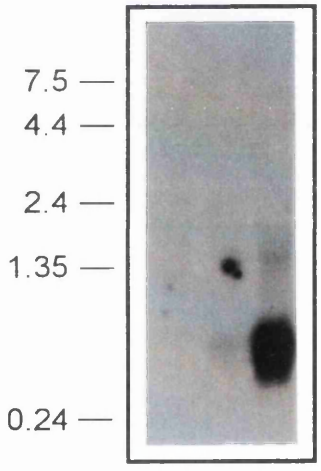
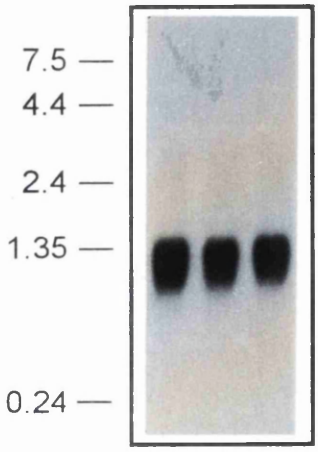
A. CAT



B. *ble*



C. Actin



and clone r10 (track 3) but could not be detected in RNA derived from wild-type trypanosomes (track 1). In transformed trypanosomes where the plasmid was integrated to the 1.22 M-VSG gene expression telomere (t8), hybridisation was weak compared to that detected for clone r10 suggesting that *ble* gene expression was low. In other studies where the PARP promoter directs transcription of an antibiotic resistance gene at a telomere, promoter activity was also found to be low in bloodstream trypanosomes (Biebinger *et al.*, 1996). However, in clone r10 where pr1.22BC is integrated to the untranscribed spacer region, *ble* transcripts are very abundant. This may be due to transcription initiated from the PARP promoter or transcriptional read-through from the upstream transcription of the CAT gene cassette initiated at the 1.22 M-VSG gene promoter region. Since CAT gene expression is high in clone r10, the 1.22 M-VSG gene promoter region must be highly active and by analogy with the results of Berberof *et al.* (1995), who showed that transcriptional read-through from an upstream promoter can inactivate a downstream promoter, the PARP promoter may be inactivated if there is significant level of transcriptional read-through from the 1.22 M-VSG gene promoter region.

To verify that each lane had been equally loaded with RNA, the same blot was stripped and reprobed with a ^{32}P-labelled DNA probe homologous to the trypanosome actin gene (from the plasmid pActine; Amar *et al.*, 1988). Hybridisation to actin mRNA was approximately equal in tracks 1, 2 and 3 that each contain approximately 5μg of total RNA from bloodstream trypanosomes (respectively wild-type, clone t8 and clone r10 total RNA; Fig. 5.2.6.1 C.).

Finally, the same blot was stripped and reprobed with the 5' *Hind*III fragment of pTcV7.1-14, a plasmid containing the cDNA of the 1.22 M-VSG gene (Cornelissen *et al.*, 1985; Graham *et al.*, 1990), to determine if any of the bloodstream trypanosomes were expressing the 1.22 M-VSG gene mRNA. The 1.22 M-VSG gene is not usually expressed in bloodstream trypanosomes because the 1.22 M-VSG gene promoter that directs transcription is inactive at this life cycle stage (Graham and Barry, 1995). However, the 1.22 M-VSG gene may be expressed in bloodstream trypanosomes following duplication and transposition of the gene to an active bloodstream VSG gene expression site (Graham *et al.*, 1990; Matthews *et al.*, 1990). Detection of 1.22 M-VSG gene transcripts in clone t8 would indicate either that the endogenous 1.22 M-VSG gene promoter was active, the 1.22 M-VSG gene was inserted in an active bloodstream expression site, or there was read-through transcription of the VSG gene from the

Figure 5.2.6.2 **RNA of transformed trypanosomes probed with the coding region of the 1.22 M-VSG gene**. The same Northern blot used in Fig. 5.2.6.1. was stripped and reprobed with a probe that contains the 5' end of the cDNA of the 1.22 M-VSG gene. The RNA loaded in each track was from wild-type trypanosomes (track 1), clone t8 transformed with pt1.22BC (track 2), and clone r10 transformed with pt1.22BC (track 3). In addition, there was also RNA from bloodstream trypanosomes that express the 1.22 M-VSG gene following duplication to a bloodstream expression site (track 4).

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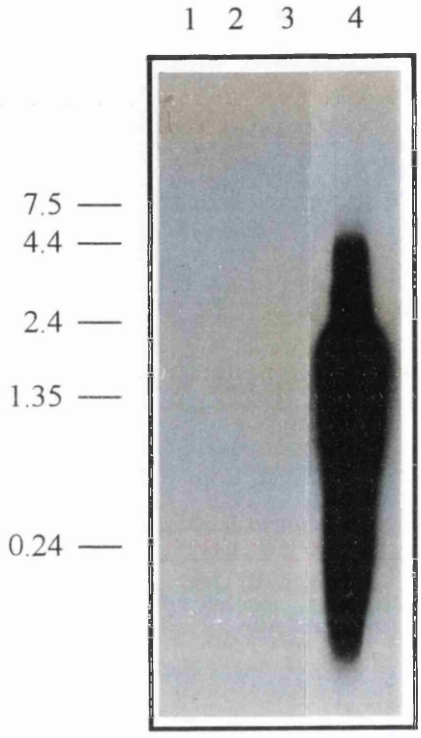
1.22 M-VSG gene



inserted PARP promoter. Included in the hybridisation was a track from a separate Northern blot that contains 1μg of total RNA from bloodstream trypanosomes that express the 1.22 M-VSG gene after duplication to a bloodstream expression site, and acts as a positive control for the hybridisation (track 4). Total RNA in track 4 hybridised very strongly with the cDNA probe whilst no hybridisation could be detected in tracks containing total RNA from wild-type, clone r10 or clone t8 bloodstream trypanosomes (tracks 1, 2 and 3 respectively, Fig. 5.2.6.2). Therefore, wild-type and transformed bloodstream trypanosomes in axenic culture do not express transcripts of the 1.22 M-VSG gene. This is in agreement with the data from indirect immunofluorescence of the VSG surface coat (see section 4.2.4) which showed that a monoclonal antibody that reacts against the 1.22 M-VSG did not react against trypanosomes in the axenic bloodstream culture. In conclusion, the absence of detectable 1.22 M-VSG gene transcripts suggests that the endogenous 1.22 M-VSG gene promoter is inactive in both the wild-type and transformed bloodstream trypanosomes, as expected, and there is no transcriptional read-through of the metacyclic VSG gene from the PARP promoter in clone t8.

The insertion of the 7 kb plasmid (pt1.22BC) to the 1.22 M-VSG gene expression telomere apparently does not affect the transcriptional inactivity of the endogenous 1.22 M-VSG gene promoter. Therefore, the mechanism that controls down-regulation of transcription initiation from the 1.22 M-VSG gene promoter at the bloodstream stage is not sensitive to large sequence insertions upstream of the promoter region or specific spacing between the promoter and elements upstream in the expression telomere. The same mechanism that represses the transcriptional activity of the endogenous 1.22 M-VSG gene promoter in bloodstream trypanosomes may also prevents transcription initiation from the integrated copy of the promoter, that lies approximately 7 kb upstream.

5.2.7 Nuclear run-on analysis of clone r10

Though transcription of the ribosomal RNA gene untranscribed spacer region is not thought to occur in trypanosomes (White *et al.*, 1986; Mike Cross, personal communication), it remained possible that transcription of the CAT gene in pr1.22BC did not initiate at the 1.22 M-VSG gene promoter region when integrated at the untranscribed spacer region. Instead, fortuitous transcription initiation from the upstream ribosomal promoter might have occurred on the opposite strand and in the

opposite orientation to transcription of the ribosomal RNA genes, resulting in high levels of transcription of the CAT and *ble* genes in clone r10. Therefore, transcription of the 1.22 M-VSG gene promoter region of plasmid pr1.22BC was analysed in clone r10 by nuclear run-on analysis.

Plasmid pMT1.22HPl contains the 1.75 kb *Hind*III-*Pst*I fragment of the 1.22 M-VSG gene promoter region and includes the entire 1.6 kb *Kpn*I-*Pst*I fragment that directs transcription of the CAT gene in pt1.22BC and pr1.22BC (Fig. 5.2.2.1 and Fig. 5.2.4.1). pMT1.22HPl was digested with *Xba*I and *Hind*III to give three bands when separated on an agarose gel that contain the pBluescript sequence (2.9 kb), a 700 bp fragment including the putative 1.22 M-VSG gene promoter and a 1 kb fragment directly upstream of the putative promoter at the metacyclic expression telomere (Fig. 5.2.7.1). Also included on the agarose gel were plasmid pR4 containing a copy of the ribosomal RNA gene repeat unit digested with *Pst*I and plasmid pTBαβ-T1 containing a repeat of the tubulin gene array digested with *Hind*III. Two identical agarose gels were run and blotted onto nylon membranes. ^{32}P-labelled nascent RNA transcripts were run-on *in vitro* in nuclei isolated from either clone r10 or wild-type trypanosomes and hybridised to Southern blots containing the tubulin gene control (track 1), ribosomal RNA gene control (track 2) and 1.22 M-VSG gene promoter region sequence (track 3). Nascent transcripts from wild-type trypanosomes hybridised to both the tubulin gene and the ribosomal RNA gene controls (tracks 1 and 2 respectively), but there was no hybridisation to the 1.22 M-VSG gene promoter region sequences (track 3). Nascent transcripts from clone r10 also hybridised to both the tubulin gene and ribosomal RNA gene controls (tracks 1 and 2 respectively). In addition, there was hybridisation to the 700 bp fragment (fragment 2, track 3) containing the putative 1.22 M-VSG gene promoter but no detectable hybridisation to the 1.1 kb fragment which is upstream of the putative promoter region (fragment 1, track 3). This shows that transcripts are initiated within the 700 bp fragment as predicted (probably close to 1.22 M-VSG gene putative promoter, see section 3.3.3) and there is no detectable transcriptional read-through of the integrated plasmid pr1.22BC from sequences outwith the inserted promoter sequence. Nascent transcripts from clone r10 also hybridised strongly to the pBluescript sequence (pBS fragment, track 3) suggesting this sequence of the inserted plasmid pr1.22BC was highly transcribed due to transcription read-through from the PARP gene or 1.22 M-VSG gene promoters.

Figure 5.2.7.1 **Nuclear run-on analysis of transcription in clone r10 transformed with pr1.22BC**. (A) Ethidium bromide stained agarose gel (EtBr) showing *Pst*I digested plasmid pR4 which contains a repeat at the ribosomal RNA gene locus (track 1), *Hind*III digested plasmid pTBαβ-T1 which contains a repeat of the tubulin gene array (track 2) and *Xba*I/*Hind*III digested plasmid pMT1.22HPl shown in B. pBS indicates pBluescript sequence, fragments marked 1 and 2 are shown in B. Clone r10 shows a Southern blot of the ethidium bromide stained gel probed with ^{32}P labelled nascent RNA transcripts run-on in nuclei prepared from clone r10 trypanosomes transformed with pr1.22BC. pBS and 2 indicate the restriction fragments that are hybridised by the probe. WT shows a duplicate Southern blot prepared from an identical agarose gel which is probed with ^{32}P labelled nascent RNA transcripts run-on in nuclei prepared from wild-type trypanosomes. (B) Diagram showing the plasmid pMT1.22HPl with restriction sites of *Xba*I (X) and *Hind*III (H) used to digest the plasmid in the nuclear run-on analysis. 1 and 2 indicate the two restriction fragments of 1 kb and 700 bp that are shown in A. (C) Diagram showing the 1.22 M-VSG gene promoter region (*Kpn*I-*Pst*I 1.6 kb fragment) that is contained in the plasmid pr1.22BC and the inserted position of the promoter at the ribosomal RNA gene repeat untranscribed spacer in clone r10.

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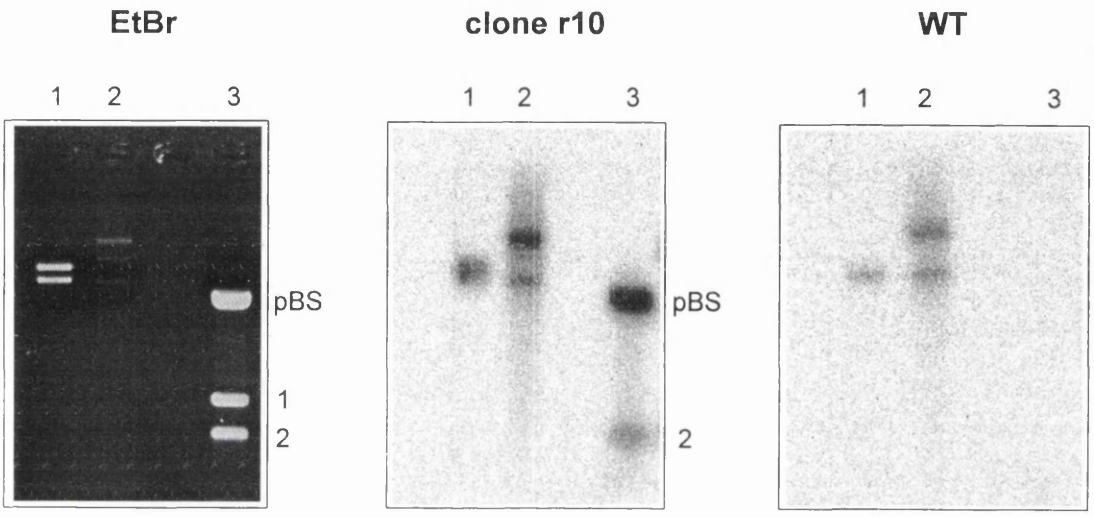
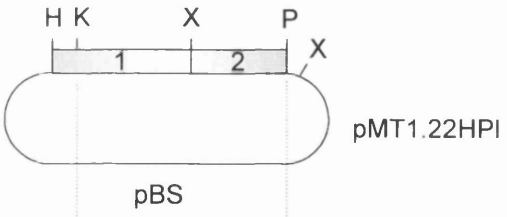
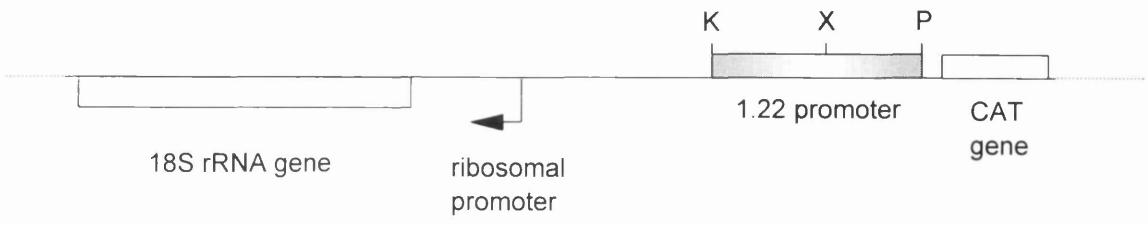
A.


EtBr
clone r10
WT
1 2 3
1 2 3
1 2 3
pBS
1
2
pBS
2
1 2 3

B.


H K
X
P
X
1
2
pMT1.22HPI
pBS

C.


K
X
P
1.22 promoter
CAT
gene
18S rRNA gene
ribosomal
promoter

5.3 DISCUSSION

There are several reasons why the use of the plasmids ptl.22BC and prl.22BC is not ideal in the stable transformation experiments I have carried out to examine the transcriptional activity of the 1.22 M-VSG gene promoter region in the genome of bloodstream trypanosomes. 1) The PARP gene 3' UTR appears to play a role in down-regulating the expression of the PARP gene in bloodstream trypanosomes (Hug *et al.*, 1993; Berberof *et al.*, 1995): it reduces reporter gene expression in transient transfections of bloodstream trypanosomes when compared directly with plasmids containing the actin gene 3' UTR (Hug *et al.*, 1993). It is reasonable to assume that a similar effect would be observed in stably transformed bloodstream trypanosomes. 2) The ble gene is under control of the PARP B locus promoter and, although this promoter is constitutively active during the life cycle it is down-regulated 5-10 fold at the bloodstream stage when assayed by transient transfection (Pays *et al.*, 1990; Vanhamme *et al.*, 1995a) or by stable transformation (Biebinger *et al.*, 1996). The combined effect of low transcription and down-regulation of gene expression exerted by the PARP 3' UTR means that expression of the *ble* gene is low as I have observed. 3) In ptl.22BC and prl.22BC the juxtaposition of the reporter gene cassette and the selectable marker gene cassette, each containing a trypanosome Pol I promoter, is not ideal since there is a possibility that the two promoters may interact. It has been shown in procyclics that the bloodstream VSG gene expression site promoter, which is minimally active at the procyclic stage, can be activated by a fragment of the PARP promoter located immediately upstream (Urmenyi and Van der Ploeg, 1995; Qi *et al.*, 1996). Since the copy of the PARP promoter in ptl.22BC and prl.22BC includes this putative activating sequence, the 1.22 M-VSG gene promoter may be activated by the downstream PARP promoter. However, the PARP promoter is not an efficient promoter at the bloodstream stage (Vanhamme *et al.*, 1995a; Biebinger *et al.*, 1996) and is located more than 1500 bp away from the 1.22 M-VSG gene promoter region making interaction between these promoters unlikely. Moreover, the results of Qi *et al.* (1996) suggest that the PARP promoter exerts this effect on a promoter downstream and, in my integration events the PARP promoter is itself downstream of the 1.22 M-VSG gene promoter region. To improve upon the experiments in this chapter, it may be necessary to carry out co-transfection experiments, using separate plasmids containing the reporter gene cassette and the selectable marker gene cassette, and targeting each of the plasmids to different positions in the genome, e.g. the selectable marker gene cassette to the tubulin locus and

the reporter gene cassette to the 1.22 M-VSG gene expression telomere. However, co-transformation of trypanosomes has so far proved to be technically difficult to achieve (Mike Cross, personal communication). In addition, the selectable marker gene and reporter gene could be flanked by the actin gene RNA processing signals, rather than the PARP gene RNA processing signals, which may increase the level of their expression in bloodstream trypanosomes and perhaps allow direct comparison between bloodstream and procyclic transformants: the actin gene RNA processing signals are not thought to regulate reporter gene expression in the trypanosome life cycle (Amar *et al.*, 1988; Hug *et al.*, 1993).

Despite the limitations of the plasmid constructs used, there were significant results achieved in these stable transformation experiments. When a single copy of pr1.22BC, a plasmid containing the CAT reporter gene, was integrated into the genome of bloodstream trypanosomes at the untranscribed spacer of a ribosomal RNA gene transcription unit, CAT gene expression was high and CAT gene steady-state transcripts could be detected readily. Nuclear run-on analysis showed that transcription of the CAT gene initiates within the putative 1.22 M-VSG gene promoter region upstream of the CAT gene in the reporter plasmid. When a single copy of a similar reporter plasmid, pt1.22BC, was integrated to the 1.22 M-VSG gene expression telomere, also transcriptionally silent in bloodstream trypanosomes, expression of CAT was very low and CAT gene steady-state transcripts could not be detected. This suggests that transcription initiation from the 1.22 M-VSG gene promoter region is down-regulated at the metacyclic expression telomere in bloodstream trypanosomes.

5.3.1 Transcriptional activity of the putative 1.22 M-VSG gene promoter at the ribosomal RNA gene untranscribed spacer

The results from integration of pr1.22BC to the ribosomal RNA gene untranscribed spacer in bloodstream trypanosomes showed there was a high level of transcriptional activity from the 1.22 M-VSG gene promoter at this position in the genome. Analysis of nascent RNA transcripts in the trypanosome clones containing a single plasmid insertion at an untranscribed spacer, showed that transcription of the CAT gene initiates within the 1.22 M-VSG gene promoter region. The untranscribed spacer, between tandemly repeated ribosomal RNA gene transcription units, was selected for integration of the reporter construct because no transcription could be detected, on either strand, by nuclear run-on analysis between the termination site of the upstream

ribosomal RNA gene transcription unit and the promoter of the downstream transcription unit (White *et al.*, 1986; Mike Cross, personal communication). Even a low level of transcriptional read-through from the upstream ribosomal RNA gene transcription unit, could not account for CAT gene expression observed in my experiments, as the reporter construct is integrated to the untranscribed spacer in the reverse orientation to transcription of the ribosomal RNA genes and therefore, such transcriptional read-through would only result in non-coding CAT gene transcripts. It is possible that the ribosomal promoter downstream of the integration event could initiate transcription in both directions, on both coding and non-coding strands and thus directing CAT gene expression (see Fig. 5.2.4.1). However, this would have to be below levels of detection by nuclear run-on experiments as transcription was not observed in nascent transcript analysis of the untranscribed spacer region (White *et al.*, 1986; Mike Cross, personal communication). Nuclear run-on analysis in this chapter clearly indicates that transcription initiates within the vicinity of the 1.22 M-VSG gene promoter region and there is no detectable transcription of the 1 kb fragment that lies between the 1.22 M-VSG gene promoter region and the ribosomal promoter (Fig. 5.2.7.1). Furthermore, the trypanosome ribosomal RNA gene promoter has been tested in the reverse orientation in transient transfection of procyclic trypanosomes and resulted in only background levels of reporter gene expression, indicating that transcription only proceeds in the forward direction from the promoter (Zomerdijk *et al.*, 1991c).

The high level of CAT gene transcripts and CAT expression in bloodstream trypanosomes transformed with pr1.22BC, can therefore most probably be attributed to transcription initiation from the 1.22 M-VSG gene promoter region. These data are consistent with the results presented in Chapter 3, where the 1.22 M-VSG gene promoter region was able to direct high levels of CAT gene transcription in transient transfection of bloodstream trypanosomes when located outwith its normal chromosomal position, on an episomal vector. Together, the results of transient transfection and stable integration at the ribosomal locus of bloodstream trypanosomes suggests the 1.22 M-VSG gene promoter region can act as a functional promoter in bloodstream trypanosomes. Unfortunately, it is not possible to directly compare the activity of the 1.22 M-VSG gene promoter region in the stable transformation experiments of this chapter with the transient transfection analysis of bloodstream trypanosomes in Chapter 3. This is because in stable transformation experiments, the number of copies of a reporter construct in each trypanosome genome can be determined by Southern analysis,

whilst in transient transfection the transfection efficiency is low and variable, and the number of plasmid copies present in each transfected trypanosome cannot be calculated. Therefore, I cannot say whether the level of transcription from the 1.22 M-VSG gene promoter region at the untranscribed spacer is equivalent to the level of transcription in transient transfection of bloodstream trypanosomes.

I have argued that transcription of the CAT gene in pr1.22BC at the ribosomal RNA gene untranscribed spacer is initiated within the 1.22 M-VSG gene promoter region, and CAT gene expression reflects the rate of transcription initiation from this putative promoter. However, the ribosomal RNA gene untranscribed spacer may not be a neutral region of the trypanosome genome for the analysis of transcription. In other organisms such as yeast (Johnson and Warner, 1989), rat (Dixit *et al.*, 1987), *Xenopus* (Moss, 1983) and *Drosophila* (Jacob, 1995) the untranscribed spacer contains enhancer elements that increase the basal rate of transcription initiation from the ribosomal promoter (Jacob, 1995) and it is believed that in some cases the transcriptional terminator may also act to enhance the rate of transcription from the downstream ribosomal promoter (Baker and Platt, 1986). These enhancers can operate at considerable distances (several kilobases) from the promoter and usually in a orientation independent fashion (Jacob, 1995). They can also increase transcription from promoters other than the ribosomal promoter that direct transcription by RNA polymerase II rather than RNA polymerase I (Ghosh *et al.*, 1994). Such enhancer elements at the untranscribed spacer have not been reported some organisms (Jacob, 1995) and may or may not exist in trypanosomes; the trypanosome ribosomal spacer region awaits more extensive study. Therefore, it is possible that elements necessary to modulate transcription from the ribosomal RNA gene promoter in trypanosomes, may also affect transcription from other promoters inserted to the untranscribed spacer. Specifically, they may increase the rate of transcription initiation from the 1.22 M-VSG gene promoter region and thereby result in an increase in CAT gene expression.

The localisation of the reporter plasmid in the nucleus of bloodstream trypanosomes, may also significantly affect the rate of transcription initiation from the 1.22 M-VSG gene promoter region. In *Trypanosoma brucei*, metacyclic VSG genes are transcribed in an α-amanitin-insensitive manner as are bloodstream VSG gene expression sites and the PARP genes; insensitivity to this drug usually indicates transcription by RNA polymerase I. Additional evidence based on the PARP and bloodstream expression site promoter structure (Janz and Clayton, 1994; Vanhamme *et al.*, 1995b) and the

ability of the ribosomal promoter to direct transcription and expression of protein-coding genes in trypanosomes (Zomerdijk *et al.*, 1991a; 1991c) as well as the entire bloodstream VSG gene expression site (Rudenko *et al.*, 1995), indicates that RNA polymerase I transcribes the VSG genes and the PARP genes. This is contrary to what happens in all other eukaryotic organisms where protein-coding genes are transcribed almost exclusively by RNA polymerase II (Jove and Manley, 1982). RNA polymerase I activity is thought to be limited to a non-membranous nuclear structure, the nucleolus, where the ribosomal RNA gene arrays are transcribed, processed and complexed with proteins to form pre-ribosomes (Shaw *et al.*, 1995). Ribosomal RNA genes are transcribed in the nucleolus by RNA polymerase I at the periphery of the fibrillar centres (Hozak *et al.*, 1994) and in this region of the nucleolus there is a high concentration of active RNA polymerase I complexes, basal transcription factors and transactivators (Scheer and Benavente, 1990). Therefore, by positioning the reporter construct between highly transcribed ribosomal RNA gene transcription units, the integrated reporter plasmid is likely be located within the nucleolus and in close proximity to RNA polymerase I transcriptional machinery. If the 1.22 M-VSG gene promoter does direct initiation of RNA polymerase I complexes, then localisation of the reporter plasmid at the nucleolus may lead to increased levels of transcription initiation from the putative metacyclic VSG gene promoter and thereby an increased level of CAT gene transcription.

It has not yet been possible to determine the site of transcription of the PARP and VSG genes in the trypanosome nucleus, but some experiments using fluorescence *in situ* hybridisation (FISH) techniques have indicated, surprisingly, that protein-coding genes may be transcribed at the nucleolus in transformed procyclic trypanosomes (Chung *et al.*, 1992). When under the transcriptional control of the ribosomal promoter, and integrated to either the ribosomal spacer region between ribosomal RNA gene transcription units (Rudenko *et al.*, 1991), or to a position upstream of the PARP gene locus (Chung *et al.*, 1992), transcripts of the neomycin phosphotransferase (*neo*) gene were co-localised with transcripts of the ribosomal RNA genes, i.e. at the nucleolus. In addition, transcription of the *neo* gene can be localised to the nucleolus in procyclic trypanosomes when the gene is transcribed from the PARP promoter and integrated at the tubulin locus (Chung *et al.*, 1992). Conversely, neo transcripts could not be detected at the nucleolus if transcription was directed by an RNA polymerase II promoter such as the tubulin or calmodulin promoter (Rudenko *et al.*, 1991). In contrast, attempts to localise the transcripts of the 221 bloodstream VSG gene expression site in the nucleus of

bloodstream trypanosomes that express the 221 VSG gene, indicated that these genes were transcribed outside the nucleolus (Zomerdijk *et al.*, 1991d). The experiments of Zomerdijk *et al.* (1991d) were, however, complicated by the use of a very long DNA probe of ~50 kb that included much or the 221 bloodstream VSG gene expression site and therefore included expression site associated genes that are multicopy in the trypanosome genome and may be transcribed from sites other than the 221 bloodstream VSG gene expression site. The analysis of bloodstream and procyclic trypanosomes by fluorescence *in situ* hybridisation has not been able to unequivocally establish the nuclear site of transcription of the PARP and VSG genes. Nevertheless, it remains a distinct possibility that trypanosome protein-coding genes, transcribed in an α-amanitin-insensitive manner (including the metacyclic VSG genes at the metacyclic stage), are transcribed at the nucleolus. If this is the case then the location of the 1.22 M-VSG gene promoter region between ribosomal RNA gene transcription units, and therefore at the nucleolus, may increase levels of transcription initiation from the putative promoter to those that may be expected at the metacyclic stage.

The variability of CAT gene expression between clones transformed with pr1.22BC, and previous observations of variability in CAT gene expression for separate clones transformed with other reporter constructs integrated to the untranscribed spacer of bloodstream trypanosomes (Biebinger *et al.*, 1996), indicates that integration to different positions in the trypanosome ribosomal RNA gene array(s) may influence reporter gene transcription. In yeast only about half of the ribosomal RNA gene repeats are transcriptionally active and the distribution of active transcription units along the tandem array seems to be random (Dammann *et al.*, 1995). Differences in the transcriptional activity of different ribosomal RNA gene units may be due to chromosomal confirmation changes such as the local density of nucleosomal arrays. If differential transcription of the ribosomal RNA gene tandem repeats does occur in trypanosomes, then integration of pr1.22BC to an untranscribed spacer adjacent to an active or inactive transcription unit may increase or reduce reporter gene expression. However, variability in CAT activity between clones did not occur when reporter constructs were integrated to the untranscribed spacer in procyclic trypanosomes (Biebinger *et al.*, 1996). This implies that variability in promoter activity is not due to location at the untranscribed spacer but may be intrinsic to bloodstream trypanosomes which can also exhibit variable promoter activity in transient transfection (Biebinger *et al.*, 1996; see section 3.2.3). Significantly, integration of a single copy of pr1.22BC to

the untranscribed spacer in procyclic trypanosomes (stock EATRO 795) gave only background levels of CAT enzyme activity (S. V. Graham, unpublished results). The 1.22 M-VSG gene promoter was also only minimally active in transient transfection of procyclic trypanosomes (Graham and Barry, 1995) and therefore simply positioning pr1.22BC at the untranscribed spacer does not result in an increase or activation of transcription from the 1.22 M-VSG gene promoter at the procyclic stage.

At present it is difficult to test for an increased rate of transcription initiation from the 1.22 M-VSG gene promoter at the untranscribed spacer, as there are only a limited numbers of transcriptionally silent regions of the trypanosome genome that may be used for targeting reporter constructs. Of these transcriptionally silent sites, the majority are located at positions upstream or within expression sites that transcribe the bloodstream or metacyclic VSG genes and may be subject to the transcriptional silencing effects observed in both procyclic and bloodstream trypanosomes (Rudenko *et al.*, 1994; 1995; Horn and Cross, 1995; this chapter). Run-on assays have indicated that regions upstream and between the mini-exon spliced leader genes are transcriptionally silent and may prove to be ideal positions for transcriptional analysis of trypanosome promoters (C. Clayton, personal communication).

5.3.2 The 1.22 M-VSG gene promoter is repressed at the metacyclic expression telomere in bloodstream trypanosomes

When assayed on an extra-chromosomal reporter plasmid in transient transfection of bloodstream trypanosomes, the 1.22 M-VSG gene promoter region is highly active (section 3.2.5). Integration of a reporter plasmid (pr1.22BC) to the ribosomal RNA gene untranscribed spacer shows that the 1.22 M-VSG gene promoter region can still direct a high level of CAT gene expression at a chromosome-internal position in bloodstream trypanosomes. However, when a similar reporter plasmid (pt1.22BC) is integrated to the 1.22 M-VSG gene expression telomere, the 1.22 M-VSG gene promoter region directs only low levels of CAT gene expression in bloodstream trypanosomes. Plasmids pt1.22BC and pr1.22BC are identical except that pr1.22BC contains an extra 700 bp of sequence upstream of the 1.22 M-VSG gene promoter region that is necessary for integration to the ribosomal RNA gene untranscribed spacer. In pr1.22BC and pt1.22BC the CAT gene is flanked by the PARP gene RNA processing signals which direct *trans*-splicing and polyadenylation of the nascent transcript to generate mature mRNAs. RNA processing of the CAT gene nascent transcripts should

proceed in the same manner and at the same rate in cloned trypanosome lines transformed with each plasmid and therefore any difference in CAT gene expression is probably due to differential levels of CAT gene transcription in the transformed bloodstream trypanosome clones. The only alternative explanation could be that the stability, processing or transport of nascent CAT gene transcripts is different in the nuclei of trypanosomes transformed with prl.22BC and ptl.22BC. This may be true if the integrated copies of prl.22BC and ptl.22BC are located at different regions of the nucleus. prl.22BC is integrated between ribosomal RNA gene transcription units and is probably located at the nucleolus (see previous section), whereas ptl.22BC is integrated to the 1.22 M-VSG gene expression telomere, the nuclear location of which is unknown. However, it seems unlikely that the stability, rate of processing or rate of transport of protein-coding gene nascent transcripts is greater at nucleolus than other regions of the trypanosome nucleus given that this subnuclear compartment is specialised for ribosomal RNA gene transcription, processing and transport.

I have argued that the differential level of CAT enzyme activity and CAT gene transcripts in bloodstream trypanosome clones transformed with prl.22BC and ptl.22BC is likely to be due to different levels of transcription initiation from the 1.22 M-VSG gene promoter at the ribosomal RNA gene locus and the 1.22 M-VSG gene expression telomere. I now propose that the reduced level of transcriptional initiation from the 1.22 M-VSG gene promoter region at the 1.22 M-VSG gene expression telomere in bloodstream trypanosomes, with respect to the 1.22 M-VSG gene promoter at the ribosomal RNA gene array, is due to transcriptional repression of the promoter at the 1.22 M-VSG gene expression telomere at the bloodstream stage.

5.3.3 A potential mechanism of transcriptional control of metacyclic expression telomeres

The positioning of M-VSG gene transcription units at the telomeres in trypanosomes could relate the control of metacyclic expression telomeres to a particular mode of reversible transcriptional repression at the telomeres in yeast and in *Drosophila* known as telomere position effect (Tartof, 1994). Positioning of a gene close to the telomere in *Drosophila* and in yeast can result in transcriptional repression of the gene in a promoter non-specific manner (Hazelrigg *et al.*, 1984; Gottschling *et al.*, 1990). The repressive effect is mitotically heritable through many generations but repression can be reversed in individual cells in a cell-cycle dependent manner (Aparicio and Gottschling,

1994). Analysis of mutants in *Drosophila* and in yeast that suppress position effect variegation, shows that there are many genes that are common to transcriptional repression at the telomere and at some chromosome-internal positions (Aparicio *et al.*, 1991; Wallrath and Elgin, 1995). However, the same studies show that transcriptional repression at the telomere involves some novel genes not associated with repressive chromatin domains at chromosome-internal positions. This suggests the mechanism for repression at the telomere is similar but distinct from repression at chromosome-internal positions. Most of our understanding of telomere position effect comes from the integration of chromosome-internal transcription units of *Saccharomyces cerevisiae* such as the URA3 gene, to a telomeric position at the left arm of chromosome VII and studying the effects of specific gene mutations, distance from the telomere and telomere structure on repression of transcription. In the yeast system, when the URA3 gene and its promoter were moved from a position 1 kb from the telomere to positions 2-6 kb from the telomere, transcriptional repression was reduced with increasing distance from the telomere (Renauld *et al.*, 1993). The positioning of an active transcription unit between the URA3 gene and the telomere resulted in disruption of repression of the URA3 gene which suggests that the repressive state is constructed in a continuous manner from the telomere (Renauld *et al.*, 1993). The distance from the telomere over which repression was effective could be increased by reducing the strength of the URA3 promoter or by increasing the dosage of SIR3 in the cell (Renauld *et al.*, 1993). SIR3 is a protein known to be involved in the association of nucleosomes to form compacted chromatin that silences transcription at chromosome-internal positions in yeast (Aparicio *et al.*, 1991). Repression of genes adjacent to telomeres was not solely dependent of the presence of telomere repeats as chromosome-internal tracts of telomere repeats failed to repress transcription and only did so when at a chromosome end (Gottschling *et al.*, 1990). Therefore, the presence of both a chromosome end and the telomere repeats was required a potential organising centre for telomeric silencing. Hecht *et al.* (1995) proposed a model for telomeric silencing based on the interaction of SIR3 and SIR4 *in vitro* with histones H3 and H4 (Hecht *et al.*, 1995) and mutations of the SIR genes and histones H3 or H4 which suppress repression at the telomere and at chromosomal-internal positions (Aparicio *et al.*, 1991). The model involved binding of the chromosome end and telomere repeats by structural proteins such as RAP1 (Conrad *et al.*, 1990) which induce the association of nucleosomes with structural proteins such as SIR3 and SIR4 to form transcriptionally silent heterochromatin-like domains. Packaging

of nucleosomes proceeds in a continuous fashion from the telomere towards the centromere until a termination signal is reached or one of the protein factors becomes limiting.

In wild-type yeast cells, genes that are known to be affected by an adjacent telomere are limited to those of the HMR mating-type locus (Thompson *et al.*, 1994). In *Trypanosoma brucei* there are up to 27 metacyclic VSG genes (Turner *et al.*, 1988) each gene probably located at a separate telomere (Barry *et al.*, 1990). In the case of the 1.22 M-VSG expression telomere the metacyclic VSG gene is located within 8 kb of the telomere repeats (S. V. Graham, personal communication). Metacyclic VSG genes are expressed at the bloodstream stage only by duplication of the gene and transposition probably to a bloodstream VSG gene expression site (Cornelissen *et al.*, 1985; Graham *et al.*, 1990; Matthews *et al.*, 1990) possibly because metacyclic VSG genes cannot be transcribed at their metacyclic expression telomeres at the bloodstream stage (Graham and Barry, 1995). The telomeric metacyclic VSG genes may be repressed at the bloodstream stage by a mechanism similar to that of repression of telomeric genes in yeast and *Drosophila*. If this mechanism is similar to that in yeast then transcriptional repression at the metacyclic expression telomeres in bloodstream trypanosomes would be promoter non-specific (Gottschling *et al.*, 1990). To date, other trypanosome promoters that are active at the bloodstream stage have not been tested at the 1.22 M-VSG gene expression telomere to determine whether transcriptional repression is promoter non-specific. A telomere position effect would also imply that transcriptional repression would be reduced if the 1.22 M-VSG gene promoter region was moved away from the telomere repeats (Renauld *et al.*, 1993).. In the bloodstream trypanosome clones that contain 3 copies of the plasmid pt1.22BC in tandem at the 1.22 M-VSG gene expression telomere (clones tA, tB and tC), there are copies of the 1.22 M-VSG gene promoter region at ~7 Kb, ~14 kb and ~21 kb upstream of the endogenous 1.22 M-VSG gene promoter region (see Fig. 5.2.2.2). A bloodstream clone with a triple insertion of pt1.22BC at the 1.22 M-VSG gene expression telomere expressed CAT at a high level that was equivalent to a bloodstream clone containing a single copy of pr1.22BC located at the ribosomal spacer region. CAT gene transcription was probably directed by the 1.22 M-VSG gene promoter region but it was not possible to determine which of the promoter regions were active (see section 5.2.5). The high level of CAT gene expression in this transformed trypanosome line may be due to an increased level of transcription initiation from copies of the 1.22 M-VSG gene promoter region that are

further from the telomere. Interestingly, recent experiments have identified an RNA polymerase II transcription unit upstream of the 1.22 M-VSG gene (S. V. Graham; K. Kobryn, unpublished results). This ESAG 1-related gene is transcribed at the bloodstream, procyclic and metacyclic stages and transcription initiates in a reverse orientation to the 1.22 M-VSG gene approximately 17 kb upstream of the 1.22 M-VSG gene promoter region. Therefore, the promoter that directs transcription of the EASG 1-related gene is not controlled in the trypanosome life cycle in the same manner as the 1.22 M-VSG gene promoter, located much closer to the telomere. Similarly, upstream copies of the 1.22 M-VSG gene promoter region, in trypanosomes containing 3 tandem copies of pt1.22BC at the 1.22 M-VSG gene expression telomere, may escape the transcriptional repression of 1.22 M-VSG gene promoter copies closer to the telomere. To determine whether the activity of the 1.22 M-VSG gene promoter region in bloodstream trypanosomes is dependent on its distance from the telomere repeats, single copies of a reporter construct would have to be integrated to different positions at the 1.22 M-VSG gene expression telomere.

Chapter 6

Concluding Discussion

The results of transient transfection of bloodstream trypanosomes suggested that the putative promoter region for the 1.22 M-VSG gene is highly active at the bloodstream stage when assayed on an extrachromosomal plasmid (see section 3.2.5). However, deletion analysis of the 1.22 M-VSG gene promoter region in transient transfection of bloodstream trypanosomes, showed that the transcriptional start site determined in metacyclic-derived trypanosomes and 70 bp upstream could be removed in the 3' to 5' direction without significantly affecting the level of reporter gene expression (see section 3.2.8). In these deleted promoter constructs, transcription must have initiated upstream of the transcriptional start site used in metacyclic-derived trypanosomes and may be signalled by a cryptic promoter just upstream of the 1.22 M-VSG gene promoter itself.

Stable transformation experiments (Chapter 5) showed that the 1.22 M-VSG gene promoter region was also highly active when positioned at a chromosome-internal position in the genome of bloodstream trypanosomes. However, at the 1.22 M-VSG gene expression telomere, the same promoter region was minimally active in bloodstream trypanosomes. I proposed that the 1.22 M-VSG gene promoter region was repressed at the 1.22 M-VSG gene expression telomere in bloodstream trypanosomes and that this effect may be related to telomere position effects similar to those observed in yeast (see section 5.3.3). Repression of transcription at the metacyclic VSG gene expression telomeres may prevent activation of metacyclic VSG genes *in situ* at the bloodstream stage and ensures expression of VSG genes exclusively from bloodstream VSG gene expression sites.

6.1 Control of the bloodstream VSG gene expression sites

Like metacyclic VSG gene transcription units, bloodstream VSG gene expression sites are also located adjacent to the telomeres of some trypanosome chromosomes and their control of expression at the bloodstream stage has been proposed to be related to telomere position effect in yeast (Horn and Cross, 1995; Rudenko *et al.*, 1995). There are up to 20 bloodstream VSG gene expression sites (Cully *et al.*, 1985; Navarro and Cross, 1996) only one of which is transcribed at the bloodstream stage (Cross, 1996). The bloodstream expression site promoters that have been identified are almost identical in sequence (Zomerdijk *et al.*, 1990; Gottesdiener *et al.*, 1991; Jefferies *et al.*, 1991) and control of expression site transcription in bloodstream trypanosomes seems to be primarily at the level of transcription initiation (Rudenko *et al.*, 1995). This is in contrast

to the control of expression site transcription at the procyclic stage, where no expression sites are transcribed due to a combination of reduced transcription initiation from the expression site promoters and attenuation of transcript elongation (Pays *et al.*, 1990; Rudenko *et al.*, 1995; see section 1.10). In bloodstream trypanosomes, the expression site promoter of an active expression site may be directly replaced by the ribosomal promoter which in turn is capable of transcribing the entire expression site (Rudenko *et al.*, 1995). The ribosomal promoter may also be controlled in the same manner as the expression site promoter in that the expression site may be silenced and the ribosomal promoter repressed and another expression site switched on (Rudenko *et al.*, 1995). Firstly, this shows the expression site promoter and the ribosomal promoter are interchangeable and therefore further suggests that the expression sites are transcribed by RNA polymerase I, and secondly, the repression of transcription initiation at the expression sites is not restricted to the expression site promoters in bloodstream trypanosomes. Similar results were achieved when trypanosome promoters were positioned just upstream of the 221 VSG gene at the 221 bloodstream VSG gene expression site, i.e. ~50 kb downstream of the endogenous expression site promoter (Horn and Cross, 1995). When the 221 bloodstream expression site was actively transcribed in bloodstream trypanosomes then the ribosomal promoter, just upstream of the 221 VSG gene, was highly active. However, when the 221 bloodstream VSG gene expression site was untranscribed, the ribosomal promoter was transcriptionally inactive, suggesting the ribosomal promoter is repressed at the silent expression site (Horn and Cross, 1995). When the bloodstream expression site promoter and PARP promoter were integrated into the same position just upstream of the 221 VSG gene at the silent 221 bloodstream VSG gene expression site, these promoters were also transcriptionally inactive suggesting that transcriptional repression is promoter non-specific (Horn and Cross, 1995). The results of Horn and Cross (1995) and Rudenko *et al.* (1995) suggests that there is promoter non-specific repression of transcription initiation at the silent bloodstream VSG gene expression sites that may extend from the VSG gene to the expression site promoter ~50 kb upstream, although it may also be possible that the effects at either end of the bloodstream VSG gene expression site are unconnected.

Both Horn and Cross (1995) and Rudenko *et al.* (1995) proposed that repression of transcription initiation at the silent bloodstream expression sites in bloodstream trypanosomes may be the result of a telomere position effect (see section 5.3.3). However, in yeast, transcriptional repression of promoters close to the telomeres extends

only about 5-8 kb upstream of the telomere repeats (Renauld *et al.*, 1993). This means that if a similar mechanism represses transcription initiation from the expression site promoters in the silent bloodstream expression sites then transcriptional repression must extend for 50-60 kb upstream of the telomere repeats. In yeast, the distance from the telomere over which transcriptional repression is effective can be increased to 22 kb by increasing the dosage of SIR3 (Renauld *et al.*, 1993), a protein thought to be involved in assembling the chromatin structure from the telomere that inhibits transcription initiation (Palladino and Gasser, 1994; Hecht *et al.*, 1995). Therefore, it is not inconceivable that a chromatin structure may be assembled from the telomeres at the silent bloodstream expression sites upstream as far as the expression site promoters to prevent transcription initiation.

In contrast to the bloodstream VSG gene expression sites, the metacyclic VSG gene expression telomeres are transcribed from promoters that are just upstream of the VSG gene, and in the case of the 1.22 M-VSG gene promoter, within 6-8 kb of the telomere repeats (S. V. Graham, unpublished results). I proposed that repression of the 1.22 M-VSG gene promoter at the 1.22 M-VSG gene expression telomere in bloodstream trypanosomes may be due to a telomere position effect. The distance of the 1.22 M-VSG gene promoter from the telomere repeats is consistent with the distance over which transcription initiation is repressed at yeast telomeres (Renauld *et al.*, 1993). Therefore, metacyclic VSG gene expression telomeres in trypanosomes represent more likely candidates for control by a telomere position effect than the promoters of bloodstream VSG gene expression sites.

6.2 Other metacyclic VSG gene expression telomeres

Donelson and co-workers have also been studying the expression of metacyclic VSG genes in *Trypanosoma brucei*. Many of their findings are very similar to those from our laboratory; they have also reported *in situ* activation (Lenardo *et al.*, 1986) and monocistronic transcription of metacyclic VSG genes (Alarcon *et al.*, 1994). However, the data that they have presented is deficient in several respects. Metacyclic VSG gene activation may not be studied directly as there are insufficient metacyclic trypanosomes produced in the salivary glands of tsetse flies for nucleic acids to be isolated and analysed. Instead, metacyclic-derived trypanosomes are used which are early bloodstream trypanosomes that are directly derived from metacyclic trypanosomes and maintain expression of metacyclic VSG genes for up to 7 days after infection of the

mammalian host (Barry *et al.*, 1979; Esser *et al.*, 1982). To study the expression of the metacyclic VSG gene, MVAT7, at the metacyclic stage, Lenardo *et al.* (1986) isolated DNA from a population of day 5 metacyclic-derived trypanosomes expressing the MVAT7 gene and showed, by Southern analysis, that the gene was not duplicated when expressed in metacyclic-derived trypanosomes. The population of metacyclic-derived trypanosomes expressing the MVAT7 gene was generated by immunolysis of all MVAT7 non-expressors in the metacyclic population and injection of metacyclic MVAT7 expressors into mice. However, the metacyclic VSG genes are activated polyclonally in the salivary glands of the tsetse fly (Tetley *et al.*, 1987) and therefore the resulting metacyclic-derived population of MVAT7 expressors used in this experiment were also polyclonal. This means that each metacyclic trypanosome expressing the MVAT7 gene, may have activated the VSG gene by a different duplication event, which might not be apparent on a Southern blot as the new copy of the MVAT7 gene may give a restriction fragment of a different size in each metacyclic trypanosome. Therefore, it is necessary to use clonal rather than polyclonal populations of metacyclic-derived trypanosomes in order to study metacyclic VSG gene activation. Graham *et al.* (1990) used clonal populations of metacyclic-derived trypanosomes to show conclusively that the metacyclic VSG genes, ILTat 1.22 and ILTat 1.61, were not duplicated when expressed at the metacyclic stage and are likely to be activated *in situ* at their metacyclic expression telomeres.

Lenardo *et al.* (1986) postulated that M-VSG gene expression was transcriptionally regulated during the parasite life cycle. They used nuclear run-on experiments to show that the metacyclic VSG gene, MVAT4, was transcribed in a polyclonal population of metacyclic-derived trypanosomes expressing the MVAT4 gene, but was not transcribed in procyclic trypanosomes. They also showed that an 8 kb region upstream of the MVAT4 gene was transcribed in MVAT4 expressing metacyclic-derived trypanosomes but did not assay this upstream region for transcription in procyclic or bloodstream trypanosomes (Lenardo *et al.*, 1986). Transcription of genes in the bloodstream VSG gene expression sites at the procyclic stage and at the PARP loci in bloodstream trypanosomes is controlled, at least in part, by attenuating transcription elongation after initiation of transcription (Pays *et al.*, 1990; Rudenko *et al.*, 1994; Vanhamme *et al.*, 1995a). Therefore, although the MVAT4 gene is not transcribed at the procyclic or bloodstream stages, the studies of Lenardo *et al.* cannot discount the possibility that transcription may initiate and be attenuated upstream of the metacyclic

VSG gene. However, Graham and Barry (1995) subsequently showed that expression of the 1.22 M-VSG gene was indeed controlled at the level of transcription initiation in the trypanosome life cycle; in clonal metacyclic-derived trypanosomes expressing the 1.22 M-VSG gene, the 1.22 M-VSG gene was transcribed as well as a region upstream as far as the putative transcriptional start site. In bloodstream and procyclic trypanosomes the 1.22 M-VSG gene and the transcriptional start site were not transcribed suggesting that transcription does not initiate from the promoter region at these life cycle stages (Graham and Barry, 1995). Significantly, a region of 13 kb 5' of the initiation site was completely transcriptionally silent at all life cycle stages tested (Graham and Barry, 1995).

More recently putative promoters for the metacyclic VSG genes, MVAT4 and MVAT5, have been identified (Alarcon *et al.*, 1994; Nagoshi *et al.*, 1995). Although both promoters are highly active in procyclic trypanosomes, neither promoter has been tested in metacyclic-derived trypanosomes, or even in bloodstream trypanosomes, and therefore it has not been confirmed that these promoters are active at the metacyclic stage. The putative promoter for MVAT4 was identified by nuclear run-on analysis in bloodstream trypanosomes that were expressing the MVAT4 gene *in situ*, i.e. without duplication (Alarcon *et al.*, 1994). Normally, metacyclic VSG genes are not expressed at the bloodstream stage, however, when this occurs they are usually activated by duplicative transposition, probably to a bloodstream VSG gene expression site (Cornelissen *et al.*, 1985; Graham *et al.*, 1990). The activation of the MVAT4 gene *in situ* at the bloodstream stage is highly unusual and these rare bloodstream form trypanosomes were selected following extensive passaging and immunoselection in mice (Alarcon *et al.*, 1994). Therefore, MVAT4 expression has been forced and may be the result of an alteration in transcriptional controls that normally prevent metacyclic VSG gene expression *in situ* at the bloodstream stage, perhaps even allowing initiation from a cryptic promoter which can be highly active in bloodstream trypanosomes. The MVAT4 putative promoter shows little homology to other trypanosome promoters that have been identified but, significantly, it is similar to the consensus sequence of the bloodstream VSG gene expression site promoter at the transcriptional start site and at a short region upstream that has been shown to be essential for transcription initiation (Vanhamme *et al.*, 1995b; Pham *et al.*, 1996; Fig. 6.2.1). The results of deletion analysis of the 1.22 M-VSG gene promoter presented in this thesis (see section 3.2.7 and 3.2.8) suggest that upstream of the 1.22M-VSG gene promoter there is a cryptic or subsidiary promoter

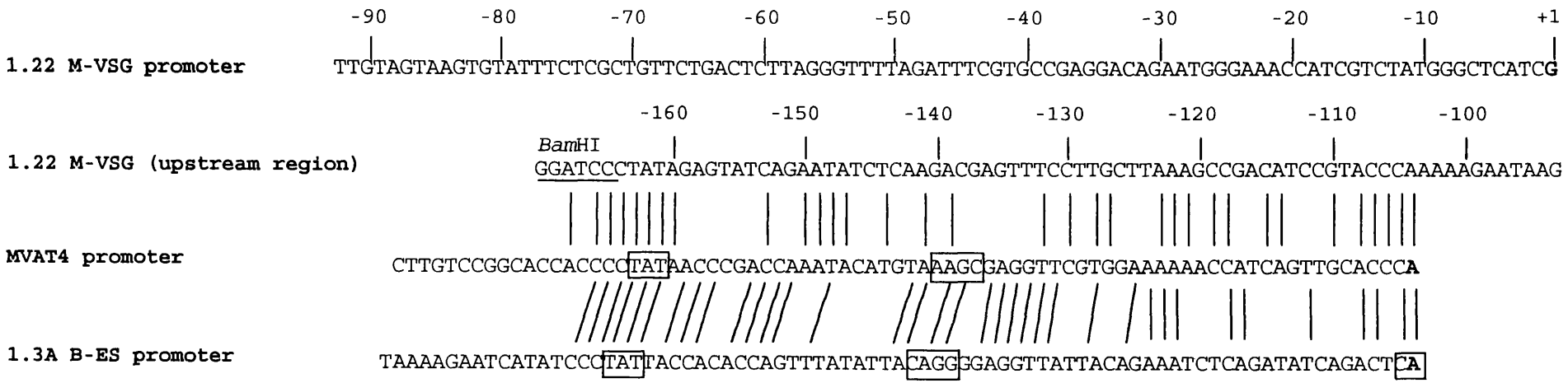


Figure 6.2.1. **Sequence homology of the upstream region of the 1.22 M-VSG gene promoter with the MVAT4 metacyclic VSG gene promoter and a bloodstream VSG gene expression site promoter.** The promoter sequence of the 1.22 M-VSG gene putative promoter is shown from the *Bam*HI restriction site to the putative start site determined in metacyclic-derived trypanosomes. Numbering is in relation to the putative start site. Regions of homology between the promoter regions are shown as vertical and diagonal lines. The transcriptional start sites of each of the promoters is shown in bold and sequences that were shown experimentally to be necessary for transcription initiation are boxed.

which can be active at the bloodstream stage: the MVAT4 promoter may be equivalent to this since it shows homology to the upstream region of the 1.22 M-VSG gene promoter that is sufficient to direct transcription initiation in bloodstream trypanosome transient transfection (Fig. 6.2.1).

When expressed at the bloodstream stage the MVAT5 gene was not activated *in situ* but activated by duplication probably to a bloodstream VSG gene expression site (Nagoshi *et al.*, 1995). The putative MVAT5 promoter was identified by cloning the region upstream of the basic copy MVAT5 gene into a reporter plasmid and testing the reporter plasmid in transient transfection of procyclic trypanosomes. The MVAT5 promoter was highly active in procyclic trypanosomes but was not tested in transfection of metacyclic-derived trypanosomes or bloodstream trypanosomes and may not be active at these life cycle stages. The MVAT5 promoter shows a limited homology to the MVAT 4 promoter. Graham and Barry (1995) showed that the 1.22 M-VSG gene promoter was active in transient transfection of metacyclic-derived trypanosomes and I have shown that the upstream region of the 1.22 M-VSG gene promoter is also active in bloodstream trypanosomes (see section 3.2.5). However, the 1.22 M-VSG gene promoter is not active in procyclic trypanosomes (Graham and Barry, 1995). The activity of the putative MVAT4 and MVAT5 promoters at the procyclic stage may be because these sequences contain cryptic promoters. These cryptic promoters may be part of the true metacyclic VSG gene promoters but lack sequences which control stage-regulated transcription initiation and are therefore not stage-regulated in the same manner as the 1.22 M-VSG gene promoter.

6.3 Future experiments

I have proposed that the 1.22 M-VSG gene promoter region is repressed when positioned at the 1.22 M-VSG gene expression telomere in bloodstream trypanosomes. However, this proposal is based on results from integrations of reporter plasmids to only two positions in the genome, the spacer region between ribosomal RNA gene transcription units and the 1.22 M-VSG gene telomere each of which might exert position specific effects on the integrated promoter. To confirm that the 1.22 M-VSG gene promoter region is normally active in the genome of bloodstream trypanosomes then it would be necessary to integrate reporter constructs containing the 1.22 M-VSG gene promoter, and possibly the ribosomal RNA gene promoter for comparison, to different regions of the genome such as the untranscribed spacer region between mini-

exon repeats (C. Clayton, personal communication) or the RNA polymerase II locus in the opposite orientation to transcription (Urmenyi and Van der Ploeg, 1995). If the activity of the 1.22 M-VSG gene promoter region is high when reporter plasmids are integrated at these positions, in relation to activity of the same promoter region at the 1.22 M-VSG gene expression telomere, then the reduced level of transcription from the promoter region at the 1.22 M-VSG gene expression telomere may be considered significant.

To determine whether repression of transcription initiation from the 1.22 M-VSG gene promoter region at the 1.22 M-VSG gene expression telomere is a phenomenon related to telomere position effect in yeast, it would be necessary to integrate different reporter plasmids to the 1.22 M-VSG gene expression telomere. In yeast repression of transcription is promoter non-specific and the effect decreases with an increase in distance from the telomere repeats (Renauld *et al.*, 1993). Integration of reporter plasmids to the 1.22 M-VSG gene expression telomere that contain different trypanosome promoters, such as the trypanosome ribosomal RNA gene promoter or the bloodstream expression site promoter, controlling transcription of the reporter gene would show whether transcriptional repression at the telomere was promoter non-specific. In addition, by integrating reporter plasmids at different positions at the 1.22 M-VSG gene expression telomere it would also be possible to determine whether the distance from the telomere repeats was a factor in transcriptional repression of the 1.22 M-VSG gene promoter.

Lastly, it may be possible to study the activity of the 1.22 M-VSG gene promoter region at the expression telomere, and at other positions in the genome, at different stages of the developmental life cycle. The reporter plasmids used in Chapter 5 for stable transformation of bloodstream trypanosomes (pr1.22BC and pt1.22BC) contained the PARP gene RNA processing signals flanking the CAT reporter gene and *ble* gene. These RNA processing signals probably lead to a reduced level of CAT and *ble* gene expression in bloodstream trypanosomes with respect to the actin gene RNA processing signals (see 5.2.2). The actin gene is also not thought to be regulated in the trypanosome developmental life cycle (Amar *et al.*, 1988). Therefore, bloodstream trypanosomes may be stably transformed with reporter plasmids containing the actin gene RNA processing signals flanking the reporter gene and differentiated to procyclic forms *in vitro*. The level of reporter gene expression directed by the 1.22 M-VSG gene promoter at the bloodstream and procyclic life cycle stages may then be directly compared in cloned,

transformed trypanosome lines at different genomic positions including the 1.22 M-VSG gene expression telomere. Results from transient transfection experiments suggest that the 1.22 M-VSG gene promoter region is inactive in procyclic trypanosomes (Graham and Barry, 1995) but highly active in bloodstream trypanosomes (see 3.2.5). Furthermore, if trypanosomes stably transformed with reporter plasmids containing the actin RNA processing signals (either bloodstream or procyclic forms) are able to be transmitted through tsetse flies then the activity of the 1.22 M-VSG gene promoter region may be directly compared at different genomic positions at the bloodstream, procyclic and metacyclic life cycle stages. This should allow a very good understanding of the activity of the 1.22 M-VSG gene promoter during the developmental cycle and perhaps an insight into the mechanism (or mechanisms) of activation of the metacyclic VSG genes at the metacyclic stage.

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Appendix 1

Primers used in sequencing the 1.3 kb *Hind*III-*Bam*HI restriction fragment directly upstream of the 1.22 M-VSG gene putative promoter.

1)	T3 1.22LA	GGA TGT CGG CTT TAA GC
2)	T3 1.22LB	CTT TGC TGA CAT GAA GG
3)	T3 1.22LC	AGT GCA TGG TGC CAT GG
4)	T3 1.22LD	TCG GCT AGT TGT GGC GA
5)	T3 1.22LE	TAT CAC TGA GAA TAT TG
6)	T7 1.22LA	TCC TTC AGT ATT TGG CA
7)	T7 1.22LB	CCA TGC ACT GCC ATG GC