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STUDIES ON THE METACYCLIC VARIANT ANTIGEN GENES OF AFRICAN TRYPANOSOMES

A thesis submitted for the
Degree of Master of Science
at the
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by

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DECLARATION

The research reported in this thesis is my own original work except where otherwise stated, and has not been submitted for any other degree.

Frances Devaney

CONTENTS

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SUMMARY

Trypanosomes contain a repertoire of a possible 100-1000 different genes for variant surface glycoproteins (VSGs). A small and strain specific fraction of these genes is expressed in the salivary glands of the tsetse fly giving rise to metacyclic variant antigen types (M-VATs). These M-VATs continue to be expressed up to day nine of infection in the bloodstream of immunosuppressed mice. The variable antigen repertoire expressed by T.b.rhodesiense metacyclics is not influenced by the anamnestic expression whereby the VAT ingested by the fly is present at high levels in early bloodstream populations of fly infected mice. This has been demonstrated by feeding tsetse flies a trypanosome line expressing a VAT, encoded by a single-copy gene which is normally a component of the metacyclic repertoire, GUTat 7.13. This VAT did not constitute a significantly increased proportion of the resultant metacyclic population in the fly, nor was it expressed at higher levels in the bloodstream of fly infected mice. Analysis of trypanosome DNA isolated from fly infected mice revealed that for those trypanosomes expressing GUTat 7.13 a new expression linked copy (ELC) of this gene was made. The presence of this new ELC was directly correlated with the presence of 7.13 expressors. It is concluded that the expression of VSG genes in the fly and in the mammal is controlled by different mechanisms.

The distribution of acute sleeping sickness caused by T.b.rhodesiense is not homogeneous throughout East Africa, rather discrete foci of infection exist where the disease is endemic. Trypanosomes with similar VAT repertoires which share predominant

VATs (those which tend to appear early in infection) can be grouped into serodemes and individual serodemes are associated with individual foci. Stocks from an endemic focus in the Luangwa Valley, Zambia were cloned and tested for relatedness in antigen repertoire to stocks isolated from an endemic focus in Kenya using serological and DNA analysis. It was concluded that stocks from these two foci in East Africa were members of different serodemes.

ABBREVIATIONS

(1) Chemicals.

BSA	Bovine Serum Albumin
CY	Cyclophosphamide
DMSO	Di-methylsulfoxide
DNA	Deoxyribonucleic acid
cDNA	Complementary DNA
EDTA	Ethylenediaminetetra-acetic acid (disodium salt)
FCS	Foetal Calf Serum
FITC	Fluorescein isothiocyanate
GPS	Guinea Pig Serum
PBS	Phosphate buffered saline
PSG	Phosphate saline glucose
PVP	Polyvinylpyrrolidine
RNA	Ribonucleic acid
mRNA	Messenger RNA
SCFSB	Single colony final sample buffer
SDS	Sodium Dodecylsulphate
SSC	Sodium Chloride, Sodium Citrate
TE	Tris, EDTA
Tris	Tris(hydroxymethyl)aminomethane

(2) Measurements.

bp	base pair
^{o}C	degrees centigrade
cm	centimetre
cpm	counts per minute
g	centrifugal force equal to gravitational acceleration
gm	gramme
kb	kilo base pair
kd	kilo dalton
kg	kilogramme
Mb	mega base pair
mg	milligramme
ml	millilitre
mm	millimetre
M	molar (moles/litre)
mM	millimolar
nm	nanometre
pH	acidity [negative $\log_{10}$ (Molar concentration H^{+} ions)]
ug	microgramme
ul	microlitre
w/v	weight /volume

(3) Techniques

IFA	indirect fluoresence assay
IFAT	indirect fluoresence antibody technique
PFGE	pulsed field gradient electrophoresis

(4) Serodemes and Antigenic Types

AnTAR	Antwerp Trypanozoon Antigenic Repertoire

AnTat	Antwerp Trypanozoon antigenic type
BoTAR	Bordeaux Trypanozoon Antigenic Repertoire
BoTat	Bordeaux Trypanozoon antigenic type
ETAR	Edinburgh Trypanozoon Antigenic Repertoire
GUTAR	Glasgow Trypanozoon Antigenic Repertoire
GUTat	Glasgow Trypanozoon antigenic type
IsTAR	Issaque Trypanozoon Antigenic Repertoire
WRATAR	Walter Reed Army Trypanozoon Antigen Repertoire

(5) Miscellaneous

BC	basic copy
CRD	cross reacting determinant
EATRO	East African Trypanosomiasis Research Organisation
ELC	expression linked copy
kDNA	kinetoplast DNA
mtDNA	mitochondrial DNA
GUPM	Glasgow University Potozoology monoclonal
McAb	monoclonal antibody
TDRC	Tropical Disease Research Centre
VAT	variable antigen type
B-VAT	bloodstream variable antigen type
I-VAT	ingested variable antigen type
M-VAT	metacyclic variable antigen type
VSG	variant surface glycoprotein
sVSG	soluble form variant surface glycoprotein
mfVSG	membrane form variant surface glycoprotein

CHAPTER 1

INTRODUCTION

INTRODUCTION

1.1 General Introduction

The African trypanosomes (genus Trypanosoma) are flagellated protozoa which present a significant health problem to humans and animals over a widespread area of Africa. Due to infestation with trypanosomes large areas of Africa cannot be used to raise livestock except poultry (Hornby,1952). An estimated area of $10^7 km^2$ is virtually devoid of cattle from this cause yet such an area could support $11x10^8$ more head of cattle than the 1962 estimate of the total cattle population of Africa-$1.14x10^8$ animals (Wilson et al,1963). As a result the protein intake of the human population is abnormally low over much of Africa as compared with European populations. The relevance of such estimates is debatable ignoring as they do socio-economic factors. However it is estimated that some 35 million people and 25 million cattle are exposed to the risk of infection, with an estimated 3 million cattle dying of trypanosomiasis each year (WHO,1979) and although only approximately 10,000 cases of human sleeping sickness are reported annually this is considered to be a gross under-estimate due to difficulties of accurate diagnosis (WHO,1979). Therefore it is true to say that trypanosomiasis has had, and continues to have, a devastating effect on the development of Africa and its people.

1.2 The Life Cycle of African Trypanosomes

African trypanosomes are digenetic parasites; their life cycle alternates between two hosts, an insect vector and vertebrates. Trypanosomes are transmitted by blood-sucking tsetse flies (*Glossina* sp.) from one mammal to another. The trypanosomes are taken up by the fly from the bloodstream of infected animals during feeding and they then undergo a period of development within the fly.

On entering the tsetse the trypanosomes lose their infectivity for the mammalian host. After approximately three weeks infectivity is regained as the trypanosomes differentiate to the infective stage in the mouthparts of the tsetse.

Trypanosomes ingested by the tsetse fly in a blood meal enter the midgut (Evans,1979), within which they differentiate into elongated procyclic forms which no longer possess the surface coat which confers on them protection in the bloodstream of the mammal. The procyclic forms divide and migrate to the anterior of the gut (proventriculus) where they differentiate into more slender proventricular forms. These differentiated forms then migrate into the oesophagus and on into the mouthparts and salivary ducts to establish infection in the salivary glands.

In *Trypanosoma brucei* the proventricular forms enter the hypopharynx (connecting the oesophagus and mouthparts) and migrate to the salivary glands (Robertson,1913). Within the salivary glands the trypanosomes transform to the multiplicative epimastigote stage which is attached to the salivary gland epithelial cells and establish a large population which then gives rise to the infective, non-multiplicative metacyclics, which have reacquired the surface coat (Vickerman,1969).

Metacyclics enter the mammalian host via saliva discharged when the tsetse feeds. At the site of entry into the mammal a local inflammatory reaction, the chancre, develops (Fiennes, 1946). Within the chancre the metacyclic trypanosomes differentiate into bloodstream forms and divide rapidly (Gordon and Willet,1956). This multiplication is accompanied by focal disorganisation and degeneration of the dermal collagen surrounding the chancre (Gray and Luckins,1980). The trypanosome population undergoes a period of division within the chancre and invades the bloodstream primarily via the lymphatic system, being detectable in the lymph by days 3-5 post infection (Barry and Emery,1984).

Bloodstream forms of T.brucei are morphologically variable ranging from long slender dividing parasites to short stumpy non-dividing forms (Ashcroft,1957). The latter are thought to be preadapted to life in the fly and initiate the cycle of development in the fly (Robertson,1912; Wijers and Willet,1960).

1.3 The Surface Coat

Development of trypanosomes in the mammalian bloodstream and the ability to maintain a chronic infection in the face of effective host antibody function is possible through the evolution of a remarkable survival mechanism, antigenic variation of the surface coat. Both the metacyclics and bloodstream forms possess a surface coat which allows their survival in the bloodstream of the infected host. It comprises a 12-15nm glycoprotein coat which overlies the entire plasma membrane and flagellum and serves to protect the trypanosomes from non-specific immune mechanisms. The uncoated insect forms are rapidly

lysed in normal serum and phagocytosed non-specifically by macrophages (reviewed by Vickerman and Barry,1982).

Each trypanosome expresses only one glycoprotein, termed the variant surface glycoprotein (VSG), in its surface coat (Cross,1975) and it has been estimated that 1.2×10^7 VSG molecules are required to cover the external surface of the trypanosome (Turner et al,1985).

1.4 The Variant Surface Glycoprotein

Variant surface glycoproteins (VSGs) have been isolated from a number of clones of T.brucei and consist of a single polypeptide of approximately 50 kd. Different VSGs vary by isoelectric focussing, peptide mapping, amino acid composition (Cross,1975; 1977), N-terminal amino acid sequencing (Bridgen et al,1976), total carbohydrate composition (Johnson and Cross,1977) and in the positions where the carbohydrate is attached (Holder and Cross,1981).

Each VSG contains approximately 500 amino acid residues and is comprised of an N terminal signal peptide (approx. 20 amino acids), a region highly variable in sequence (approx. 360 amino acids) and a C terminal homology region (approx. 120 amino acids) which by inference from nucleic acid sequencing includes a hydrophobic tail (approx. 20 amino acids). In the surface coat the VSG molecules are organised with the C terminus oriented towards the membrane and the N-terminal domain exposed (Cross and Johnson,1976).

VSGs are made as precursor molecules containing, like other membrane bound proteins, N-terminal hydrophobic signal peptides which show no particular homology in sequence apart from their

hydrophobicity (Boothroyd et al,1981). This leader sequence is consistent with the VSG's status as a cell surface protein. Such sequences are thought to be signals for transport of surface proteins through membranes (Davis and Tai,1980).

The precursor protein contains a C terminal hydrophobic tail which is absent from the mature VSG (Boothroyd et al, 1980). The C terminal hydrophobic tails show a high degree of conservation and sequence information has allowed VSGs to be classified into two groups, or isotypes, on this basis (Rice-Ficht et al,1981). Unlike other membrane glycoproteins, this hydrophobic tail lacks any charged amino acids which are thought, in other proteins, to provide anchorage in the correct position by interactions with polar groups in the lipid bilayer. It is now known, however, that the C terminal extension is replaced by a glycolipid which serves to anchor the glycoprotein in the membrane (Ferguson and Cross,1984).

All VSGs contain two types of oligosaccharide side chain (Holder and Cross,1981). The first, which is asparagine-linked, is derived from internal sites in the polypeptide chain and contains mannose and glucosamine. The second, which is attached to the C terminal amino acid of the mature protein , contains mannose, galactose and glucosamine and forms part of the glycolipid structure which holds the VSG in position in the plasma membrane. This carbohydrate is referred to as the cross reacting determinant (CRD) as it is immunogenic and the antibody directed against it cross reacts with all purified soluble form VSGs (Barbet and M^{c}Guire,1978).

Holder (1983), having found the CRD was attached through ethanolamine via an amide linkage with the carboxyl group of

the terminal amino acid residue, proposed that the VSG precursor is processed to replace the hydrophobic tail with the ethanolamine linked oligosaccharide. The ethanolamine is in turn attached to a glycolipid which in T. brucei contains myristic acid (Ferguson and Cross,1984).

Thus biosynthesis of VSGs involves several post translational modifications: the removal of the N terminal signal peptide; the addition of the asparagine linked oligosaccharides; processing of the C terminal hydrophobic peptide and its replacement by a glycolipid containing the CRD.

Cardoso de Almeida and Turner (1983) discovered that VSG release may be effected by an enzyme which modifies the glycoprotein. Purification of VSG in a conventional manner is thought to activate this enzyme, releasing soluble form VSG (sVSG), whereas boiling in detergent releases the "membrane form" VSG (mfVSG) which has an apparent difference in molecular weight. It was also demonstrated that anti-CRD antibody preferentially bound to the soluble form whereas recognition of the CRD in the membrane form was impaired , indicating the difference between the two forms involves the C terminal domain. The putative enzyme has the characteristics of a phospholipase C (Jackson and Voorheis,1985) and on release of the sVSG leaves the glycolipid anchor within the membrane (Ferguson et al,1985). It is possible that this enzyme may play a role in coat turnover during antigenic switching or at the stage in the trypanosome life cycle where the surface coat is shed.

1.5 Antigenic Variation

The amino terminal domain of VSGs is exposed to the

environment and rapidly stimulates an immune response, leading to antibody mediated removal of trypanosomes from the bloodstream. However trypanosomes can consecutively express a large number of different variable antigen types (VATs) which do not share exposed antigenic determinants and by VSG switching they can survive for prolonged periods in the bloodstream of the host.

The ability to switch from one surface antigen to another results in a characteristic undulating parasitaemia consisting of alternating patent parasitaemias and remissions as successive VATs are expressed. The remissions result from antibody mediated removal of trypanosomes expressing the major VAT present in the bloodstream at a particular time and the high parasitaemias from the subsequent overgrowth of one or more of the minor VATs. Such a relapsing parasitaemia suggests that population survival is at the expense of the majority of individual trypanosomes, with 99.9% or higher individual trypanosome destruction (Vickerman, 1978).

Antigenic switch rates have been estimated to occur at the rate of 10^5-10^6 per generation (Doyle 1977). Similar switch rates have been found *in vitro* (Doyle *et al*, 1980), in the presumed absence of host antibody. Such evidence suggests antigenic variation is an intrinsic property of trypanosomes, requiring no obvious host induction mechanisms, which allows them to avoid complete destruction and maintain a chronic infection in the face of effective host antibody function.

Chronic infection requires an extensive number of immunologically different VSGs. Capbern *et al* (1977) showed that a single clone of *T.equiperdum* gave rise to 101 different antigenic types in several chronically infected rabbits. Each

different VSG is encoded by a separate gene (Hoiejmakers et al,1980a) and it has been estimated that there are as many as 10^3 VSG genes per trypanosome nucleus (Van der Ploeg et al,1982a).

Throughout a chronic infection the expression of VSG genes is not completely random. Evidence suggests there is a loosely defined order of expression of VATs, with some VATs expressed early in infection and others later (Gray,1965a; Van Meirvenne et al,1975a,1975b; Capbern et al,1977; Kosinski,1980). The sequential appearance of VATs in this loosely defined manner does not require cyclical transmission through the tsetse fly. Syringe passaging to a non-immune host is sufficient to reset the programme: early VATs are again the first to appear (Capbern et al,1977). Similar VATs expressed by different repertoires are referred to as isoVATs or isotypes. Similar repertoires sharing all predominant isotypes can be grouped into serodemes, where a serodeme is defined as a stable immunologically distinct set of strains which does not exhibit cross-immunity with other immunologically distinct sets.

The mechanism of programming that determines the order of VAT appearance is not yet understood. It could result from different growth rates of trypanosomes expressing different VATs. Competition effects between trypanosomes of the same stock expressing different VATs have been demonstrated (Seed,1978; Miller and Turner,1981). However some form of programming at the genetic level seems likely. The order of appearance of VATs does not depend solely on relative growth rates (Van Meirvenne et al,1975b; Miller and Turner,1981). Van Meirvenne et al (1975b) demonstrated that different trypanosome stocks could express the same set of VSGs in a different order. Also some VSGs tend to be

expressed with high probability after one another in a given stock (Miller and Turner,1981). These observations suggest that programming may occur at the genetic level.

Further evidence supporting genetic programming of the VAT repertoire is seen in the metacyclic population found in the salivary glands at the end of cyclical development in the fly. The metacyclics are antigenically heterogeneous but a predictable and limited set of VSGs is produced by this population which is uninfluenced by the VAT originally ingested by the fly (Le Ray et al,1978; Barry et al,1979; Hajduk et al,1981;Hajduk and Vickerman 1981; Crowe et al,1983).

1.6 Karyotype of Trypanosomes and Chromosomal Distribution of VSG genes

Trypanosome chromosomes, like those in other lower eukaryotes, do not condense at any point in the life cycle, thus precluding conventional cytological staining techniques to estimate chromosome number. However evidence from other types of experiment suggest that trypanosomes are diploid organisms with means of genetic exchange. Data for diploidy are based on measurements of DNA content per cell as compared to its kinetic complexity. Borst et al (1980a) estimated by renaturation and Cot analysis that the haploid genome size of T.brucei nuclear DNA was 3.7×10^4 kb with 68% of the genome present as single copy DNA. The DNA content per nucleus in bloodstream forms, determined by quantitative absorption and fluorescence cytophotometry of individual Feuglen stained cells, is 0.091 pg or 8.2×10^4 kb (Borst et al,1982) i.e. the nuclear DNA content was estimated to be twice that estimated for the haploid genome indicating that

trypanosomes are diploid. Tait (1980), investigating enzyme electrophoretic variation, found that for dimeric enzymes the relative proportion of homodimers and heterodimers was exactly that predicted for a diploid organism with means of genetic exchange. More recently Gibson et al (1985), studying restriction enzyme polymorphisms flanking the genes for several glycolytic enzymes have confirmed that trypanosomes are diploid for house-keeping genes.

However, it has been known for some time that trypanosomes are haploid with respect to at least some VSG genes. In a quantitative hybridisation analysis only one copy of the 117 gene was found per nucleus (Borst et al,1980a) and only one copy of the 118 gene (Hoeijmakers et al,1980a). Similar analysis reveals only one copy of the 221 gene (Bernards et al,1984a).

Recently the technique of pulsed field gradient electrophoresis (PFGE) (Schwartz and Cantor,1984) has been applied to trypanosome nuclear DNA. Using this technique DNA molecules in the range of approximately 25 to 2000 kb can be separated allowing the trypanosome nuclear DNA to be resolved into four general size classes (Van der Ploeg et al,1984a).

An unknown number of chromosome-length DNA molecules do not enter the gel either because of their size or perhaps because of structural constraints. It has been estimated that this DNA represents roughly 60% of the trypanosome genome. There are estimated to be at least three chromosomes of about 2000 kb, a set of about six chromosomes of 200-700 kb and a mini-chromosomal fraction containing about 100 DNA molecules ranging from 50-150 kb. As these mini-chromosomes were not found in a related species Crithidia fasciculata (Van der Ploeg et al,1984a), which does not

undergo antigenic variation, it was initially proposed they may be related in some way to the molecular mechanism of antigenic variation. However extension of PFGE analysis to other trypanosomes with antigenic variation has indicated that T.vivax contains no mini-chromosomes and T.equiperdum contains very few indicating therefore that mini-chromosomes are not essential for antigenic variation (Van der Ploeg et al,1984b).

Using PFGE for Southern analysis and probing with a DNA sequence corresponding to the C terminal homology region of VSGs, under low stringency conditions, revealed hybridisation to all four size classes of DNA molecules indicating that VSG genes are scattered throughout the genome. Evidence from an analysis of cosmid clones containing trypanosome DNA inserts of approximately 40 kb, using probes corresponding to both the 3' end and upstream regions of VSG genes, indicated that the clones frequently contained more than one region which hybridised under low stringency conditions, indicating that VSG genes were clustered in the genome (Van der Ploeg et al,1982a).

1.7 The Molecular Basis of Antigenic Variation: Duplicative Activation of VSG Genes

Evasion of the host's immune response by trypanosomes is accomplished by the process of antigenic variation. Each single trypanosome can make more than 100 VSGs each differing in amino acid sequence such that host antibody raised against one VSG will not react effectively against other VSGs in the repertoire. Each trypanosome in a stock contains the entire VSG gene repertoire of that stock and each VSG is encoded by a separate gene (Hoeijmakers et al,1980a). Therefore diversity of VSGs is not

generated, as with antibody genes, by piecing together of gene segments, and chronic infection is maintained by the successive expression of a large number of different antigen genes.

VSG genes can be broadly classified into two groups according to their genomic location: the majority of genes are chromosomal internal, while others are found at chromosome ends. It has been recognised for some time that for some VSG genes activation is accompanied by a duplication and transposition event. For these genes a silent basic copy (BC) gene is duplicated and inserted into an expression site to yield an expression linked copy (ELC) (Hoeijmakers et al,1980a; Borst et al ,1980b ; Pays et al ,1981a). The BC and ELC can be distinguished in blots of nuclear DNA by large differences in the surrounding DNA sequences (Michels et al ,1983). The ELC has been identified as the gene copy which is probably transcribed due to preferential sensitivity to DNase 1 (Pays et al ,1981b).

The transposition units of some of the genes activated by this mechanism have been sequenced (Liu et al,1983). For VSG gene 118 of T.brucei stock 427 the transposed sequence is approximately 3.5 kb. The coding region comprises only 1.8 kb and in this case an upstream sequence of more than 1 kb is cotransposed with the coding region. The duplicated segment is characterised on both sides by the presence of conserved sequences found for all VSG genes sequenced to date which are believed to mediate integration of the VSG genes into the expression site (Michels et al,1983; Liu et al ,1983). At the 5' border of the transposed segment there are a series of imperfect repeats of approximately 70 bp which are found 5' to other VSG genes. At the 3' end of the transposed sequence the conserved

sequences are mainly in the 3' untranslated part of the gene, the most obvious being a 13 bp sequence which is present adjacent to all VSG genes analysed (Majumder et al ,1981; Boothroyd et al ,1981; Matthyssens et al,1981; Rice-Ficht et al, 1981).

Incorporation of the transposed sequence into the expression site involves a crossover at a variable position in the 3' end of VSG genes (Michels et al,1983; Bernards et al,1981). Michels et al (1983) found no differences in the restriction maps of several 118 ELCs except at the 3' ends and on comparison of the sequences of these ELCs concluded that the duplicative transposition of a VSG gene may lead to recombinational replacement of the 3' end occurring anywhere within the final 150 bp of the gene. The exchange of 3' ends gives rise to an ELC with restriction sites in the 3' end that differ from the corresponding BC. In such cases the maps of the cDNA and the ELC are identical providing further evidence that the ELC is the gene copy transcribed.

The upstream crossover point occurs at a variable position within the tandem array of 70 bp repeats found 5' to VSG genes. This region has been referred to as the "barren" region due to lack of restriction enzyme sites. This barren region has been shown to be of variable length for four 118 ELCs (Michels et al, 1983). Such variability can be explained by the finding that integration into an expression site may occur by a recombination at a variable position within this tandem array (Campbell et al, 1984a).

1.8 Trypanosome Telomeres and their Role in Antigenic Variation

ELCs are located next to chromosome ends. This was first

recognised by the observation that the region downstream of the ELC ended at a position where all restriction enzymes apparently cut. Incubation of trypanosome DNA with Bal31, an exonuclease which progressively degrades DNA from the ends with increasing incubation time, was found to progressively shorten the DNA fragments that extend to the universal apparent restriction site downstream of the ELC (De Lange and Borst,1982).

In addition to ELC genes other VSG genes have been located to telomeres as recognised by the above criteria (Williams et al,1979; Williams et al,1982; Raibaud et al,1983; Parsons et al, 1983a).

The size of the region downstream of the VSG gene is variable. This is due to continuous growth of trypanosome telomeres. Van der Ploeg et al (1984c) cloned a telomeric DNA segment and determined the sequence of trypanosome telomeres. This clone contained approximately 50 tandemly repeated copies of the hexamer $^{5'}$ CCCTAA $^{3'}$ and it was proposed that this sequence was added on to telomeres during trypanosome multiplication resulting in the variable length of telomeric DNA segments. Bernards et al (1983) investigated the telomere carrying the 118 ELC over many generations and found that it grew by approximately 10 bp per generation. Another telomere harbouring the 221 VSG gene, which was not active, grew more slowly at the rate of approximately 7 bp per generation. Pays et al (1983a) also found that telomeres with transcribed VSG genes grew slightly faster than those with silent genes. Such telomere growth has to be compensated for by occasional deletions and deletions have also been found to occur preferentially at telomeres in which actively transcribed VSG genes reside. It is possible that the chromatin

structure of active VSG genes, which shows increased sensitivity to DNase 1 , could influence the rate and extent of size increases and decreases in telomeric DNA.

Bernards et al (1984b) found modifications in telomeric DNA that contained silent VSG genes. Some restriction enzyme sites were found to be partially uncleavable in silent telomeric antigen genes, presumably as a result of DNA modifications. These modifications were not found in transcribed genes but did return after inactivation of the gene. They were always absent from chromosome internal genes regardless of their transcriptional status. The level of modification in the gene was highest towards the telomere and was influenced by the size of the telomeric DNA segment downstream of the gene suggesting modifications of this kind are specific for telomeric DNA. Whether they are a cause or consequence of telomere gene inactivation remains speculative.

Similar results have been reported by Pays et al (1984). Apparent modifications of GC dinucleotides were found in silent telomeric genes but not in actively transcribed telomeric genes . However no modifications were found in procyclic trypanosomes where VSG synthesis is shut down completely . There is therefore no absolute correlation between the absence of such modifications and gene transcription. However it is likely that the mechanisms controlling VSG gene switch off at particular stages in the life cycle differ from mechanisms controlling VSG gene switching in the bloodstream and the absence of base modifications in the procyclics may reflect such a difference.

1.9 Telomeric VSG Genes

VSG genes can be classified broadly into two groups: chromosome internal genes which use the duplicative mode of activation previously described and telomeric genes which can be activated by other mechanisms which do not always involve gene duplication. The non-duplicative mode of activation was first described by Williams and co-workers (Williams et al,1979; Williams et al,1980; Young et al,1982; Majiwa et al,1982; Donelson et al,1982; Young et al,1983a;1983b) and has also been described by others (Borst et al,1980b; Bernards et al,1984a; Laurent et al,1984a).

All genes so far found to utilise the non-duplicative mode of activation are located within telomeres and initially it was proposed that these genes may be activated via a reciprocal translocation event between two telomeres, namely the one in which the expression site resides and the other containing the VSG gene. Such a model would agree with there being only one expression site from which VSG genes can be transcribed. However Bernards et al (1984a) found the telomeric VSG gene 221 could be activated by both the duplicative and non-duplicative mechanisms. When the former mechanism was used the 221 ELC was found in an expression site identical to the one used by the 117 and 118 VSG genes suggesting duplicative activation of this gene is similar to activation of chromosome internal genes. Van der Ploeg et al (1984a) using PFGE analysed the 221 gene during both modes of activation. The BC of the gene resides in the large DNA which remains in the slot. When activated by duplication the ELC appeared in the fraction containing chromosomes of 2000kb in length. However when the gene is activated without duplication it

remains in the large DNA and is not transferred to the dominant expression site chromosome used by other VSG genes i.e. genes 117 and 118. Although transfer of the gene to another large chromosome cannot be excluded this result does indicate the presence of multiple expression sites and apparently refutes the model of reciprocal translocation which was proposed.

However more recently Pays et al (1985a) have demonstrated that reciprocal translocation can occur. In following the switch from AnTat 1.3 to AnTat 1.10 they found the AnTat 1.10 gene was activated without duplication although its environment was completely altered. From restriction mapping and determination of the chromosomal location of the two telomeres involved they found that during this switching event the AnTat 1.10 gene had recombined into the AnTat 1.3 active telomere and the previously expressed 1.3 gene had recombined into the old 1.10 telomere where it was inactivated. The crossover point in this event occurred within the 70 bp repeat units upstream of the gene and was readily detected in Southern blots as it occurred within 10 kb of the gene. This provides the first direct evidence that telomeric genes can be activated by a reciprocal translocation event. A model of reciprocal translocation involves two predictions for which evidence now exists. The first is that when a VSG gene is activated by this mechanism the previous ELC will be retained in an inactive conformation and secondly that the gene activated by reciprocal translocation may be lost in the ensuing variant, if a third VSG gene enters the same expression site.

Laurent et al (1984a) found that the VSG gene AnTat 1.6 of T.brucei stock EATRO 1125 was a telomeric gene which could be

expressed without a concomitant duplication. In clones expressing AnTat 1.6 the ELC of the preceding variant was conserved in an inactive configuration. Also, in ensuing variants derived from the AnTat 1.6 clone, the 1.6 gene was lost. In those variants where the VSG gene was using an ELC the 1.6 gene was replaced by the incoming ELC. Pays et al (1983b) found a similar situation in the switch from AnTat 1.16 to AnTat 1.6. In this case the 1.16 ELC was conserved and the 1.6 gene lost in ensuing variants. Similar reports of the retention of inactive ELCs have been made (Young et al,1983b; Buck et al,1984). These reports provide indirect evidence of reciprocal recombination between two telomeres during non-duplicative activation.

Retention of the ELC can also occur when another gene is activated in situ. Michels et al (1984) followed the switch from VSG gene 118, activated by an ELC, to VSG gene 1.8, activated without duplication, and found that the 1.8 expressors retained the 118 ELC. Yet one prediction of a reciprocal recombination model is that there should be differences in restriction enzyme sites in front of the putative crossover points. No such differences were found for at least 28 kb upstream of the inactive and active ELC genes which would suggest that activation of the 1.8 gene had not occurred by reciprocal translocation but rather that the 1.8 gene had been activated in situ. Bernards et al (1984a) when studying the non-duplicative activation of gene 221 found no upstream rearrangements for 55 kb in front of the gene accompanied its expression. The maps for active and inactive 221 genes were identical for this distance upstream. Such evidence would suggest that telomeric VSG genes can be activated in situ.

In four of the five populations studied by Bernards et al (1984a) where the 221 gene was switched off the gene was deleted. It was suggested that this deletion occurred by conversion of this telomere by another. Telomeric gene conversion has been proposed as the mechanism whereby telomeric genes can be activated with concomitant duplication. Pays et al (1983c) have shown that for the telomeric VSG gene AnTat 1.3 duplication in one trypanosome clone involved the co-duplication of at least 40 kb of upstream DNA and they have suggested that sequence homologies between the previously expressed gene AnTat 1.1c and AnTat 1.3 were restricted to a region far upstream from the coding sequence so that the duplicated segment would have to be very long to reach the region of homology required for a conversion event to occur. In this case the AnTat 1.1c gene was lost, being replaced by an extra copy of the AnTat 1.3 containing telomere. Telomere conversion has been found in other cases. Pays et al (1983d) found in the AnTat 1.1 gene expression site that the ELC was associated with another sequence, in front of the coding region which they referred to as the "companion sequence". They found that the companion sequence was a transposed copy of a sequence also located in a telomere and concluded that it represented a 5' residual fragment of a former ELC.

Pays et al (1983c) found further evidence of gene conversion when investigating two trypanosome clones derived from AnTat 1.1. They proposed the mechanism of DNA rearrangement in this case was a gene conversion taking place between different members of the same gene family. Again in this instance the three sequences involved were all telomeric.

De Lange et al (1983a) found an aberrant 118 ELC gene which

was flanked at the 3' side by at least 1 kb of DNA containing restriction enzyme sites. They showed this DNA and the 3' end of the 118 gene were derived from another telomeric VSG gene, 1.1006 and suggested that this sequence may have arisen by the co-transposition of a whole chromosome end, including part of the 1.1006 telomere linked gene, into an expression site. They referred to this as a telomere conversion which duplicated the entire region between the gene and the end of the chromosome.

Recent evidence suggests that end points for telomeric gene conversions are non-randomly distributed (Pays et al,1985b). On investigating switching between two telomeric genes AnTat 1.1 and AnTat 1.10, which share more than 70% homology, it was found that although the limits of the gene conversion events were not identical they were not distributed at random. This clustering of conversion limits could indicate the existence of specific sequences which act as targets for specific recombinases or that certain gene rearrangements are selected for in the process of gene conversion.

In the yeast mating type interconversion system a site specific endonuclease, acting on a particular target sequence has been found to trigger gene conversion (Kostriken et al,1983). However in the conversion investigated by Pays et al (1985b) no target sequence, similar to that found in yeast, could be located. The authors proposed that the gene conversion events analysed are not stimulated by a site specific recombinase but rather are performed by the general recombination machinery which is operating at recombinational "hotspots", in this case telomeres. It had been suggested previously that telomeres can act as "hotspots" for recombination due to the presence of

homologous repeated sequences and single stranded nicks (Van der Ploeg and Cornelissen,1984; Pays et al,1985a).

The evidence presented from this analysis of gene conversion endpoints would seem to suggest that telomeric gene conversion occurs via the general recombinational machinery and that a post recombinational selection system may operate such that conversion events leading to unsuitable changes within the VSG protein would be selected against.

In summary, trypanosomes have two types of VSG gene; the internal chromosomal genes activated by duplication to a telomeric expression site and telomeric genes activated by several mechanisms which include in situ activation, gene conversion and reciprocal translocation.

1.10 Multiple Expression Sites

Given there are an estimated 10^3 VSG genes per trypanosome nucleus and the assumption that only one VSG is expressed at a given time there must be a process, akin to allelic exclusion, whereby only one gene is selected for transcription in a trypanosome at a particular time.

Initially a simple model of control of VSG gene expression involving only one expression site within the genome was proposed. To be transcribed a VSG gene had to be transposed to this site. However more recent evidence clearly indicates that multiple expression sites do exist. In the BoTAR serodeme of T. equiperdum restriction mapping showed that VSG gene BoTat 1 can be expressed in at least three different expression sites (Longacre et al,1983). Pays et al (1983b) investigating VSG genes of the AnTAR serodeme have found that two genes, AnTat 1.6 and

AnTat 1.16 were expressed from the same site but AnTat 1.1c was found within an expression site with a very different restriction enzyme map upstream of the 5' barren region. Also, evidence from PFGE analysis of trypanosome chromosomes and examination of the positions of the 221 gene of the MITAR serodeme of T.brucei shows that this gene can be expressed from two different telomeric sites (Van der Ploeg et al,1984a).

The presence of multiple VSG gene expression sites within each nucleus requires a more complex model of control such that only one expression site can be activated at a time. This has led to the idea of a mobile activator element which can transpose from one telomere to another. However no evidence for this exists. Analysis of VSG genes far upstream from the coding region has not yet revealed any such element. Bernards et al (1984a) have shown for VSG gene 221 that no major rearrangements occur within 55 kb upstream of the coding region when this gene is activated without duplication. Activation of other genes without detectable DNA alterations has been reported (Pays et al,1983b; Laurent et al,1984a;1984b). Detailed sequence analysis of many upstream regions of transcribed VSG genes would be required before the mobile activator model can be definitely discarded as a possible control mechanism for VSG gene expression. In particular restriction enzyme mapping of upstream regions could conceivably miss very small insertions.

Bernards et al (1984b) and Pays et al (1984) have suggested a variation to the mobile activator model such that the activator comprises a unique site on the nuclear matrix with which only active telomeres are associated. No evidence yet exists in trypanosomes to support this theory, however it has been reported

that certain eukaryotic genes are associated with the nuclear matrix when transcribed (Robinson et al,1982; Ciejek et al,1983).

A second model proposes that VSG gene expression could be controlled by the DNA modifications which are found in silent telomeric genes (Bernards et al ,1984b; Pays et al,1984). When telomeric genes are activated the modifications disappear. The fact that these modifications do not exist in procyclics where VSG gene expression is switched off could suggest that they may not be the cause of gene switch off. However it seems likely that switch off at this stage in the life cycle could be regulated in a different way from regulation of switching events in the bloodstream. Absence of modifications in procyclics does not necessarily refute their role in control of VSG gene switching.

Liu et al (1985) suggest that there is a hierarchy within the telomeres with respect to their activation or switch off. On studying the predominant genes of the MITAR serodeme of T.brucei they found that the 1.8 gene was switched on most frequently- about 50 % of all switches led to expression of this gene. The preference for expression of this gene did not depend on its predecessor nor was it due to differential growth rates of trypanosomes expressing this gene. They suggested that this gene lies within a telomere which is easily activated in comparison to others. There is therefore an order of preference in telomeric activation. Differences which may exist between telomeres such that a hierarchy exists have not been found.

The control of multiple expression sites in the trypanosome remains one of the central issues in research on antigenic variation particularly in the light of recent research which suggests that the assumption that only one VSG gene can be

expressed at a given time may not be valid. Esser and Schoenbechler (1985) studying individual T.b.rhodesiense trypanosomes during switching in early bloodstream populations showed that trypanosomes could simultaneously express both pre- and post-switch VSGs uniformly over their cell surface as detected with monoclonal antibodies. More recently Baltz et al (1986) have provided evidence that a clone of T.equiperdum can stably express two VSG genes in vitro and both antigens are present on the cell surface. This analysis goes further than that of Esser and Schoenbechler in that the genes corresponding to these two stably expressed antigens have been analysed and have been shown to reside in different telomeric expression sites, and both genes are active in double expressors. Double antigen expression was found to be relatively stable in vitro though the population slowly drifted towards single expression. In vivo however, when double expressors were inoculated into mice the population rapidly changes to single expression.

As pointed out by the authors the fact that two VSG genes are actively transcribed from different expression sites simultaneously demonstrates that activation of one site is not mutually exclusive of activation of others and provides evidence against a model of VSG gene regulation involving a single mobile regulatory element. They also suggest that trypanosomes express only one antigen in vivo because of selection against double antigen expression in the animal rather than because of an intrinsic genetic mechanism in the parasite. The existence of double expressors suggests that expression sites can be switched on and off independently of each other therefore future models for control of VSG gene expression need not accommodate a

mutually exclusive mechanism for VSG gene transcription.

1.11 VSG Gene Transcription

Control of VSG gene expression occurs at the level of transcription. Lheureux *et al* (1979) isolated RNA from cloned trypanosome populations and found that it directed the translation *in vitro* of a VSG recognised only by antisera which had been raised against the clone. Hoeijmakers *et al* (1980a;1980b) found that a given VSG cDNA hybridised only to RNA from trypanosomes expressing that VSG. Such evidence indicates that control of VSG expression occurs at the level of RNA synthesis.

Sequence determination of cDNAs corresponding to VSG mRNA revealed that all VSG mRNAs contain an identical sequence of 35 nucleotides at their 5' ends, referred to as the 35-mer or spliced leader sequence (Boothroyd and Cross,1982; Van der Ploeg *et al*, 1982b). This 35-mer was found at the 5' termini of mRNA from VSG genes activated by different mechanisms.

Initially it was proposed that this sequence could be involved in the control of VSG gene expression in that the BC remained silent because it was incomplete, lacking the 35-mer, and that this sequence was provided by the expression site and added onto the mRNA via splicing. However De Lange *et al* (1983) using a specific probe for the 35-mer sequence were unable to find a single spliced leader sequenc within 10 kb of a transposed VSG gene in the expression site. Also the mRNA of VSG gene 221, activated without diplication, contains the 35-mer sequence but no 35-mer was found within 8 kb of this gene (Bernards *et al*,1984a). It has since been found that the probe corresponding

to the spliced leader hybridises to many clones in a trypanosome cDNA library besides the VSG cDNAs, as well as hybridising to many RNA species in procyclic trypanosomes where VSG genes are no longer transcribed (De Lange et al,1984; Parsons et al,1984). Furthermore other trypanosomatids which do not undergo antigenic variation but are related to African trypanosomes also possess DNA sequences containing the 35-mer (Nelson et al,1984). This evidence therefore implies that the presence of this sequence is not a unique feature of VSG gene transcription. Using the specific probe for the 35-mer sequence to determine the copy number and genomic location of the spliced leader sequences De Lange et al (1983b) found the sequence was not present in the 5' barren regions found upstream of the VSG genes and further that the 35-mer was part of a 1.35 kb repetitive element present at about 200 copies per nucleus. These were found in tandem arrays of linked units. These tandem arrays are located both in the very large chromosomes and in the 2000 kb chromosomes but are undetectable in the mini-chromosomes (Van der Ploeg et al,1984a).

Van der Ploeg and Cornelissen (1984) found that the ELC of the VSG gene 1.8 was located on a 550 kb chromosome which lacks the spliced leader yet the 1.8 mRNA contained the sequence at the 5' end. Similar results were obtained by Rothwell et al (1985). Investigation of the single copy IsTat 1.1 VSG gene of the IsTAR serodeme revealed that it is located on a mini-chromosome and is activated in situ yet there is no 35mer sequence found on the chromosome. This provided evidence that the 35-mer sequence was joined to the mRNA by unconvential means. Recently indirect evidence suggests that this unconventional mechanism is discontinuous transcription.

It is not only VSG mRNAs which contain the spliced leader sequence. Therefore to provide each mRNA with the 35-mer sequence two possible mechanisms exist: either discontinuous transcription or production of very large mRNA precursors which could then be processed. As yet no such precursors have been found, however this does not necessarily mean they are not made but rather, could reflect either rapid processing or instability of the RNA species. Campbell et al (1984b) identified the transcriptional products from the 1.35 kb repeats and found they directed the synthesis of discrete 137 nucleotide transcripts referred to as mini-exon derived RNA (med RNA) which contain the 35 nucleotide sequence at the 5' end. Kooter et al (1984a) found the med RNA was synthesised at a very high rate, 700 times higher than the transcription of the remainder of the 1.35 kb sequence, indicating it was not made as part of a larger precurser but as a discrete species. Transcription of med RNA is sensitive to α-amanitin, an inhibitor of RNA polymerase II, (Laird et al,1985), whereas VSG gene transcription is insensitive (Kooter et al,1984b). It is unlikely therefore that both RNAs are transcribed by the same polymerase, further supporting the hypothesis that VSG gene transcription is a discontinuous process. This evidence, though indirect, supports the view that discontinuous transcription is a feature of transcription of trypanosome VSG genes.

In conclusion there are many interesting features exhibited by trypanosomes particularly with regard to the gene expression and regulation mechanisms employed during antigenic variation. The consecutive but non-random expression of a large number of VSG genes and the predominant expression of metacyclic VSG genes

is of particular interest. The evolution of VSG gene repertoires is also intriguing and provides the researcher with a large gene family amenable to analysis.

CHAPTER 2

Analysis of a metacyclic VSG gene during cyclical transmission through the tsetse fly

INTRODUCTION

Bloodstream trypanosomes are pleomorphic, ranging from long slender dividing trypomastigotes to stumpy non-dividing trypomastigotes, the latter being infective for the tsetse and developing in the midgut, initiating the cycle in the fly (Robertson,1912; Wijers and Willet,1960).

On ingestion by the tsetse fly the stumpy trypanosomes differentiate into non-infective procyclics and rapidly lose their surface coat (Vickerman,1969; Steiger,1973; Brown et al,1973). After a period of development the trypanosomes migrate to the salivary glands where they ultimately develop into infective metacyclic forms that have reacquired the surface coat. The metacyclic developmental stage is the only insect form which possesses the surface coat (Vickerman,1969).

Trypanosome clones belonging to the same serodeme (i.e. which have the same repertoire of VSG genes) give rise to the same restricted subset of VSGs in the metacyclic population (Hajduk et al,1981). Why a specific subset of VSG genes is expressed at this stage in the life cycle is still unknown. The number of metacyclics extruded at one time by the tsetse fly is very small, estimated to be approximately 3,000/proboscis (Harley et al,1966) which prevents direct investigation of the metacyclic population at the DNA level. However metacyclic variable antigen types (M-VATs) continue to be expressed by trypanosomes up to day nine in fly infected mice, thus collection of trypanosomes early in infection allows investigation of metacyclic VSG gene expression albeit indirectly.

Initially it was proposed that the trypanosome population reverted to a single basic antigenic type at the end of cyclical

development in the fly (Gray,1965b). However heterogeneity within the metacyclic population with respect to VAT has been demonstrated using indirect means (Le Ray et al,1978) and monospecific antisera raised against metacyclic variable antigen types (M-VATs) (Barry et al,1979; Hajduk et al,1981;Hajduk and Vickerman,1981).

Le Ray et al (1978) found sera collected from rabbits one month after infection with a syringe passaged trypanosome line showed lytic activity against most of the metacyclics extruded at one time by the tsetse fly, indicating therefore that M-VATs are not specific to the fly stages of the trypanosome life cycle. This observation led to the preparation of monospecific antisera against individual M-VATs (Barry et al,1979). Rabbits were infected with a syringe passaged trypanosome line. Serum and trypanosomes were collected from infected animals frequently and the serum tested for lytic activity against metacyclics allowing the time of appearance of M-VATs in the bloodstream to be estimated. Blood collected at that time should contain trypanosomes expressing M-VATs and indeed cloning of such trypanosomes gave rise to antigenically stable trypanosome populations expressing M-VATs. These populations were used for the production of antisera directed against individual M-VATs.

Application of these monospecific antisera in immunofluoresence tests on metacyclic trypanosomes revealed that the metacyclic population is heterogeneous with respect to VAT as were trypanosomes in the first patent parasitaemia (FPP) in mice, following cyclical transmission, which were found stillto be expressing M-VATs. The first non M-VATs were detected after day four or five of infection (Barry et al, 1979).

There is a constancy in M-VAT expression: Hajduk *et al* (1981) found that three M-VATs were present in constant proportions in metacyclic populations obtained at different times from individual flies. Also, the VAT ingested by the fly was not present in the metacyclic population, and did not influence the VAT composition of the metacyclics.

In a further study Hajduk and Vickerman (1981), again using monospecific antisera, analysed the VATs present in mice following cyclical transmission. The first VATs detectable in the bloodstream were M-VATs which were present until at least day five post infection. Barry *et al* (1985) found neutralisation of specific M-VATs did not prevent their expression in early bloodstream populations, suggesting that metacyclic trypanosomes can switch to the expression of other M-VATs. This evidence indicates that M-VAT expression continues in early bloodstream populations and switching from one M-VAT to another can occur.

Recently for a West African stock of *T. congolense* all the metacyclic VATs have been characterised with monoclonal antibodies (McAbs) raised against exposed epitopes on living metacyclic forms. Twenty-one McAbs recognised epitopes on living metacyclics. Using these in indirect immunofluoresence assays (IFA) it was found that twelve of the twenty-one McAbs separately labelled unique VATs. When pooled these twelve McAbs labelled the entire metacyclic population and could neutralise infectivity of the metacyclics for mice, indicating the M-VAT repertoire of this stock is limited to twelve VATs (Crowe *et al*,1983). For one *T.b.rhodesiense* cloned line it has been found that eight M-VATs constituted 60-80% of the metacyclic population (Barry *et al*,1983). A pool of sixteen McAbs can label all the M-VATs of

the WRATAR serodeme of T.b.rhodesiense (Esser and Schoenbechler, 1985). These observations indicate that of a repertoire of a possible 100-1000 VSG genes (Capbern et al,1977; Van der Ploeg et al ,1982a), there is a specific subset expressed in the metacyclic stages. However Barry et al (1983) presented evidence that, though the metacyclic VAT repertoire is limited, it is unstable. The M-VAT repertoire in a series of T.b.rhodesiense cloned isolates collected from an East African focus of sleeping sickness over a period of twenty years was found to be unstable. Of a total of eleven M-VATs investigated by indirect immunofluoresence three were found to be present in all stocks tested, two M-VATs were present in stocks isolated early but absent from those isolated in later years. The remaining six VATs were not present in all stocks consistently but no correlation could be made between their absence and the time of isolation.

Sequential transmission of one of the clones through tsetse flies allowed a more controlled investigation of the M-VAT repertoire. Most of the M-VATs analysed were consistently expressed throughout ten sequential transmissions. However one M-VAT, GUTat 7.15, was lost after the third transmission and expression of this M-VAT did not recur throughout the remaining seven transmissions. This evidence shows that although the metacyclic VAT repertoire is restricted, it is not necessarily stable over a period of time.

Hajduk and Vickerman (1981) carried out a detailed study on the influence of the ingested VAT (I-VAT) on bloodstream populations derived from infected flies and found that the ingested VAT, although having no effect on M-VAT expression, was always present early in infection, during the first patent

parasitaemia (FPP), being detected from day 4 post infection. The ingested VAT did not necessarily become the major VAT in the FPP, perhaps because of differences in growth rate of trypanosomes expressing different M-VATs (Seed,1978) or because of differential gene activation. This phenomenon is called the "anamnestic" effect and a molecular explanation of this memory effect has been proposed.

Overath et al (1983) analysed the repression of glycoprotein synthesis during in vitro transformation from bloodstream forms to procyclic forms, a process thought to mimic the in vivo situation in the midgut of the fly. After transfer to the transformation medium VSG synthesis was repressed rapidly, this was not concomitant with rapid excision of the ELC of the gene which was expressed in the bloodstream prior to transformation. Rather the ELC was retained in the genome but not transcribed. Parsons et al (1983b) reported a similar observation of lingering yet inactive ELCs in procyclic culture forms. In bloodstream trypanosomes retention of ELCs in inactive forms, after a switching event involving a VSG gene activated by the non duplicative mode of activation, have been reported (Laurent et al,1984a; Pays et al,1983b; Young et al,1983b; Buck et al,1984; Michels et al,1984; Pays et al,1985a). In bloodstream trypanosomes the retained ELC is reactivated preferentially (Michels et al,1984; Laurent et al,1984b).

These findings provide an hypothesis at the molecular level for the "anamnestic" effect and also provide supportive evidence that the control of VSG gene expression may differ in the metacyclic and bloodstream populations. It is possible that the ELC of the ingested VAT, though not expressed in the metacyclic

population may reside in a "bloodstream expression site" which could be easily reactivable when the trypanosomes next meet bloodstream conditions. On entering the bloodstream M-VATs continue to be expressed for up to five days in immunocompetent hosts during which "early" or "predominant" VATs including the ingested VAT are activated. This would indicate that the expression of M-VATs is dominant over the expression of bloodstream VATs and would further suggest that the expression of metacyclic and bloodstream VSG genes may be under separate control mechanisms.

To test this hypothesis a stable bloodstream population expressing the M-VAT GUTat 7.13 was used in cyclical transmission through the tsetse fly. Thus, an M-VAT was used as the ingested VAT, thereby creating a situation where this VAT could be expressed preferentially by the anamnestic effect. If VAT expression at both stages of the trypanosome life cycle (i.e. in the fly and in the bloodstream) is controlled by the same mechanism one would expect to see the anamnestic effect direct expression of the M-VAT, GUTat 7.13, at an increased level in the metacyclic population. If, on the other hand VAT expression is under separate control at different stages in the life cycle then no increase in the expression of GUTat 7.13 would be observed. The expression of this VAT in early bloodstream populations was investigated using IFA and the DNA analysed via Southern Blot Analysis.

MATERIALS AND METHODS

Trypanosomes and Tsetse Flies

Variable antigen type (VAT) GUTat (Glasgow University Trypanozoon antigen type) 7.13 which is derived from stock EATRO (East African Trypanosomiasis Research Organisation) 2340 was investigated in these experiments. The pedigree of GUTat 7.13 is given in Figure 1 (see also Cornelissen et al,1985). GUTat 7.13 is a cloned metacyclic form which has undergone 27 rapid syringe passages in mice and has been recloned 10 times to ensure that it is antigenically stable and homogeneous.

Trypanosomes were transmitted through Glossina morsitans essentially as described previously (Le Ray et al,1978). Recently hatched flies were fed on female CFLP mice harbouring patent infections of GUTat 7.13. All mice were cyclophosphamide (CY) treated (250mg/kg) 24 hours before infection as a means of suppressing the immune system. The bloodstream populations used were screened by indirect fluorescent antibody technique (IFAT) to confirm antigenic homogeneity. GUTat 7.13 was found to constitute 92-97% of the populations used for fly feeds. Following the infective feed maintenance blood meals were on citrated sheep blood (Gibco Ltd.,Paisley) three times weekly. Three weeks after the initial infective feed tsetse flies were induced to probe saliva onto heated microscope slides which were examined for the presence of metacyclic trypanosomes. Infected flies were fed on CY treated female CFLP mice either directly, or by probing into 500ul of fresh Guinea Pig Serum (GPS) heated to 37°C. The GPS was then divided into two aliquots and each used to infect one mouse by intraperitoneal injection.

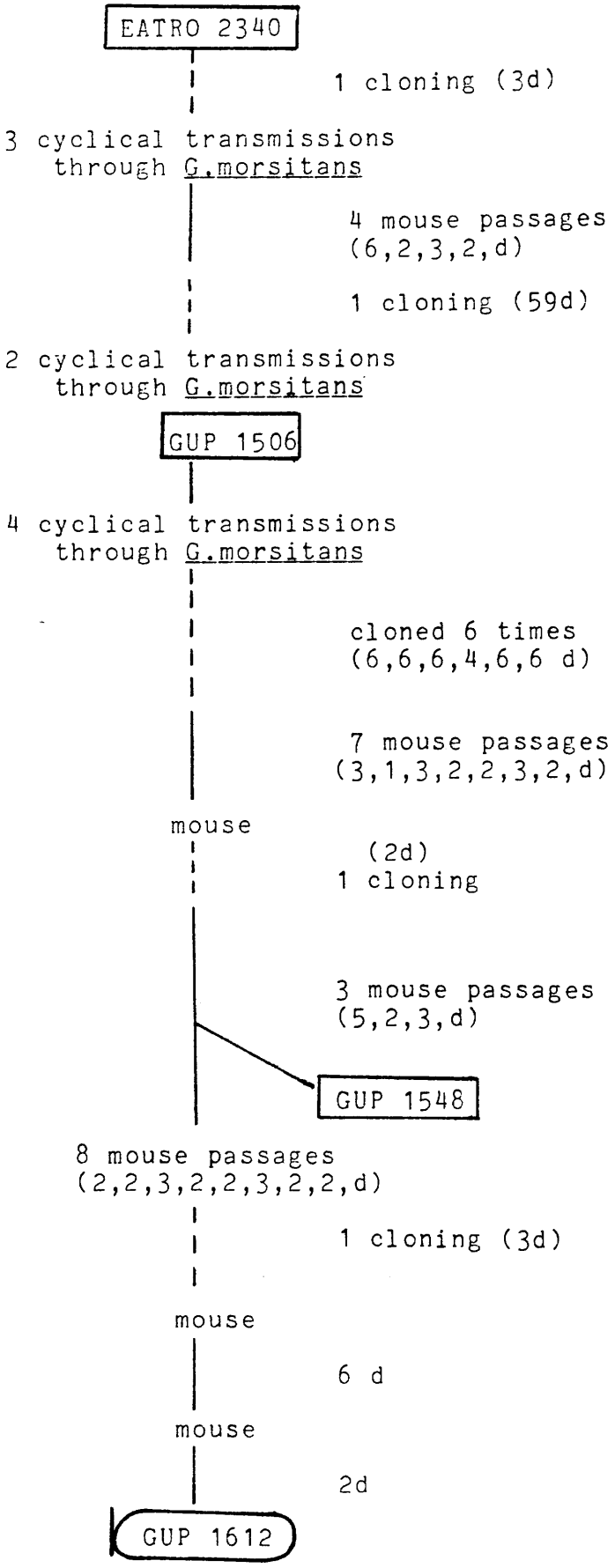

EATRO 2340
1 cloning (3d)
3 cyclical transmissions through G.morsitans
4 mouse passages (6,2,3,2,d)
1 cloning (59d)
2 cyclical transmissions through G.morsitans
GUP 1506
4 cyclical transmissions through G.morsitans
cloned 6 times (6,6,6,4,6,6 d)
7 mouse passages (3,1,3,2,2,3,2,d)
mouse
(2d)
1 cloning
3 mouse passages (5,2,3,d)
GUP 1548
8 mouse passages (2,2,3,2,2,3,2,2,d)
1 cloning (3d)
mouse
6 d
mouse
2d
GUP 1612

Figure 1 **Pedigree of the syringe passaged GUTat 7.13 population**
This population was used to obtain an antigenically stable clone corresponding to the metacyclic variable antigen type 7.13 drawn according to the conventions of Lumsden, Herbert & M^{c}Neillage (1973).
Stabilate numbers are in boxes.
VAT reference stabilates in cartouches.
Broken lines indicate cloning.
Solid lines indicate syringe passaging.
EATRO- East African Trypanosomiasis Research Organisation.
GUP- Glasgow University Protozoology.

Bloodstream to Procyclic Transformation

Female CFLP mice were inoculated intraperitoneally with approximately 10^4 trypanosomes and bled by cardiac puncture on day 7 post infection. Approximately 10^7 trypanosomes in 0.1 ml blood were added to 10 mls of Hill's medium which isessentially SM-medium (Cunningham,1977) but with slightly different buffering, supplemented with 15% heat inactivated foetal calf serum (FCS) (Gibco Ltd.,Paisley) and Gentamycin (15ug/ml) and incubated at 26°C. After 48 hours the trypanosomes were transferred to $25cm^2$ tissue culture flasks (Lux, Gibco Ltd., Paisley) in which they were maintained at a concentration of approximately $1x10^7$/ml, and were continually sub-passaged until enough procyclics were obtained for DNA isolation.

Antisera and Monoclonal Antibodies

Monospecific rabbit antiserum was prepared against GUTat 7.13 using a standard protocol (Van Meirvenne et al,1975a). Monoclonal antibodies (McAbs) specific for four different metacyclic variable antigen types (M-VATs) were produced by the procedure described previously (Crowe et al,1983).

The McAbs were GUPM (Glasgow University Protozoology Monoclonal) 17.2 for GUTat 7.1, GUPM 18.7 for GUTat7.2 and GUPM 17.1 for GUTat 7.13.

Immunofluoresence Reactions

Bloodsmears taken from mice on day 7-9 post infection were air dried and fixed in acetone at room temperature for 15 minutes. The smears were rehydrated in phosphate buffered saline (PBS) pH 7.2, for 15 minutes after which the antibody was applied

to reaction zones and the slides incubated in a humid chamber at room temperature for 30 minutes. After two 5 minute washes in PBS, Fluorescein Isothiocyanate (FITC) conjugated rabbit anti-mouse IgG (whole molecule) (Sigma, Poole) was applied at a 1:100 (v/v) dilution in PBS containing 1:10,000 w/v Evans Blue (Merck) and the slides incubated for a further 30 minutes in a humid chamber. The slides were washed twice in PBS and excess liquid drained off before being mounted in 50% (w/v) glycerol/PBS. The reaction zones were examined using a Leitz Ortholux II microscope with incident light fluoresence, an HB50 high pressure mercury vapour lamp, a TK510 dichroic mirror, 2XKP490 (exciting) and K515 (suppressing) filter.

Trypanolysis Reactions

Trypanolysis reactions were performed on trypanosomes isolated from whole blood on day 7-9 of infection. The reactions were performed in 5mls fresh Guinea Pig Serum (GPS) using a monospecific rabbit antiserum directed against GUTat 7.13 at a 1:50 dilution. The trypanosomes (at $2x10^8$/ml) were incubated at room temperature for 2-3 hours after which samples were taken and examined for lysis by phase contrast miroscopy.

Isolation of Trypanosomes from Blood

CY-treated female CFLP mice infected by tsetse bite were exsanguinated on day 7-9 post infection. Trypanosomes were separated from blood cells by ion-exchange chromatography on DE52 cellulose (Lanham and Godfrey,1970) and concentrated by centrifugation at 1000g for 15 minutes at room temperature and resuspended in the appropriate buffer.

Trypanosome Lysates, DNA Isolation and Purification

One volume of purified trypanosomes containing 10^{10} trypanosomes was gently suspended in 10 volumes of NET buffer (100mM NaCl, 100mM EDTA, 10mM Tris, pH8.0) at room temperature and lysed by the addition of Sarkosyl to a concentration of 3%. After lysis proteinase K (Sigma, Poole) was added to a concentration of 100 ug/ml and the lysate incubated for 30 minutes at 37°C after which it was stored at room temperature until a total of 10^9-10^{10} trypanosomes had been isolated.

DNA isolation was performed essentially as described by Bernards et al (1981). Briefly, the individual lysates were pooled and an equal volume of Phenol: Chloroform: Iso-amyl alcohol, (50:50:1) added. After 15 minutes gentle rocking extraction was carried out by centrifugation at 1160g, 15 minutes at room temperature. After careful removal and storage of the aqueous phase the interface was diluted by addition an equal volume of 1xTE (10mM Tris HCl,pH8.0, 1mM EDTA) and re-extracted with phenol. The two aqueous phases were pooled and Ribonuclease A (Sigma, Poole) added to a final concentration of 100ug/ml for 30 minutes at 37°C. The solution was incubated for an additional 30 minutes at 37°C in the presence of 100ug/ml Proteinase K. A third phenol extraction followed, after which the DNA was precipitated in 2 volumes of Ethanol after addition of 3M Sodium Acetate, pH 5.6, to a final concentration of 300mM. The precipitation was carried out at room temperature, the DNA being spooled out carefully and washed in 70% Ethanol before resuspension in an appropriate volume of 1xTE.

The DNA was further purified by centrifugation in a CsCl gradient. 20.5 gm of CsCl was added to 20 mls DNA in 1xTE and

mixed by gentle inversion until the CsCl dissolved. 2 ml Ethidium Bromide (10 mg/ml) was added and the solution centrifuged to equilibrium at room temperature, (16 hr at 196,408g in a Beckman VTi50 rotor). The gradient was examined under short wave UV light and the DNA removed using a 1ml syringe and 18g needle. The Ethidium Bromide was removed by extraction with isopropanol and the DNA exhaustively dialysed against 1xTE to remove the CsCl.

Production of Competent Escherichia coli Cells

E.coli 1440 was cultured in 20 ml L-broth at 37°C overnight in a shaking water bath. 1.25 ml was then added to 250 ml L-broth and incubated at 37°C for 3 hours in a shaking incubator. The culture was then cooled on ice for 10 minutes after which the cells were pelleted by centrifugation at 959g, 4°C for 5 minutes. The cells were resuspended in 15 ml 100mM $CaCl_2$ and incubated at 4°C for 30 minutes. After centrifugation at 1160g, 4°C for 5 minutes the cells were resuspended in 2.5 ml $CaCl_2$. A sample was removed and used for transformation. The remainder of the cells were stored at -20°C in 100mM $CaCl_2$ containing 17.6% glycerol.

Transformation of E.coli with Recombinant Plasmid DNA

Recombinant plasmids containing DNA complementary to VSG mRNA corresponding to GUTat 7.13 have been described previously (Cornelissen et al,1985). The cDNAs were inserted into the Pst1 site of the vector pBr322 by GC tailing. The recombinant plasmid TcV 7.13.25 was used to transform E.coli 1440.

Fifty ul of competent E.coli 1440 cells were incubated on ice for 15 minutes with 10ng of TcV 7.13.25 plasmid DNA followed by a 5 minute incubation at 37°C. One ml of L-broth was added

and the mixture incubated at 37°C for 35 minutes. A serial dilution of the transformation mix was made and 0.1 ml aliquots from each dilution plated out on L-broth agar plates containing Tetracycline at 15 ug/ml and incubated overnight at 37°C. Single colonies were picked from the plates and single colony lysates prepared as follows: the colonies were suspended in 80 ul distilled H_2O, 20 ul 5x single colony final sample buffer (SCFSB) (2.5% Ficoll(w/v), 1.25% SDS(w/v), 0.015% Bromophenol blue(w/v), 0.015% Orange G(w/v), made up with buffer E (0.04M Tris, 0.02M Sodium Acetate, 0.001M EDTA,pH 8.2)), 1ul RNase (5mg/ml) and incubated at room temperature for 15 minutes. The lysates were spun for 5 minutes at 12,000g in a Centaur microfuge and 20 ul of the supernatant run on 1% agarose gels to check for presence of the plasmid. Colonies in which the plasmid was present were picked from the plates and used for plasmid isolation.

Isolation of Plasmid DNA

A 1 litre culture of transformed cells was prepared in L-broth containing Tetracycline to a final concentration of 15 ug/ml and incubated at 37°C overnight in a shaking incubator. Cells were pelleted by centrifugation at 15300g, 4°C for 10 minutes and resuspended in 3.3 mls of 25% Sucrose in 50mM Tris HCl, pH 8.0. 6.7 mls of lysozyme (20 mg/ml in 250 mM Tris HCl,pH 8.0) was added and the mixture swirled gently on ice for 15 minutes. After the addition of 13 mls of 250 mM EDTA the mix was incubated on ice for a further 5 minutes after which 53 mls of lytic mix was added and after gently inverting several times lysis was allowed to proceed for at least 30 minutes on ice.

After lysis was completed the mix was centrifuged at 48,400g, 4°C for 25 minutes and the cleared lysate was then

incubated at 37°C for 15 minutes with 500 ul of Ribonuclease A (1 mg/ml) followed by a further incubation with 500 ul Proteinase K (1 mg/ml) for 15 minutes at 37°C.

The lysate was then phenol extracted by the addition of an equal volume of Phenol: Chloroform: Isoamyl alcohol, 50:50:1, followed by centrifugation at 1,160g for 15 minutes at room temperature. The plasmid DNA in the aqueous phase was then precipitated by the addition of 3M Sodium Acetate, pH 5.6 to a final concentration of 0.3 M followed by 1 volume of isopropanol. The mix was then incubated on ice for 20 minutes after which the DNA was pelleted by centrifugation at 27,200g, 4°C for 30 minutes. The pellet was washed in 65% isopropanol, dried and resuspended in 5 mls 1xTE. The plasmid DNA was further purified by centrifugation in a CsCl gradient. 5 gm CsCl was added to 5 mls DNA in 1xTE and mixed by gentle inversion until dissolved. 0.33 ml Ethidium Bromide (3 mg/ml) was added and the solution centrifuged to equilibrium at 193,687g, 15°C for 16 hours.

The gradient was examined under UV illumination and the plasmid band removed with a 1 ml syringe and 22g needle. The Ethidium Bromide was removed by repeated Butanol extraction and the DNA diluted in 3 volumes 1xTE before precipitation by the addition of 2 volumes of Ethanol followed by a 1 hour incubation on ice. The DNA was pelleted by centrifugation at 27,200g, 4°C for 15 minutes. The pellet was then washed in 70% Ethanol after which it was dried and resuspended in an appropriate volume of 1xTE and stored at 4°C.

Isolation of cDNA Fragments

Fragments of the recombinant plasmid TcV 7.13.25 were isolated following complete digestion with the restriction endonuclease PstI to excise the cDNA fragment. 50ug of plasmid DNA was digested overnight at 30°C with 5 units of PstI in medium salt buffer (50mM NaCl, 10mM Tris HCl, pH7.5, 10mM $MgCl_2$, 1mM Dithiothreitol). Following electrophoresis of the digestion products on low melting point agarose gels (1.5%) containing 0.5 ug/ml Ethidium Bromide in Borate buffer (0.089M Tris HCl, 0.089M Boric Acid, 0.002M EDTA, pH8.3) the bands were visualised under short wave UV light and excised from the gel.

The agarose was incubated at 70°C for 15 minutes in 10 volumes of NET buffer (150mM NaCl, 5mM EDTA, 50mM Tris HCl, pH 8.0). A short column consisting of 1-2mm Sephadex G100 overlaid with 1-2mm DE52 cellulose was prepared in a siliconised pasteur pipette plugged with siliconised glass wool. The column was equilibrated with several volumes of NET buffer at 70°C before the agarose solution was loaded. The column was kept at 70°C to prevent the agarose solution resolidifying. The flow through from the column was collected and reapplied after which the DNA was eluted from the column using high salt NET buffer (1.5M NaCl, 5mM EDTA, 50mM Tris HCl, pH8.0). Six 500ul fractions were collected (in general the DNA was found in the first two fractions). One ml of Ethanol was added to each and the DNA precipitated overnight at -70°C. The DNA was pelleted by centrifugation at 4°C for 15 minutes in an Eppendorf centrifuge. The pellet was washed in 70% Ethanol after which it was dried at 37°C. The DNA was then resuspended in 100ul 1xTE and phenol extracted by the addition of an equal volume of Phenol: Chloroform: Isoamyl Alcohol,

(50:50:1), and centrifugation for five minutes at room temperature in an Eppendorf centrifuge. The aqueous phase was removed and the DNA precipitated by the addition of 3M Sodium Acetate, pH5.6 to a final concentration of 300mM, followed by 2 volumes of Ethanol and incubation at -70°C for at least 30 minutes. The DNA was pelleted by centrifugation for 15 minutes at 4°C in an Eppendorf centrifuge and the pellet washed in 70% Ethanol before being dried at 37°C after which the DNA was resuspended in 50ul 1xTE.

Restriction Endonuclease Digestion, Electrophoresis and Transfer of Genomic DNA to Filters

DNA digestion by restriction endonucleases was performed essentially as recommended by the manufacturers (Bethesda Research Laboratories). 2ug of genomic DNA was digested overnight at 37°C with 5 units of EcoRI in high salt buffer (100mM NaCl, 50mM Tris HCl, pH 7.5, 10mM $MgCl_2$, 1mM Dithiothreitol). To test the completeness of DNA digestions 100ng of bacteriophage λ DNA was added to a sample of the restriction mixture, and incubated overnight, followed by size fractionation in 0.5% agarose gels containing 0.5ug/ml Ethidium Bromide and visualisation of the discrete λ bands under UV illumination.

Electrophoresis of the DNA digestion products was performed in Borate buffer on 0.5% agarose horizontal gels at 4V/cm overnight.

Southern transfer of the DNA was performed by a modification of the procedure described by Southern (1979). After electrophoresis, the gel was stained in Borate buffer containing 0.5ug/ml Ethidium Bromide and photographed. The gel was then

placed in an excess of denaturing solution (0.5M NaOH, 1.5M NaCl) for 30 minutes at room temperature with gentle agitation. The denaturing solution was replaced with an excess of neutralising solution (1.5M NaCl, 3M Sodium Acetate, pH5.5) and the gel gently agitated for a further 30 minutes. Excess solution was drained from the gel which was placed on a perspex support covered with Whatman 3MM filter paper, the ends of which lay in a reservoir containing the transfer sclution, 20x SSC (3M NaCl, 0.3M Sodium Citrate, pH 7.0).

A piece of dry Biodyne A nylon membrane (Pall Ultrafine Filtration Corporation, NY) cut to the size of the gel was placed carefully on the gel surface, ensuring any trapped air bubbles were removed. Two sheets of Whatman 3MM filter paper were placed over the membrane followed by a three inch stack of paper towels, a glass plate and a 1 kg weight. Transfer was allowed to proceed overnight after which the filter was baked for one hour at 80°C.

Nick Translation and Hybridisation

^{32}P labelled probes were prepared by nick translation essentially as previously described (Rigby et al,1977). Generally a specific activity of approximately10^7 cpm/ug was obtained.

Hybridisation of the Southern Blots was carried out as recommended (Pall Ultrafine Filtration Corporation). Briefly, the filters were preincubated for a minimum of one hour at 65°C in 5x Denhardt's solution (1mg/ml BSA, 1mg/ml Ficoll400, 1mg/ml PVP), 5xSSPE (0.9M NaCl, 50mM Sodium Phosphate, pH8.3, 5mM EDTA), 0.2% (w/v) Sodium Dodecyl Sulphate (SDS), 500ug/ml herring sperm DNA. The heterologous DNA was denatured by the addition of 1/10 volume 1N NaOH, heating at 65°C for 10 minutes followed by

neutralisation with 1/10 volume of 1N HCl, 4ml of hybridisation solution was used per 100cm^2 of filter. After preincubation the hybridisation solution was removed and fresh hybridisation solution added, 2ml/100cm^2, along with the labelled probe which had been denatured and neutralised as described above. Hybridisation was allowed to proceed overnight at 65^{o}C.

The filters were initially washed for 30 minutes at room temperature in wash buffer (5mM Sodium Phosphate, pH 6.8, 1mM EDTA, 0.2% (w/v) SDS) with vigorous agitation followed by a final post hybridisational wash in 0.1x SSC, at 65^{o}C for 30 minutes.

RESULTS

Immunofluoresence

The mean percentage labelling of trypanosomes expressing GUTat 7.1, GUTat 7.2 and GUTat 7.13 in the metacyclic population and in the bloodstream of fly infected mice is shown in Tables 1 and 2. There was no increase in the presence of GUTat 7.13 which was the VAT originally ingested by the fly when compared to the other two M-VATs. All three M-VATs persisted at high levels up to day nine post infection in immunosuppressed mice.

Removal of GUTat 7.13 Expressors from Purified Trypanosome Populations

Trypanosomes were purified from the blood of fly infected mice on days 6-9 post infection. Trypanosomes expressing GUTat 7.13 were removed from the population by lysis with a monospecific rabbit antiserum. The lytic reaction removed the expected numbers of trypanosomes, as observed by comparison of Table 2 (level of 7.13 in bloodstream populations assessed by immunofluoresence) with Table 3 (percentage level of lysis).

TABLE 1. Mean percentage labelling of metacyclic trypanosome populations from flies infected with GUTat 7.13 ($x \pm 1SE$).

Variable antigen type	Antibody	Flies fed on GUTat 7.13 N=9	Flies fed on controls N=4
7.13	Rabbit	9.4±1.4	9.4±4.6
7.13	GUPM 17.1	10.4±4.1	14.9±12.2
7.1	GUPM 27.1	31.5±4.5	16.5±6.8
7.2	GUPM 18.7	15.2±3.7	18.5±14.0
5.1	GUPM 10.1	14.4±4.1	15.5±8.7

Figures refer to the mean percentage labelling of trypanosomes in acetone fixed salivary probes from infected flies. As a control GUTat 7.13 infected mice were sub-curatively treated with a single injection of 380mg/ml SHAM plus 3.8gm/kg glycerol and flies fed on the subsequent relapse population in which GUTat 7.13 could not be detected by immunofluoresence (see Turner et al,1985).

TABLE 2.Percentage labelling of three metacyclic VATs in early bloodstream populations of immunosuppressed mice bitten by GUTat 7.13 infected tsetse flies (x ± 1SE).

Days after fly bite	N	M-VAT		
		7.1	7.2	7.13
6	8	9.5±3.5	14.6±1.6	19.6±1.7
7	11	11.1±2.3	16.8±3.3	11.2±2.1
8	8	4.7±2.2	9.9±2.6	12.9±1.9
9	3	7.7±1.5	24.7±8.6	20.3±7.1

Figures refer to the mean percentage labelling of trypanosomes in acetone fixed blood smears taken from several immunosupressed mice on days 6-9 post infection. A total of 200 trypanosomes were counted for each VAT in each sample.

TABLE 3. Percentage of GUTat 7.13 trypanosomes in early bloodstream populations of immunosuppressed mice bitten by GUTat 7.13 infected tsetse flies assessed by an *in vitro* immune lysis assay.

Days after fly bite	%lysis
6	18.7
7	16.6
8	19.5
9	19.1

Figures refer to the mean percentage lysis of trypanosomes from three separate samples where a total of 200 trypanosomes were counted in each sample.

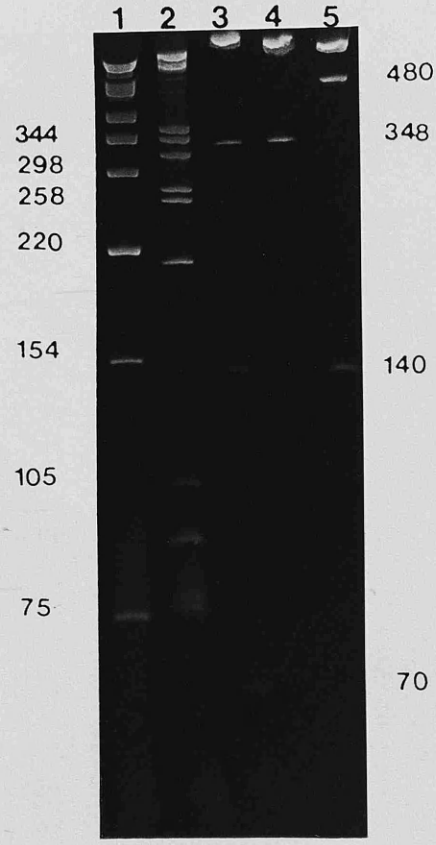


Figure 2 **Mapping of recombinant plasmid TcV 7.13.25**
Electrophoresis was carried out in a 8% polyacrylamide vertical gel and run in Borate buffer. Enzyme digestion was performed with 1 enzyme unit /ug DNA at 37°C overnight.
Track1 pBr322 HinfI
Track2 pBr322 Sau3A
Track3 TcV 7.13.25 PvuII/PstI
Track4 TcV 7.13.25 PvuII
Track5 TcV 7.13.25 PstI

Mapping of Recombinant Plasmid TcV 7.13.25

The recombinant plasmid TcV 7.13.25 contains DNA complementary to VSG mRNA of GUTat 7.13. The cDNA was inserted into the Pst I site of the vector pBR322; the size of the cloned insert is 620 bp (Cornelissen et al,1985). Mapping of this plasmid was carried out using two restriction enzymes Pst I and Pvu II (Figure 2).

Track 5 contains the plasmid DNA digested with Pst I alone. In addition to the large plasmid band two small fragments of 480 and 140 bp can be visualised suggesting the presence of a Pst I site within the cDNA. Track 4 contains the plasmid DNA digested with Pvu II alone and one fragment, 348 bp in length, can be visualised, besides the large plasmid band. The presence of this band suggests there are two Pvu II sites within the cDNA. Given the map of pBR322, which has only one Pvu II site, 2817 bp and 1545 bp from the PstI site, the 348 bp fragment from the Pvu II single digest can only be explained if there are two Pvu II sites within the cDNA. Track 3 contains the plasmid DNA digested with both enzymes. The 348 and 140 bp fragments are present in the double digest. The only way in which these results can be explained is by assuming that there are two smaller fragments of approximately 70 bp comigrating. Due to poor resolution of fragments this size they cannot be seen distinctly on this gel. However in Track 3, containing the double digest, one can see a faint and diffuse band at the correct position.

The resulting map of the cDNA insert in TcV 7.13.25, constructed using the results from the gel in Figure 2 and other mapping gels (evidence not presented), is given in Figure 3. The total length of the insert is 620 bp and it is proposed that

there is a Pst I site approximately 70 bp downstream from the 3' Pvu II site. However there is no corresponding Pst I site in the map of the basic copy (BC) or expression linked copy (ELC) of the 7.13 gene (See Figure 4 and Cornelissen et al,1985). The map produced by Cornelissen et al (1985), was constructed using a different recombinant plasmid, TcV 7.13.28 as a probe. It is possible the discrepancy between the two maps may have arisen from a cloning artifact which has resulted in the production of a new Pst I site in the cDNA.

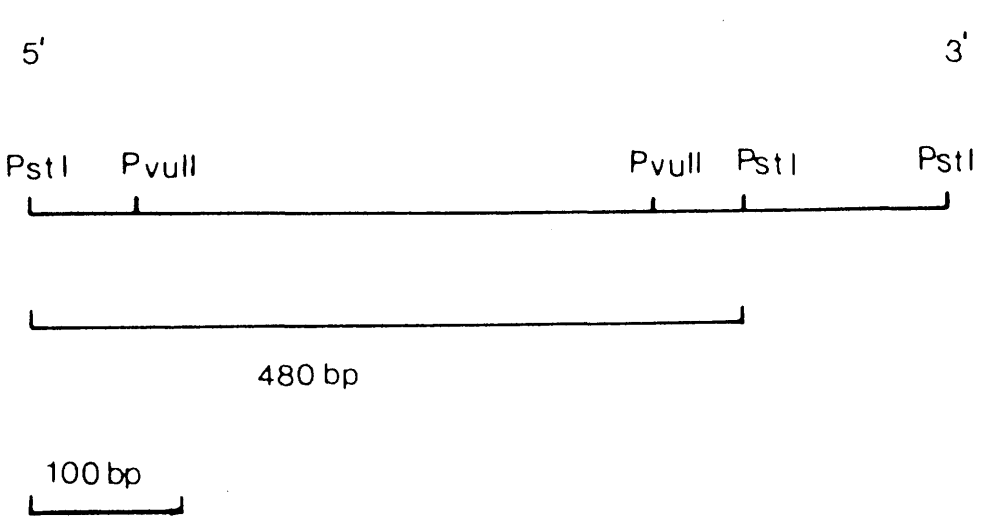


Figure 3 **Physical map of cDNA plasmid TcV 7.13.25.**
Only the insert DNA is shown. 5'/3' orientation derived from Cornelissen et al (1985). The sub-probe isolated from low melting point agarose gels and used for hybridisation extends from the 5' PstI site to the PstI site internal to the cDNA.

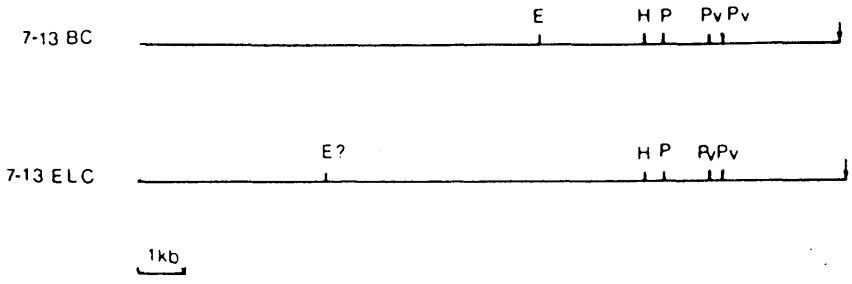


Figure 4 **Physical map of gene copies for VSG 7.13**
The restriction sites were mapped using the cDNA insert from the recombinant plasmid TcV 7.13.28. (See Cornelissen et al,1985).
Arrows indicate the chromosome end.
? indicates site position is uncertain.
Abbreviations of restriction enzymes: E-EcoRI; H-HindIII; P-PstI; Pv-PvuII.

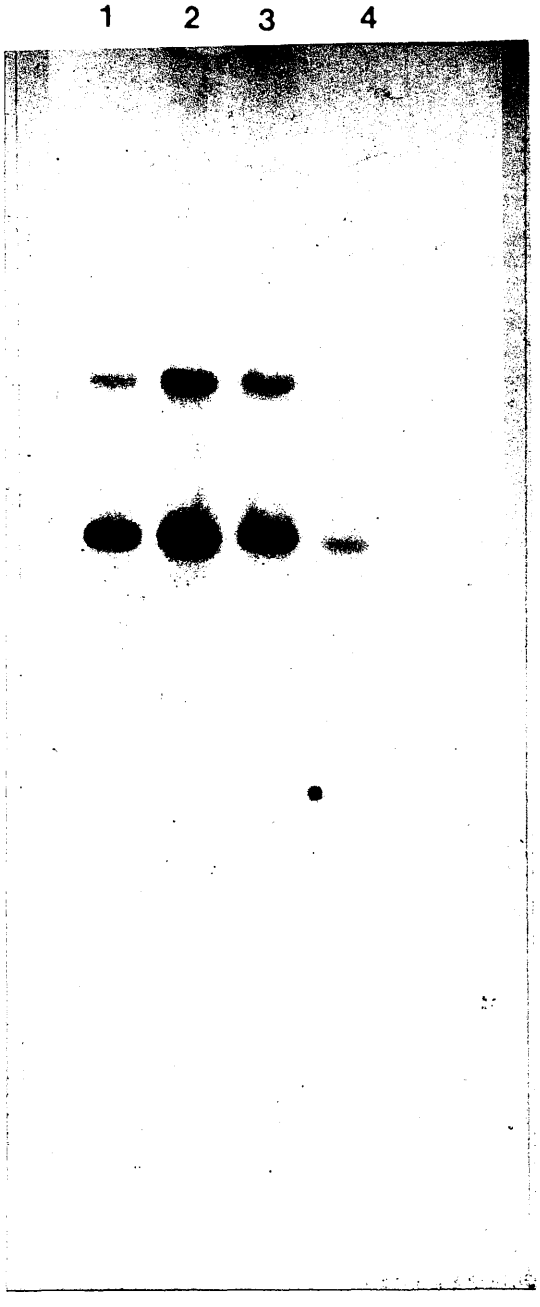
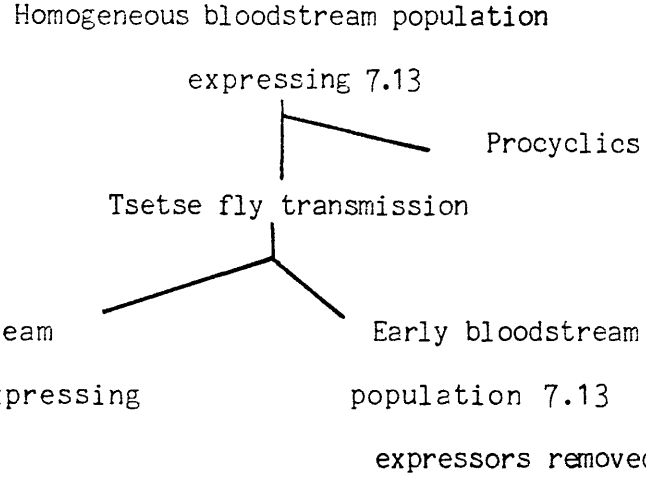


Figure5 **Analysis of GUTat 7.13 after cyclical transmission through the fly.**
Southern blot analysis of the 7.13 gene in four different populations using a cDNA subprobe isolated from the recombinant plasmid TcV 7.13.25 (see figure 2).

Track1 Homogeneous bloodstream population expressing GUTat 7.13.
Track2 Procyclics derived from GUTat 7.13 bloodstream population.
Track3 Early bloodstream population derived from infected fly bites including the 7.13 expressors.
Track4 Early bloodstream population as above but with the 7.13 expressors removed.

Southern Analysis of Genomic DNA

The cDNA subprobe isolated from the recombinant plasmid TcV 7.13.25 and hybridised to EcoR1 digests of genomic DNA's is shown in Figure 2. The four populations used for DNA isolation are:



The results obtained from the hybridisation are given in Figure 5. In all four populations there are two fragments which hybridise with the probe confirming previous results which show 7.13 to be a single copy gene activated by the duplication transposition mechanism of activation (Cornelissen et al,1985). The smaller of the two fragments (10 kb) represents the basic copy (BC) of the gene, the larger (20 kb) represents the expression linked copy (ELC).

Track 1 contains DNA isolated from the population initially ingested by the tsetse flies. Both the BC and ELC are present in this population. Track 2 contains DNA from procyclics derived from the GUTat 7.13 bloodstream population, and both fragments are present. Although VSG gene expression ceases at this stage in the life cycle the ELC is not lost but remains as a non-expressed "lingering ELC" (Overath et al,1983).

Track 3 represents the early bloodstream population (days 6-9) in which 7.13 expressors constituted less than 20% of the

population (See Tables 1 and 2). An extra fragment (8 kb) hybridises with the probe. However this new fragment is at a much lower intensity than any of the other bands. If this new fragment represents a new ELC made by the trypanosomes expressing 7.13 then the lower intensity of this band is explained by the fact that less than 20% of the population are expressing 7.13. The previous two tracks contain DNA derived from populations where almost 100% of the population either were (Track 1) or had been (Track 2) expressing 7.13.

Track 4 represents the same early bloodstream population as in Track 3 except the 7.13 expressors have been removed; the extra fragment is no longer present and thus seems to be specific to trypanosomes expressing 7.13. This suggests that in early bloodstream populations, derived from infected fly bites, a new copy of the 7.13 gene has been made and transposed to a different expression site.

DISCUSSION

An antigenically stable bloodstream population expressing an M-VAT,GUTat 7.13, was cyclically transmitted through tsetse flies and the infected flies used to infect immunosuppressed mice. Tables 1 and 2 show that ingestion of GUTat 7.13 by the fly did not cause it to constitute 100% of the metacyclic or early bloodstream population. The VAT composition of the metacyclic population was investigated directly by screening salivary probes from flies which had been infected with a population homogeneous for 7.13 expression as compared to flies which had been infected with a control population which did not contain 7.13 expressors. No difference in the percentage labelling of the metacyclic population with a McAb directed against 7.13 was observed. Ingestion of GUTat 7.13 by the fly did not lead to 100% of the metacyclic population expressing this VAT. Investigation of early bloodstream populations in mice derived from GUTat 7.13 infected flies indicated that GUTat 7.13 was still present at high levels until day nine in immunosuppressed mice.

From Southern Analysis of genomic DNAs (See Figure 5) the results of Cornelissen *et al* (1985) are confirmed, in that 7.13 is shown to be a single copy gene which is activated in bloodstream forms by duplicative transposition of the BC to an expression site resulting in the presence of two bands, one representing the BC, the other the ELC. As predicted the ELC is retained in the procyclic population and in the two bloodstream populations derived from fly infected mice. In the first of these populations where the 7.13 expressors are retained an extra band is visible. In the population from which 7.13 expressors have

been removed this third band is absent. Therefore the presence of this third band is related to the expression of the 7.13 gene. The intensity of the band is significantly reduced in comparison to those representing the BC and retained ELC suggesting there is only a proportion of the population in which this new copy is made, and as is shown (Table 1) less than 20% of the total population examined are 7.13 expressors.

If the expression of M-VATs and bloodstream VATs (B-VATs) is controlled by the same mechanism one might have expected to see an increase in the expression of 7.13 due to the anamnestic effect whereby expression of the ingested VAT (I-VAT) in the first patent parasitaemia (FPP) is thought to be due to preferential reactivation of a lingering ELC. As the population used to infect the tsetse flies was homogeneous with respect to 7.13 expression (92-97% of the population expressed GUTat 7.13) the majority of trypanosomes entering the fly should have had a 7.13 ELC which would have been inactivated on transformation to procyclics, and as discussed previously, preferentially reactivated following fly transmission. Both metacyclic and early bloodstream populations derived from fly bites were heterogeneous with respect to VAT expression i.e. 7.13 did not constitute 100% of either population. If the ex-ELC is reactivated in preference to other VSG genes one would expect a significantly higher percentage of the population to express 7.13 as all members of that population would contain an ex-ELC. However this is clearly not the case indicating that activation of M-VAT genes prevails over reactivation of an ex-ELC.

Barry et al (1985) have shown that preferential expression of M-VATs continues in early bloodstream populations. As

discussed in the previous chapter VAT expression is loosely programmed in that certain B-VATs always appear early in infection, others later. Amongst the early or predominant VATs the I-VAT is always expressed. How this preferential activation of M-VATs and the non-random expression of B-VATs is achieved remains unknown. However several observations have indicated that the position of a VSG gene on a chromosome can affect its activation frequency, in that those VSG genes activated early in infection are invariably telomeric.

Michels et al (1984) carried out an extensive analysis of the phenomenon of lingering ELCs, studying VSG gene 118, a chromosomal internal gene activated by the duplicative mode of activation. A trypanosome population expressing 118 was allowed to switch to the expression of a telomeric gene 1.8. Trypanosomes expressing 1.8 retained the previous 118 ELC in an inactive form. Those variants where the 118 ELC had been retained were found to switch back to expression of 118 at a high frequency. The authors suggest that the ability to retain an ELC in an inactive form results in a new telomeric gene, which by virtue of its new chromosomal location can be activated early in infection and thus become a predominant gene. Therefore the trypanosome has the ability to reset the programmed order of gene expression.

Laurent et al (1984a) made similar observations studying the fate of AnTat 1.13 which appears late in infection and is a chromosome internal gene activated via the production of an ELC. Again a trypanosome population expressing AnTat 1.13 was allowed to switch to expression of a telomeric gene AnTat 1.6. This led to the conservation of the 1.13 ELC as a new telomeric gene and the authors found this ELC was preferentially activated. In this

case the ex-ELC was reactivated via duplicative transposition. In the case described by Michels et al (1984) reactivation of the ex-ELC involved no duplication. Laurent et al (1984a) found that in those trypanosomes which retained the AnTat 1.13 ELC as a new telomeric gene AnTat 1.13 had become a predominant VAT which again suggests that predominance is linked to a telomeric position in the genome.

It has been suggested that the telomeric position of VSG genes leads to their predominance because of increased ability to recombine with telomeric expression sites. However not all telomeres can undergo recombination with equal efficiency. Pays et al (1983b) investigating a variant AnTat 1.3B found there were two copies of the 1.3 gene, both telomeric. One was the BC, the other an ex-ELC. Yet on reactivation of the AnTat 1.3 gene it was the ex-ELC which was duplicated to form a new ELC. Therefore predominance is not simply as a result of residing within a telomere.

What controls the preferential reactivation of an ex-ELC remains unknown. In looking at putative modifications of GC dinucleotides in telomeric sequences (which are thought to prevent expression of inactive telomeres) Pays et al (1984) found that the ex-ELC showed identical modifications to those found in the BC. Also testing for differences in chromatin structure via DNase 1 sensitivity has shown no significant differences between the BC and ex-ELC Pays et al (1983b). The fact that transfer of a late gene to a telomere in the form of an ex-ELC can result in the transformation of that gene to an early gene suggests preferential activation of predominant genes is a function of the telomere in which the gene resides rather than a function of the

gene itself.

Laurent et al (1984b) have suggested that the degree of sequence homology between the gene and the expression site could play a role in timing. These homologies are normally provided by the 70 bp repeats found upstream of VSG genes and a common sequence at the 3' border of the gene. However both the ex-ELC and BC of AnTat 1.3B have ample 5' repeats upstream of the gene so in this case differences in the extent of homologies surrounding the gene cannot explain the preferential reactivation of the ex-ELC.

Thus the timing of expression of bloodstream VSG genes appears to be influenced by their genomic location, telomeric genes being expressed early in infection. Metacyclic VSG genes however show dominance of expression over predominant early bloodstream genes. The origin of this dominance is unknown.

It is possible that the metacyclic VSG genes could be located within specialised genomic regions which allows for their selective activation in the salivary glands of the fly or alternatively the mode or site of activation used by these genes could differ from that utilised by bloodstream genes.

Recently Lenardo et al (1984) have characterised two metacyclic genes of the WRATAR serodeme of T.b.rhodesiense. The mRNAs corresponding to these metacyclic VSGs have all the characteristics of mRNAs corresponding to bloodstream VSGs. Both genes are single copy telomeric genes as judged by Southern Blot Analysis and digestion of DNA with Bal 31. Unlike telomeric linked bloodstream VSG genes these two metacyclic genes are not preceded by upstream barren regions. In both cases a continuous distribution of restriction enzyme sites was found upstream from

the gene. No ELC could be found for either gene suggesting they were activated by a non duplicative mode of activation.

Cornelissen et al (1985) have carried out a similar study of four bloodstream VATs that correspond to four VATs (including GUTat 7.13) of the metacyclic repertoire of the GUTAR serodeme of T.b.rhodesiense. Three of these B-VATs contain a single gene for its VSG implying the same gene can be expressed both in the bloodstream and metacyclic repertoires. All genes were telomeric and in the three cases where the chromosomal location of the genes was identified they were found in the large DNA fraction which remains in the slot in PFGE analysis. All four genes were activated by duplicative transposition in bloodstream trypanosomes.

In the case of GUTat 7.13 described in this work the presence of a third extra band specific to trypanosomes expressing GUTat 7.13 indicates a new copy of the 7.13 gene has been made and transposed to a new expression site. This new site could in some way be specific for metacyclic genes. The new copy observed could be a duplicate of either the BC or the retained ELC which has been reactivated. The presence of one expression site specific for metacyclic genes implies this site must be different in some way from other expression sites to allow specific activation in the fly and also to allow only a particular subset of genes to be transposed into the site. If insertion into expression sites is brought about by telomere conversion involving sequence homologies, as has been suggested, then why a particular subset of genes can be inserted into this site is not obvious. The sequence homologies involved in this conversion are thought to be provided, in part, by the 70 bp

repeats found upstream of VSG genes. No such barren region has been found upstream of the metacyclic genes of the WRATAR serodeme however neither of these genes were activated via the production of an ELC. It is possible the absence of the 70 bp repeats indicates that these genes are restricted to activation without a concomitant duplication.

Cornelissen et al (1985) report that the BCs of the four M-VAT genes they have investigated do not contain as large barren regions as are found upstream of ELCs active in the bloodstream. They have however reported from Southern blotting and hybridisation analysis that for the 7.1 gene there are 70 bp repeats 5' to this gene, suggesting therefore that the absence of such repeats is not common to all metacyclic genes. They also point out that often 70 bp repeats are imperfect in terms of sequence which can lead to occasional restriction enzyme sites being formed. As Lenardo et al (1984) did not carry out direct hybridisation with probes containing the 70 bp repeat unit, their evidence for the absence of barren regions upstream from the two genes analysed is indirect, being based on restriction enzyme analysis and it is possible therefore that imperfect repeats are present in front of these genes.

Because of the difficulty involved in looking at the metacyclic population directly it is necessary to analyse early bloodstream populations where M-VAT genes are still being expressed. It remains a possibility therefore that in the salivary glands of the fly the metacyclic VSG genes are being activated in situ and on entry into the mammalian bloodstream these genes are transposed into bloodstream expression sites. If in situ activation of metacyclic genes does occur this implies

that there are a limited number of telomeres activated specifically in the fly and that some form of exclusion mechanism must exist such that only one telomere is activated in each trypanosome. Lenardo et al (1984) investigated populations derived on day five post infection and found no evidence that the two metacyclic genes were activated by the production of an ELC. This would suggest that duplicative transposition to a "bloodstream" expression site on entry into the mammal is not essential for continual expression of the two M-VATs.

Lenardo et al (1984) also found that the telomeres containing metacyclic genes did not undergo size variation to the same extent as other telomeric VSG genes. In the present study there was also no apparent variation in size between the BCs and ELCs present in all four populations analysed.

Cornelissen et al (1985) found that for the 7.2 gene this was also the case. However the remaining three genes did show similar size variations to other telomeric genes so it is unlikely that this is a general feature of all telomeres which carry metacyclic genes.

The present results suggest that for the metacyclic gene 7.13 a new copy of the gene is made either in the metacyclic population in the fly, or in early bloodstream populations, after cyclical transmission. Extensive mapping or sequencing would be necessary to find whether this new copy is a duplicate of the BC or the ex-ELC. Also DNase 1 analysis of this new copy should be carried out to elucidate whether or not it is apparently transcribed. Telomeric gene conversions do not always correlate with activation of the gene converted (Liu et al,1985). However the presence of this new copy of the 7.13 gene was directly

correlated with the presence of 7.13 expressors within the population. The intensity of the band suggests only a proportion of the population make this new copy, and as only approximately 20% of the population examined are 7.13 expressors this would support the suggestion that the presence of the new copy is indeed correlated with expression of the gene.

The experiments described were carried out in an attempt to elucidate the nature of control of metacyclic VSG gene expression as compared to the expression of bloodstream VSG genes. Are the two sets of genes controlled by the same mechanism ? As pointed out previously by using a metacyclic gene as an ingested VAT it is possible, by looking at early bloodstream populations derived from GUTat 7.13 infected flies to answer this question. The hypothesis on which the experiments were based is as follows: if the same control mechanisms are in operation for both sets of genes then one would expect to see a higher proportion of the metacyclic population expressing 7.13, the ingested VAT, due to the preferential reactivation of the ex-ELC.

Not only was no increase of 7.13 expression observed, but in the early bloodstream populations, derived from fly infected mice, in which 7.13 expressors remained, a new ELC was made, while the same population, in which the 7.13 expressors had been removed, this new ELC is no longer present, correlating the presence of the new ELC directly with the presence of 7.13 expressors.

It could be argued that the new ELC observed is the retained ELC which has been reactivated. However all the trypanosomes entering the fly should have this retained ELC and the fact that only a proportion of the population should reactivate it, when

evidence exists to suggest that ex-ELCs show preferential reactivation over other genes is difficult to explain. It would seem more likely that that proportion of the metacyclic population which is expressing GUTat 7.13 does so by the production of a new ELC. Perhaps this new ELC is residing in an expression site which is specific for metacyclic genes.

The 7.13 population described here is a metacyclic clone i.e. was cloned directly from the metacyclic population which would therefore imply that its ELC should be occupying a metacyclic specific site, if one exists. Also transposition into a specific site does not account for the observations of Lenardo et al,(1984) where neither of the genes investigated were activated by duplication. Obviously the proposal of an expression site specific for M-VAT genes implies not only that this site must in some way differ from others but also that only a specific set of genes can be transposed into it. As yet no obvious difference can be found between M-VAT genes and B-VAT genes such that a mechanism may operate to allow for specific transposition of M-VAT genes into a "metacyclic" expression site.

In conclusion the evidence presented does support the view that the metacyclic genes are expressed preferentially over other genes and it is therefore likely that they are controlled by different mechanisms.

CHAPTER 3

Comparison of M-VAT genes in *T.b.rhodesiense* from two different foci

INTRODUCTION

Trypanosomes of the sub-genus Trypanozoon are causitive agents of trypanosomiasis in Africa.In medical and economic terms the trypanosomes transmitted by the tsetse fly (Glossina sp.) are the most important. These include the agents of human trypanosomiasis, Trypanosoma brucei gambiense and T.b.rhodesiense, and T.b.brucei which causes trypanosomiasis of domestic and game animals but is not human-infective. Two other species, which are also found outwith Africa, are T.evansi and T.equiperdum which are closely related to T.b.brucei and are thought to have descended from T.b.brucei but have replaced transmission by the tsetse vector with mechanical means of transmission, Tabanid flies in the case of T.evansi and in the case of T.equiperdum complete loss of the vector, T.equiperdum being transmitted directly from one mammal to another via sexual contact. The loss of dependence on the insect vector for transmission of these two species has allowed their spread outwith the tsetse fly belts of Africa. For those species transmitted by the tsetse their distribution coincides with, and is confined to, that of the fly.

For many years T.b.gambiense, T.b.rhodesiense and T.b.brucei have been awarded separate sub-species status. They are morphologically identical but differ in their geographical distribution, host specificity and in the nature of the disease they cause (Hoare,1972).

T.b.brucei is widespread throughout Africa and infects domestic and wild animals but not man. The main vectors are

tsetse flies belonging to the G.morsitans group but transmission by flies of other groups has been demonstrated (Ford,1971).

T.b.gambiense is confined mainly to West and Central Africa but has been found as far east as Uganda and Tanzania. It is transmitted in West Africa mainly by the "riverine" tsetse flies, (the G.palpalis group, G.palpalis and G.tachinoides) and in East Africa by G.fuscipes (Ford,1971). T.b.gambiense is thought to be maintained mainly where there is close human-fly contact and for this reason is considered to be a "peridomestic" disease i.e. the infection is usually acquired near the victim's home. For many years it was believed that there was no non-human reservoir and the parasite was dependent wholly on human-fly contact though experimental infection of wild and domestic animals with trypanosomes isolated from humans suggested there was a potential for certain animals to act as reservoir hosts (Van Hoof,1947) and recent biochemical analysis suggests that stocks isolated from domestic animals may indeed be T.b.gambiense (Tait et al,1984; Paindavoine et al,1986). T.b.gambiense causes a chronic form of sleeping sickness in humans, the patient often surviving a number of years without treatment though it is invariably fatal if left untreated. One of the features of chronic sleeping sickness is the low parasitaemias found in the patients suggesting T.b.gambiense is well adapted, in parasitic terms, to utilising humans as a host.

T.b.rhodesiense is limited in distribution to East Africa. The chief vectors belong to the G.morsitans group. They include G.morsitans, G.swynnertoni and G.pallidipes which are less susceptible to dessication and inhabit mainly the savannah-like woodland abundant in East Africa (Ford,1971). Habitation on these

plains tends to be scattered and often causes the driving away of both the tsetse and game from the vicinity of humans. Therefore fly-human contact is low except in those situations where some activity or occupation requires prolonged absence from the community. Often the incidence of trypanosomiasis is found to be higher in men as they are traditionally the ones who leave the villages to hunt etc. Unlike T.b.gambiense, T.b.rhodesiense is found naturally in a large selection of game animals which act as reservoirs for the disease. T.b.rhodesiense causes acute sleeping sickness which results in high parasitaemias and is fatal within months if untreated.

Since the original definition that T.b.brucei, T.b.rhodesiense and T.b.gambiense were separate sub-species was made (Hoare,1972) observations have suggested this may not be the case and the division of this group of trypanosomes on the basis of geographical distribution, host specificity and the course of the disease in humans is inaccurate. The trypanosomes of the T.b.brucei group are identical morphologically and in the past discrimination of these species was based on their ability to infect human volunteers, which as well as being questionable on ethical grounds often gave equivocal results. However Rickman and Robinson (1970) designed a test, based on observations of other workers, that human blood or plasma had a trypanocidal effect on T.b.brucei but not T.b.rhodesiense, to differentiate between the two. This test is of limited use however, and again often leads to equivocal results (see Gibson et al,1980).

More recently attempts have been made to characterise the trypanosomes of the T.b.brucei group by biochemical means. Gibson et al (1980) looking at electrophoretic variation in twelve

enzymes in a number of stocks of the subgenus Trypanozoon and using statistical analysis to test the relatedness of the stocks concluded they were too similar to be awarded separate species status. However enzyme variants specific to T.b.gambiense have been found (Godfrey and Kilgour,1976).

Tait et al (1984) also found enzyme variants specific for T.b.gambiense stocks allowing them to be distinguished from "non-T.b.gambiense" stocks. On analysing resistance to human serum and correlating it with the presence or absence of T.b.gambiense specific variants stocks which showed resistance to human serum contained T.b.gambiense specific alleles. However three of the stocks analysed in this way were isolated from domestic animals in Zaire and the Congo. All showed resistance to human serum and all had T.b.gambiense specific enzyme variants suggesting that domestic animals can act as reservoirs for T.b.gambiense. The conclusion drawn from such analysis is that T.b.gambiense can be distinguished from T.b.rhodesiense and T.b.brucei but does not constitute a separate species, but rather could be considered a sibling species.

In a similar analysis looking at enzyme variation in groups of stocks designated as T.b.brucei (isolated from cattle in Nigeria and tsetse flies in Kiboko, Kenya) and T.b.rhodesiense (isolated from humans or tsetse flies in Central Nyanza, Kenya) no enzyme variants were found to be specific to the the T.b.rhodesiense stocks (Tait et al,1985). It was concluded that T.b.rhodesiense and T.b.brucei were not seperate sub-species but rather, the T.b.rhodesiense stocks constituted a set of variants of T.b.brucei which have the ability to infect man. Another interesting result was that the T.b.rhodesiense stocks analysed

were found to be more homogeneous than the T.b.brucei stocks. This homogeneity was also detected within two collections of stocks from Zambia (Gibson et al,1980) and Uganda (Gibson and Gashumba,1983). It would therefore appear that T.b.rhodesiense stocks isolated from particular areas in Africa show marked homogeneity with respect to enzyme variants.

Serological evidence has been presented which suggests that within a given area many T.b.rhodesiense populations belong to a single serodeme (Barry et al ,1983). In that study sixteen of twenty-six stocks isolated from man, tsetse flies and game animals in the sleeping sickness focus along the North-east shore of Lake Victoria between 1958 and 1979 were found to belong to the same serodeme suggesting that stocks of T.b.rhodesiense isolated from a given area are homogeneous with respect to VAT. The remaining ten stocks need not necessarily belong to a different serodeme: the limited extent of antigenic screening was insufficient in this respect. Isharaza (1985) found that stocks isolated in Busoga, Uganda and Kagera Park, Rwanda did not share any VATs and were postulated to belong to two different serodemes whereas stocks from Busoga shared a number of VATs and were classed into three serodemes, each sharing several isotypes (similar VATs expressed in different antigen repertoires) with the others. Therefore homogeneity found in different T.b.rhodesiense stocks isolated from the same geographical area, in terms of enzyme polymorphisms is also found to a degree with regard to antigen repertoire.

DNA analysis has also been used in an attempt to differentiate between members of the T.b.brucei group. Borst et al (1980a) compared DNA from several T.b.brucei stocks with DNA

from T.evansi and T.equiperdum using restriction enzyme digestion and electrophoresis. Digestion of genomic DNAs from the different species gave a characteristic and reproducible series of fragments for each species, and even between different T.b.brucei strains but not between three different antigenic variants from the same T.b.brucei clone.

This approach was also used in the analysis of kinetoplast DNA (kDNA) found in trypanosomes. This is an unusual form of mitochondrial DNA (mtDNA) consisting of networks of 10^4 catenated circles of two types, the mini-circle (0.9 kb) which constitute 90% or more of the network, and are heterogeneous in sequence and maxi-circles (20 kb) which are akin to mtDNA in other organisms, and are highly conserved in sequence. Borst et al (1980c;1981) used restriction enzyme analysis of kDNA as a means of differentiating different T.b.brucei strains. The mini-circle DNA gave rise to a complex series of bands and it was suggested that mini-circle DNA evolves so rapidly that major sequence differences exist between strains of T.b.brucei. In contrast the maxi-circle DNA from all stocks tested was so similar in sequence it precluded their differentiation by this means. Gibson et al (1985) extended this analysis and found there were two maxi-circle sub-types: "Kiboko" which is widespread in East Africa and "Sindo" which originate from one locality. However as a means of differentiating between the three sub-species analysis of kDNA is not sufficient due to sequence similarities between the three sub-species.

Analysis of trypanosome DNA has been extended by the use of Southern hybridisation with DNA probes corresponding to specific trypanosome genes. Pays et al (1981c;1983e) used two VSG cDNAs

cloned from T.b.brucei (AnTat 1.1 and AnTat 1.8) in hybridisation analysis. The 1.8 genes were found in T.b.brucei, T.b.gambiense, T.b.rhodesiense and T.evansi. In contrast, the 1.1 gene was missing from all fifteen T.b.gambiense stocks analysed. It was however found in the two T.b.rhodesiense and the one T.evansi stock tested although the sequence was modified in comparison to that found in T.b.brucei. Hybridisation with the AnTat 1.8 probe revealed a pattern of bands in the T.b.gambiense stocks that was characteristic for these stocks and differed from the pattern of bands found in the other sub-species. Therefore the use of VAT-specific probes in hybridisation analysis could be a useful means of discriminating different sub-species. The 1.1 probe could be used as a non-T.b.gambiense probe whereas the 1.8 probe revealed a restriction enzyme digestion pattern specific to the T.b.gambiense stocks.

Paindavoine et al (1986) also applied this technique in a study of stocks of the T.b.brucei group. The T.b.brucei, T.b.rhodesiense and T.evansi stocks were designated as "non-T.b.gambiense". Use of three probes corresponding to different VATs revealed that the pattern of VSG specific bands was highly conserved in all the T.b.gambiense stocks but was highly variable in the non-T.b.gambiense stocks, in none of which was the pattern similar to that of the T.b.gambiense stocks. Additionally two undefined probes, obtained from a T.b.gambiense genomic DNA bank screened in such a way as to increase the chance of selection of T.b.gambiense specific sequences were used. One hybridised to DNA of all stocks tested though in the non-T.b.gambiense stocks again the banding pattern was more variable than in the T.b.gambiense stocks. The other hybridised only very

weakly with the non-T.b.gambiense stocks. Also certain stocks isolated from domestic animals in West Africa were identical, with respect to the analysis carried out here, to T.b.gambiense, providing further support that non-human reservoirs of T.b.gambiense exist. The relationships between the different stocks were expressed as dendograms. The conclusions drawn were in support of those of Tait et al (1984), that T.b.gambiense stocks were more homogeneous than the non-T.b.gambiense stocks, and further it should be awarded sub-species or sibling species status within the T.b.brucei group. In contrast T.b.rhodesiense and T.b.brucei were indistinguishable by the criteria used and no evidence exists to suggest that T.b.rhodesiense is a separate sub-species, but rather is a variant of T.b.brucei able to infect humans.

In the light of this more recent evidence based on biochemical and DNA hybridisation analysis the validity of separating the T.b.brucei group into three distinct sub-species is questioned, the evidence suggesting rather that T.b.gambiense is a distinct sub-species and T.b.brucei and T.b.rhodesiense are variants of the same species, the latter having the ability to infect man.

The question of species status of the T.b.brucei group is relevant when considering the epidemiology and origin of T.b.rhodesiense. Human trypanosomiasis has been known in West Africa for more than 600 years (Nash,1960). However before colonisation by European powers few reports, and those almost exclusively from West Africa, were made of the disease. This is not to suppose that epidemic outbreaks of the disease did not occur, however it is likely that with the relative lack of

movement of populations and communities such oubreaks as did occur were smaller in scale to those recorded in the early 20th century. Todd (1906) gives an account which demonstrates that European development of the Congo River and its many tributaries led to the accelerated spread of the disease into regions in which it had previously been unknown. By 1905 chronic sleeping sickness had reached the Northern and Eastern shores of Lake Tanganyika, and it is estimated that during this advance approximately half a million people died of the disease (Duggan,1970). Simultaneously an epidemic broke out in Uganda reaching the Southern-most shores of Lake Victoria by 1903 (Ashcroft,1959).

In contrast to the long recorded history of T.b.gambiense in Africa the first record of T.b.rhodesiense came from the Luangwa Valley in North Rhodesia (now Zambia) early this century (Stephen and Fantham,1910). Ormerod (1961) mentions an early report suggesting that sleeping sickness was not new to this area but may have existed in the Luangwa Valley for many years but had not been diagnosed. The trypanosomes isolated by Stephen and Fantham caused a more acute form of the disease in laboratory animals than those causing chronic sleeping sickness in West Africa. They named this strain of trypanosome T.b.rhodesiense.

In Tanganyika (now Tanzania) no reports of T.b.rhodesiense occured until the 1930's. However incidences were recorded at the border of Tanzania and Mozambique, but by and large reports of sleeping sickness in the south were sporadic in nature. In 1920 an epidemic broke out in the North of the country which did not spread greatly and eventually ceased after 10 years . At the same time, towards Central Tanzania a similar epidemic broke out and

spread to the shores of Lake Victoria and Lake Tanganyika (Fairbairn,1948). Ormerod (1961) points out that the initial cases of T.b.rhodesiense sleeping sickness recorded from the Luangwa Valley and Nyasaland (now Malawi) were fairly chronic whereas those recorded in the epidemic in Tanzania were more acute. As the disease spread Northwards the virulence of the parasite apparently increased. It was expected that the epidemic which broke out in South and Central Tanzania would spread into Uganda but it was not until 1940 that T.b.rhodesiense was first recorded in a region near Kampala, called Busoga. The disease was particularly acute, victims dying within 4-6 weeks of diagnosis. The disease spread from Busoga and into Kenya and is now endemic in both areas (Apted,1970).

As mentioned previously, the existence of chronic sleeping sickness has been recorded for many years in West Africa whereas the first recorded incidence of acute sleeping sickness was at the beginning of this century. This has led to much speculation as to the origin of T.b.rhodesiense. It is believed T.b.gambiense is the older of the two types in evolutionary terms. This stems not only from the long historical records of chronic sleeping sickness but also from a biological perspective; because of its lower virulence in humans it is thought to be better adapted to human hosts. High virulence resulting in rapid death of the host is not conducive to either spread or surival of the parasite. Also, the presumed lack of non-human hosts in the transmission of T.b.gambiense has suggested better adaptation to human hosts due to longer association with them. It is assumed that both T.b.gambiense and T.b.rhodesiense arose initially from T.b.brucei, and thus two possibilities exist for the evolutionary

origin of T.b.rhodesiense, either it arose from T.b.gambiense or both evolved from T.b.brucei independently.

Willet (1965) supports the former view, suggesting T.b.rhodesiense evolved when T.b.gambiense was introduced to the savannah areas of South East Africa from the G.palpalis fly belts of the Congo to the G.morsitans belts of Zambia and Malawi. He suggests that virulent strains of T.b.gambiense, capable of infecting game animals, on which G.morsitans feeds, were selected for, allowing the establishment of an animal-fly-animal cycle and in those situations where flies fed on humans these virulent strains caused acute sleeping sickness.

Ormerod (1961) supports the independent origin of T.b.rhodesiense and T.b.gambiense and suggests the original strains of T.b.rhodesiense which eventually gave rise to the epidemics in Malawi, Tanzania and Uganda probably arose in the Zambezi Basin, being first detected in the Luangwa Valley. He suggests that the remoteness of the Zambezi Basin from any known T.b.gambiense focus makes it most unlikely that these strains arose from T.b.gambiense but rather they arose independently from T.b.brucei.

If the two nosodemes arose from T.b.brucei independently a possible scenario can be postulated whereby a mutant or variant population capable of infecting humans arose and, after many years association with humans, selection pressure would operate towards a reduction in virulence to humans in those areas where direct man-fly contact was possible. Reduction in virulence resulting in prolonged survival of the human host would increase the chances of transmission of the parasite from host to fly and in those areas where non-human reservoirs are scarce this low

virulence trend would be of obvious benefit to the parasite. In those areas where non-human hosts were prevalent i.e. in the East African savannah selection pressure favouring lower virulence towards humans would not be necessary. In essence the origin of T.b.rhodesiense and T.b.gambiense could be regarded as an evolutionary response of the one species T.b.brucei to two different environments. Ormerod (1961) points out the northward spread of T.b.rhodesiense is correlated with increased virulence. Generally the most chronic forms of sleeping sickness caused by T..rhodesiense are found in the south of East Africa i.e.Botswana, the most virulent in Ethiopia (Baker,1970). Those strains which are geographically intermediate also exhibit intermediate virulence thus supporting the view that longer association with humans has induced a reduction in virulence. In the light of more recent evidence based on biochemical analysis of the T.b.brucei group the proposal of a separate origin of the two species of trypanosome which cause human sleeping sickness seems the more likely.

Throughout the fly belt of Africa sleeping sickness is not distributed homogeneously but rather discrete foci of infection exist where the disease is endemic. It is from these foci of infection that epidemics arise. Many of the T..gambiense foci have been in existence for so long that knowledge of them has passed into tribal folklore (see Duggan,1970). In contrast the endemic foci of acute sleeping sickness in East Africa have arisen more recently and one of the central issues regarding the origin of T.b.rhodesiense is whether all the endemic foci have originated from southern T.b.rhodesiense strains or whether they have arisen de novo from T.b.brucei.

Ormerod (1961) gives evidence to suggest T.b.rhodesiense can arise at individual foci which are particularly apparent when they lie outside the T.b.rhodesiense distribution area. From biochemical evidence it is clear that T.b.rhodesiense isolated from the same areas in East Africa exhibit great homogeneity both serologically and enzymatically yet differences have been found between the northern and southern stocks both in kDNA and enzyme variation (Borst et al,1981; Gibson et al,1980). This evidence would therefore support the view that northern T.b.rhodesiense strains have arisen de novo from T.b.brucei rather than spreading from southern strains.

In this study a collection of nine stocks isolated from a sleeping sickness focus in the Luangwa Valley, Zambia have been cloned and used in comparative serodeme analysis. Serological and DNA hybridisation analysis using end infection antisera (repertoire antisera) and cDNA probes from trypanosomes isolated in the Kenyan focus were carried out to investigate the degree of relatedness and whether stocks from these two East African foci belong to the same serodeme.

MATERIALS AND METHODS

Trypanosomes

Nine stocks of T.b.rhodesiense which were isolated from an endemic focus of sleeping sickness in the Luangwa Valley, Zambia in 1982-83 and cryopreserved at the Tropical Disease Research Centre (TDRC) at Ndola were used in this study and are listed below:

CODE	LOCATION	HOST	DATE
DA/ZM/83/TRPZ/267	Zambia,Kasyasya,Katyetye	Goat	1983
DA/ZM/83/TRPZ/273	Zambia,Kasyasya,Katyetye	Goat	1983
MAN/ZM/82/TRPZ/182	Zambia,Kasyasya,Katyetye	Human	1982
MAN/ZM/82/TRPZ/199	Zambia,Kasyasya,Katyetye	Human	1982
MAN/ZM/82/TRPZ/203	Zambia,Kasyasya,Katyetye	Human	1982
MAN/ZM/82/TRPZ/220	Zambia,Kasyasya,Katyetye	Human	1982
MAN/ZM/82/TRPZ/221	Zambia,Kasyasya,Katyetye	Human	1982
MAN/ZM/82/TRPZ/231	Zambia,Kasyasya,Katyetye	Human	1982
MAN/ZM/82/TRPZ/244	Zambia,Kasyasya,Katyetye	Human	1982

DA-isolated from domestic animal.

ZM-Zambia.

Stocks were obtained courtesy of Dr.P.Dukes (see also Dukes et al,1983).

Growth of Trypanosomes

Each stock was inoculated from stabilate intraperitoneally into female CFLP mice treated with Cyclophosphamide (CY) (250mg/kg body weight) 24 hours before infection. When high levels of parasitaemia were observed blood samples were taken and used for cryopreservation and cloning.

Cryopreservation of Trypanosomes

Dimethylsulfoxide (DMSO) was mixed with heparinized blood to a final concentration of 7.5%. Blood was then injected into 0.63mm diameter plastic tubing (Portex) and the tubing cut into 2cm lengths and placed in 1.8ml cryotubes (Nunc). The cryotubes were wrapped in cotton wool and placed in a small polystyrene box which was transferred to the vapour phase of a liquid Nitrogen container and left overnight, then transferred directly into liquid Nitrogen.

Cloning of Trypanosomes

Trypanosomes were cloned using direct observation of isolated single cells in Terasaki plates (Flow Labs.,Irvine) maintained humid with wet tissue paper inserts. Single drops of infected blood appropriately diluted with guinea pig serum (GPS) were dispensed with a pin into each well and the plate examined at x200 magnification on an inverted microscope. Wells containing one trypanosome were identified, witnessed by another observer and immediately filled with 10ul of GPS. The samples were then taken up with a micropipette ensuring all liquid was removed from each well and inoculated intraperitoneally into CY-treated CFLP mice.

Antisera

Repertoire antisera directed against large numbers of VATs from each cloned Zambian stock were produced by infecting single mice with each stock and collecting sera at least one month after infection. Such repertoire antisera directed against a series of cloned Kenyan and Ugandan stocks prepared in rabbits as described

by Van Meirvenne et al (1975) were also used. These repertoire antisera are listed below:

CODE	LOCATION	HOST	DATE
A	Uganda	Vector	1960
B	Uganda	Vector	1960
C	Uganda	Vector	1960
D	Uganda	Vector	1960
E	Kenya	Human	1961
F	Kenya	Human	1977
G	Kenya	Human	1977
H	Kenya	Human	1979

It should be noted that these locations span the border and are within one focus of acute sleeping sickness.

Immune Lysis Tests

Immune lysis tests, as described by Van Meirvenne et al (1975) were performed on the cloned Zambian stocks using the Ugandan and Kenyan repertoire antisera. The Zambian cloned populations were tested during the first patent parasitaemia usually about day 5-6 post infection, and trypanosomes from two or sometimes four ensuing peaks throughout the course of infection were also tested. The reciprocal tests were also done, testing the Zambian repertoire antisera against two antigenically stable clones derived from stock EATRO 2340, isolated in the Kenyan focus in 1977. These two clones correspond to two M-VATs from the GUTAR 7 serodeme, GUTat 7.1 and GUTat 7.2.

Dot Blot Analysis of DNA

Trypanosomes were grown in CY treated CFLP mice and isolated from blood by ion-exchange chromatography (Lanham and Godfrey,1970). The trypanosomes were concentrated by centrifugation at 1000g,15 minutes at room temperature and gently resuspended at a concentration of 10^8-10^9/ml in 6:4 phosphate saline glucose (PSG), pH 8.0 + 15% glycerol. The suspensions were stored in 1ml Eppendorf tubes at -20°C.

The spot test method used was a modification of that described by Massamba and Williams (1984). Ten-fold serial dilutions of the trypanosome suspensions (10^3-10^9/ml) were prepared in PSG + 15% glycerol. Aliquots of 10ul of each dilution were applied to dry Biodyne A nylon membrane (Pall Ultrafine Filtration Company, Glen Cove, NY 11542).

The membranes were dried at room temperature for one hour. The samples were denatured in an excess of 0.5M NaOH, 1M HCl for 30 minutes with gentle agitation, neutralised for one hour in an excess of 1M Tris HCl, pH 7.4, 3M NaCl at room temperature and baked for two hours at 80°C.

Before hybridisation with cDNA probes the filters were preincubated for at least one hour at 65°C in the hybridisation solution which consisted of 5x Denhardt's solution (1mg/ml BSA, 1mg/ml Ficoll-400, 1mg/ml PVP), 5x SSPE (0.9M NaCl, 50mM Sodium Phosphate, pH 8.3, 5mM EDTA), 0.2% w/v sodium dodecyl sulphate (SDS), 500ug/ml herring sperm DNA.

Nick translation of the cDNA probes was as described in Chapter 2.

After preincubation the hybridisation solution was removed and fresh hybridisation solution containing the ^{32}P labelled

probe, which had been denatured and neutralised, was added. Hybridisation was carried out at 65°C overnight. The filters were washed twice at room temperature in wash buffer (5mM Sodium Phosphate, pH 6.8, 1mM EDTA, 0.2% w/v SDS) with vigorous agitation followed by a final post hybridisational wash in 0.1x SSC at 65°C for 30 minutes.

Isolation and Purification of DNA from Trypanosome Lysates

CY treated CFLP mice were inoculated intraperitoneally with the cloned stocks and exsanguiated by cardiac puncture at high parasitaemia. Trypanosomes were isolated from blood and lysates prepared as described in Chapter 2.

High molecular weight genomic DNA was isolated and purified on CsCl gradients. The lysates were suspended in 1xTE (10mM Tris HCl, pH 8.0, 1mM EDTA) to a total volume of 20mls. 20 gms CsCl were added and dissolved by gently inverting the mixture. 2ml Ethidium Bromide (10mg/ml) was added and Beckman VTi 50 tubes filled with the solution and centrifuged to equilibrium at room temperature, 167,150g, for 16 hours. The DNA was removed from the gradient using an 18G needle in approximately 5mls of solution and put into Beckman VTi 65 tubes and centrifuged at room temperature, 266,635g for 4 hours. The DNA band was removed as described above and the Ethidium Bromide removed by repeated extraction with isopropanol. The DNA was then exhaustively dialysed against 1xTE to remove excess salt and stored at 4°C.

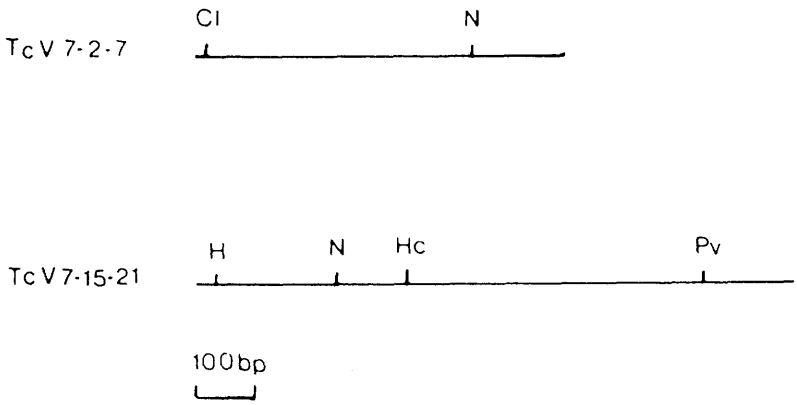


Figure 6 **Physical maps of cDNA probes used in dot blot analysis**
Physical maps of cDNA plasmids TcV 7.2-7, corresponding to VSG gene 7.2 and TcV 7.15-21, corresponding to VSG gene 7.15. Only the insert DNA is shown.
Abbreviations of restriction enzymes: Cl-ClaI; H-HindIII; Hc-HincII; N-NaeI; Pv-PvuII.

Southern Analysis of DNA

Restriction endonuclease digestion, electrophoresis and transfer of genomic DNA to filters and hybridisation of probes to filters were carried out as described in Chapter 2. Post hybridisational washing was carried out in 2xSSC and the filters exposed for 48 hours initially, then for a further 72 hours.

cDNA Probes

The probes used in Southern blot and dot blot analysis are cDNA probes corresponding to mRNA specific for two M-VAT genes of the GUTAR 7 serodeme, namely GUTat 7.2 and GUTat 7.15. Both inserts were cloned into pBR322 and have been previously described (Cornelissen et al,1985). The maps of these inserts are given in Figure 6. Only the insert DNA was used in hybridisation.

RESULTS

Immune Lysis Tests

Immune lysis tests were carried out using the cloned Zambian stocks and the Kenyan and Ugandan repertoire antisera. This test was carried out to obtain a crude estimate of the similarity in antigen repertoire between the two collections of stocks. All nine cloned Zambian stocks were tested. The populations were tested during the first peak of infection and during ensuing peaks throughout infection. Both negative and positive controls were included. The former consisted of trypanosomes incubated in GPS alone, the latter consisted of two homogeneous monomorphic cloned Kenyan populations derived from EATRO 2340, namely GUTat 7.1 and GUTat 7.2.

In all cases the lysed percentage in the negative control was negligible. The lysed percentage in the positive control preparations tested against Kenyan repertoire antisera was in all cases high, 85-100%.

For all nine of the cloned Zambian stocks tested against Kenyan repertoire antisera lysis was less than 5% and was recorded as negative.

The reciprocal reaction testing Zambian repertoire antisera from all nine cloned stocks against the two Kenyan populations GUTat 7.1 and GUTat 7.2 was also carried out. Again lysed percentages were less than 5% in all cases and therefore recorded as negative.

These results indicate that with respect to antigen repertoire there is little detectable similarity between the Kenyan and Zambian stocks.

Dot Blot Analysis

Various numbers of trypanosomes (10^3-10^7) from six of the cloned Zambian stocks were applied to nylon membranes and the membranes treated as described. The membranes were then hybridised with either Probe A which corresponds to the 7.2 gene or to Probe B which corresponds to the 7.15 gene (See Figure 6). Probe A had a specific activity of 10^7 cpm/ug and the filters were exposed for 5 days. Probe B had a specific activity of 10^8 cpm/ug and the filters were exposed overnight.

Both positive and negative controls were included. The former consisted of the two Kenyan populations GUTat 7.1 and GUTat 7.2, both of which contain the 7.2 and 7.15 genes. The negative control consisted of a third Kenyan population referred to here as transmission 12 (T 12) from which both the 7.2 and 7.15 genes have been deleted (Barry, unpublished results).

Using Probe A (see Figures 7 & 8), specific for the 7.2 gene, all Zambian stocks tested gave strong positive signals. For cloned populations 182, 231 and 199 a minimum of 10^6 trypanosomes gave a positive signal in comparison to both the positive controls and the remaining three cloned Zambian populations where a minimum of 10^7 trypanosomes were required to give a positive signal. No strong positive signal was observed for any of the control populations. In Figure 7 the T12 population did not give a positive signal as expected. However, nor did the GUTat 7.1 population which was included as a positive control as it does contain the 7.2 gene, as does the GUTat 7.2 population which did give a positive signal though not as intense as the signals obtained from the cloned Zambian stocks. In Figure 8 anomolous results were again obtained with both the positive and negative

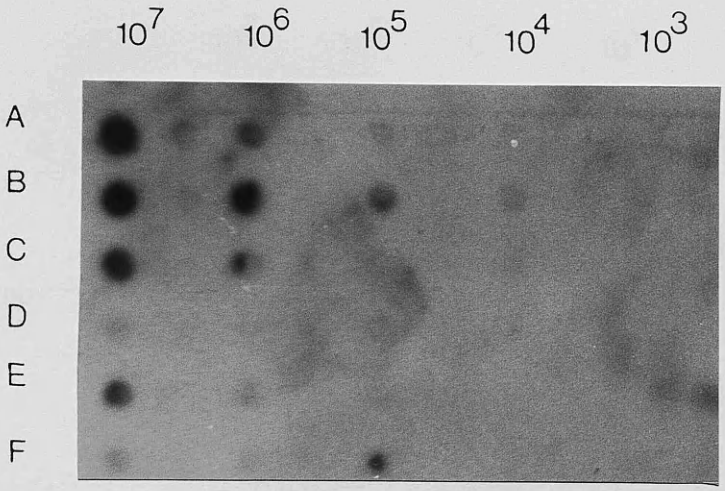


Figure 7 **Dot blot analysis of Zambian stocks using Probe A.**
10ul of 10 fold serial dilutions of trypanosomes were applied to Pall membrane and processed as described in the Materials & Methods section. The filters were hybridised with Probe A, corresponding to the 7.2 gene. The number of trypanosomes in each spot is indicated above the figure and different trypanosome strains indicated on the side of the figure.

A cloned Zambian stock 244
B cloned Zambian stock 182
C cloned Zambian stock 203
D GUTat 7.1
E GUTat 7.2
F T 12

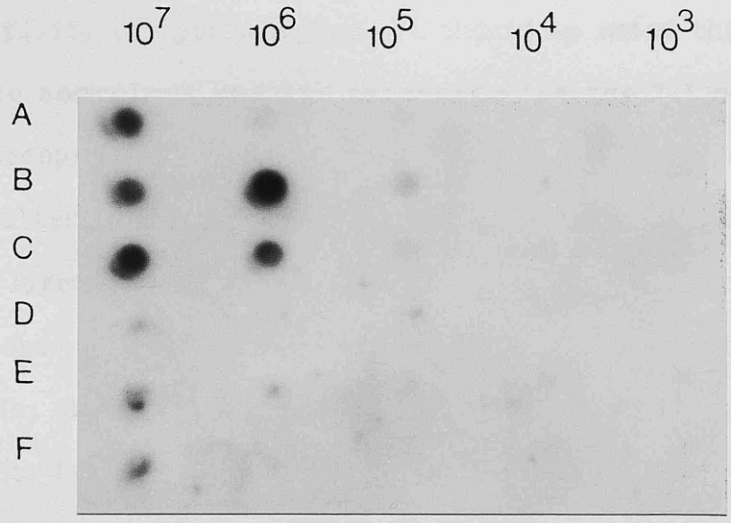


Figure 8 **Dot blot analysis of Zambian stocks using Probe A.**
The filters were treated as those in Figure 7 .
A cloned Zambian stock 220
B cloned Zambian stock 231
C cloned Zambian stock 199
D GUTat 7.1
E GUTat 7.2
F T 12

control populations. Again GUTat 7.1 gave no signal. Both GUTat 7.2 and the T12 populations gave weak positive signals. The reasons for these equivocal results obtained from the control populations are not clear however, they cast doubt upon the specificity of hybridisation. It should be noted that with regard to the anomolous results obtained with the 7.1 population, on microscopic examination of the suspensions before application to the filter a large number of the trypanosomes in this preparation were lysed, thus the DNA from this preparation was likely to be degraded. The other trypanosome suspensions,including the Zambian stocks, also showed a degree of lysis but to a much lesser extent. It is thought the lysis was due to repeated thawing and refreezing of the suspensions.

Using Probe B specific for gene 7.15 five of the six Zambian stocks gave strong positive signals (see Figures 9 & 10). For clones 182 and 199 as few as 10^5 trypanosomes gave a positive signal. For the remaining clones 203, 220, and 231 10^7 trypanosomes were required to give a strong positive signal and cloned population 244 did not give a positive signal. Again equivocal results were obtained with the control populations. In Figure 9 the two positive control populations, GUTat 7.1 and GUTat 7.2 both give fairly strong positive signals and the T12 population, the negative control gives no signal. However, in Figure 10 no hybridisation is seen with the GUTat 7.1 population and a positive signal is obtained with the GUTat 7.2 population though of a much lower intensity than that seen in Figure 9. Again no signal was obtained with the T12 population.

Given the false negative results obtained with the control population GUTat 7.1 and the equivocal results obtained with the

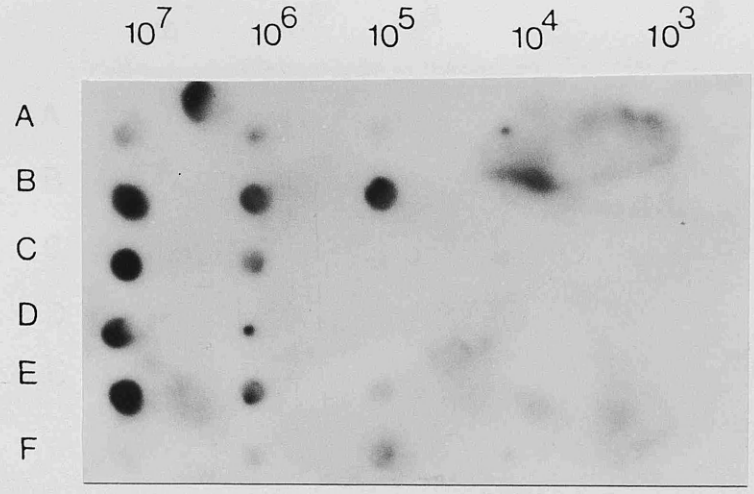


Figure 9 Dot blot analysis of Zambian stocks using probe B.
The fliters were treated as those in previous Figures except that they were hybridised with probe B corresponding to the 7.15 gene.
A cloned Zambian stock 244
B cloned Zambian stock 182
C cloned Zambian stock 203
D GUTat 7.1
E GUTat 7.2
F T 12

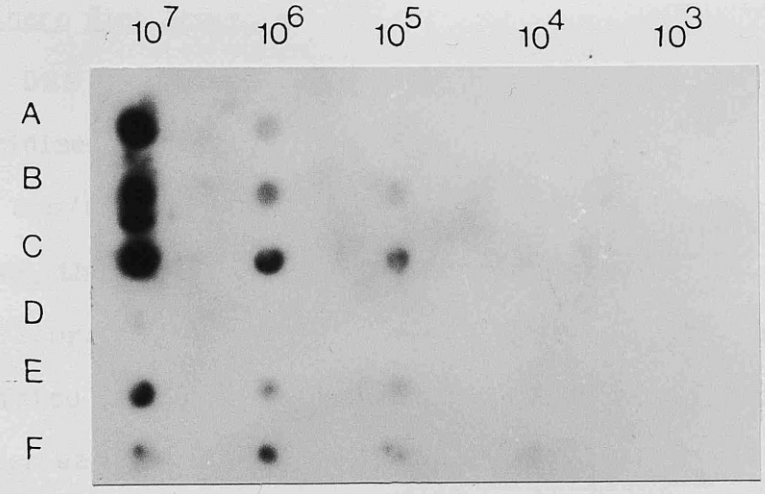


Figure 10 **Dot blot analysis of Zambian stocks using probe B.**
The filters were treated as those in Figure 9.
A cloned Zambian stock 220
B cloned Zambian stock 231
C cloned Zambian stock 199
D GUTat 7.1
E GUTat 7.2
F T 12

T12 population no conclusion could be drawn from these results with respect to antigen similarities which may exist between the two groups of stocks.

Southern Blot Analysis

DNA was isolated from all nine stocks, blotted and hybridised as described. Both probes had a specific activity of 10^8 cpm/ug. The first autoradiograph film was exposed for 48 hours, the second for a further 72 hours. The results are shown in Figures 11 and 12. It should be noted that in the filter depicted in Figure 12 probe A was not melted off before the filter was reprobed with probe B.

For this analysis AnTat 1.3 DNA was used as the negative control as it is known to contain neither the 7.2 nor the 7.15 genes. Positive controls were the GUTat 7.1 and GUTat 7.3 populations both of which contain the 7.1 and 7.15 genes. Genomic DNA was digested with EcoRI, PstI and HindIII. Only the EcoRI results are shown but similar results were obtained with the other two enzymes.

Neither probe was seen to hybridise with any of the Zambian stocks yet both probes hybridised with the positive control DNA. The EcoRI band observed using the 7.2 probe is 18-20 kb in length, that visualised by the 7.15 probe is 8-9 kb in length. These sizes agree with the maps of the basic copies of these genes deduced by Cornelissen et al,1985. It is concluded that neither the 7.2 nor the 7.15 gene is present in any of the cloned Zambian stocks.

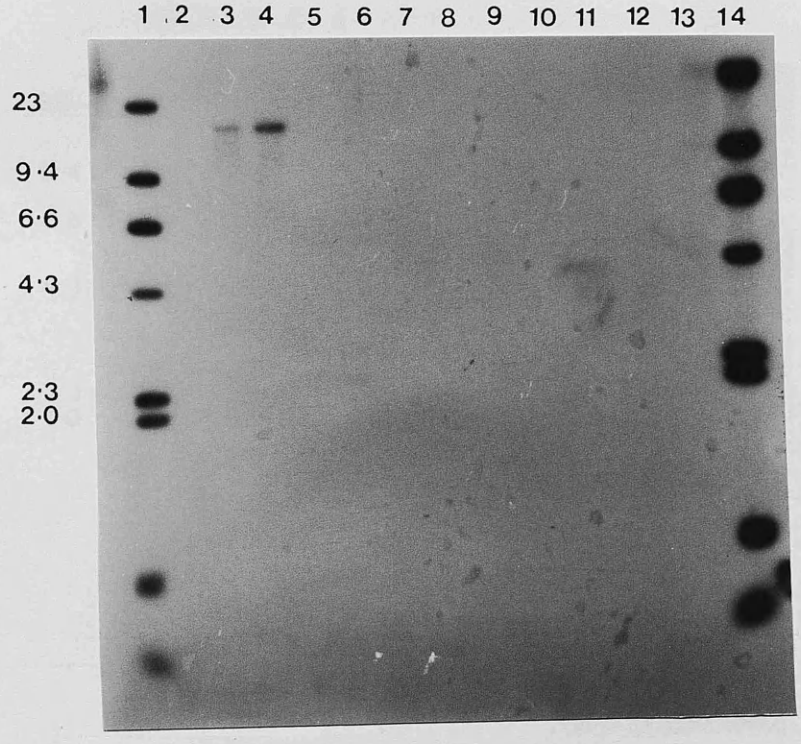


Figure 11 **Southern blot analysis of genomic DNA's from the nine cloned Zambian stocks.**
Nuclear DNA's were digested with EcoRI. The blot was hybridised with Probe A, washed to 2xSSC and exposed for 48 hours. Different genomic DNA's used are indicated below. HindIII digest was used as a marker.

Track1 λ HindIII
Track2 AnTat 1.3
Track3 GUTat 7.1
Track4 GUTat 7.3
Track5 Cloned Zambian stock 182
Track6 Cloned Zambian stock 199
Track7 Cloned Zambian stock 203
Track8 Cloned Zambian stock 220
Track9 Cloned Zambian stock 221
Track10 Cloned Zambian stock 231
Track11 Cloned Zambian stock 244
Track12 Cloned Zambian stock 273
Track13 Cloned Zambian stock 267
Track14 λ HindIII

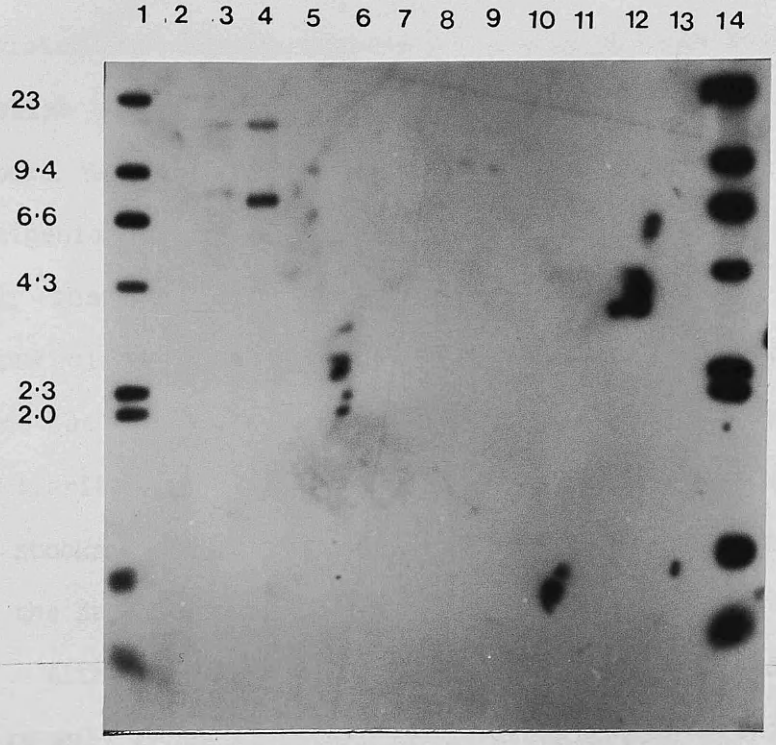


Figure 12 **Southern blot analysis of genomic DNA's from the nine cloned Zambian stocks.**
Nuclear DNA's were digested with EcoRI. The blot was hybridised with Probe B, washed to 2xSSC and exposed for 48 hours. Different genomic DNA's used are indicated below. HindIII digest was used as a marker.

Track1 λ HindIII
Track2 AnTat 1.3
Track3 GUTat 7.1
Track4 GUTat 7.3
Track5 Cloned Zambian stock182
Track6 Cloned Zambian stock199
Track7 Cloned Zambian stock203
Track8 Cloned Zambian stock 220
Track9 Cloned Zambian stock 221
Track10 Cloned Zambian stock 231
Track11 Cloned Zambian stock 244
Track12 Cloned Zambian stock 273
Track13 Cloned Zambian stock 267
Track14 λ Hind III

DISCUSSION

Trypanolysis reactions were used as an estimate of antigenic similarity between the stocks isolated from Kenya and those isolated from Zambia. Repertoire antisera from the nine cloned Zambian stocks showed no cross reaction with the cloned Kenyan stocks. However only two cloned Kenyan stocks were used. Both are antigenically stable clones corresponding to GUTat 7.1 and GUTat 7.2, therefore effectively only two VATs were tested for. Repertoire antisera from the Kenyan stocks tested against all nine Zambian stocks also resulted in no lysis, suggesting no similarity exists in the antigen repertoires of these two groups of stocks. In this test a greater number of VATs were tested for as the Zambian stocks were tested throughout infection.

Although trypanolysis reactions using repertoire antisera give only an approximate estimate of the antigenic similarity that may exist between different trypanosome stocks they have been used previously in comparative analysis of serodemes. Van Meirvenne et al (1975a; 1975b), in examining cloned populations of the ETAR 1 and ANTAR 1 serodemes, using repertoire antisera concluded that, with the exception of two VATs which showed similar serological activity, these serodemes were quite distinct. In a more extensive study Van Meirvenne et al (1977) used trypanolysis tests to study eight cloned stocks of trypanosomes belonging to different species and sub-species and found each clone produced its own characteristic series of predominant variants. Barry et al (1983) used this test to screen twenty-six stocks selected from the North East shore of Lake Victoria between 1958-69 and concluded sixteen of the twenty-six

stocks were antigenically similar. Therefore trypanolysis tests have been used successfully to distinguish between different stocks and serodemes of trypanosomes. The results of the trypanolysis reactions described here indicate that there is little antigenic similarity between the two collections of stocks.

This evidence is confirmed by Southern Blot analysis. Using cDNA probes corresponding to mRNA from two distinct M-VATs of the GUTAR 7 serodeme isolated in 1977 from the Kenyan focus in hybridisation analysis of all nine cloned Zambian stocks no cross hybridisation was observed indicating that neither of these M-VAT genes are present in any of the cloned Zambian stocks. The blots were initially washed to 2xSSC, 65°C, a fairly low stringency of washing, and exposed for 48 hours initially, then a further 72 hours, yet no hybridisation was observed.

The results from the Southern Blot analysis contrast with those obtained from the Dot Blot analysis where hybridisation with all of the Zambian stocks was observed. These blots were washed at various stringencies but had to brought down to 0.1xSSC to reduce background sufficiently in order to visualise the hybridisation pattern. Also it was felt that washing at 0.1xSSC would significantly reduce any non-specific hybridisation.

The results obtained with this technique were obviously not consistent with those obtained from the Southern Blot analysis. In the dot blot analysis the positive signals observed with the Zambian stocks are presumably not a consequence of non-specific hybridisation as the strength of the signal is, to some degree, correlated with the number of trypanosomes spotted onto the filter. If the positive signals observed with the Zambian stocks

had been due to non-specific binding of the probe then presumably this would have been observed with all populations at all concentrations. The lack of hybridisation with the T12 population in three of the four filters (Figures 7-10) also suggests there was little non-specific hybridisation. On one filter where a positive signal was obtained with this population the signal was very weak and not particularly convincing. Also the filters were washed to 0.1xSSC, a stringency which should have decreased to a minimum non-specific binding of the probe to the material on the filter.

Of the two techniques the dot blot analysis could be considered a more sensitive test. It is possible there are distantly related sequences in the Zambian stocks which can be picked up when the genomic DNA is intact, and on endonuclease digestion and electrophoretic separation of genomic DNA the degree of sequence homology may not be high enough to be observed in Southern analysis, particularly if the related sequences are present as single copies. However given that the dot blot filters were washed at 0.1xSSC, 65^{o}C which is a high stringency wash, it seems unlikely that distantly related sequences would be detected. In the Southern blot analysis described here the post-hybridisational wash was carried out in 2xSSC at 65^{o}C. This was permissive enough to allow related sequences to hybridise, however it is possible that if the filters had been exposed for a longer period hybridisation with the Zambian stocks may have been observed.

In conclusion the results obtained from these two methods, combined with the serological analysis, which detects expressed functional genes, it can be tentatively concluded, bearing in

mind the limited extent of the analysis, that the Zambian and Kenyan stocks do not share functional surface antigen genes, though they may share distantly related sequences.

Investigations of the antigenic composition of metacyclic populations has indicated that the population is heterogeneous with respect to VAT (Le Ray et al,1978; Barry et al,1979; Hajduk et al,1981; Crowe et al,1983). However the same mixture of VATs are expressed regardless of the VAT originally ingested by the fly (Hajduk et al,1981). Although a heterogeneous mixture of VATs is expressed in this population the number of VATs which can be expressed at this stage in the life cycle is limited (Crowe et al,1983) and is serodeme specific (Hajduk et al,1981; Barry et al,1983). The M-VATs therefore can be considered as serodeme specific markers. However instability in the M-VAT repertoire has been reported (Barry et al,1983). In that study seven cloned stocks isolated between 1961 and 1979 from the Kenyan focus investigated here were analysed for similarities in their M-VAT repertoires. Of a total of eleven M-VATs investigated only three were present in all stocks, two being present in stocks isolated from 1961-64 but absent from those isolated after 1964. The remainder of the M-VATs were not found consistently in all stocks but no correlation between their date of isolation and the presence or absence of the M-VAT could be made.

Given the reported instability of the M-VAT repertoire more M-VAT probes would need to be used in analysing the Zambian stocks described here before any conclusion regarding the serodeme of these stocks could be made. However from the limited evidence presented here the proposal that the stocks isolated from the Zambian focus and those isolated from the Kenyan focus

belong to different serodemes can be tentatively made.

With reference to the origin of T.b.rhodesiense strains circulating within the different foci of infection throughout East Africa and the proposal that those strains in the North arose from the Southern strains, the limited analysis described here would suggest that this may not be the case, because if it were then it would not be unreasonable to expect to find similarities in antigen repertoire between strains isolated in the North of East Africa and those isolated in the South. However, it has been reported that VSG genes may be subject to preferential alterations resulting in rapid evolution of VSG gene sequences. Frasch et al (1982) suggest some VSG genes are rapidly evolving whereas others are highly conserved and the former set are located within hypermutagenic regions of the genome i.e. telomeres. It has been suggested that telomeres may act as recombinational "hotspots" (Van der Ploeg and Cornelissen,1984; Pays et al,1985a) and also telomeric genes are frequently replaced by partial or total gene conversions (Pays et al,1983b;1983c;1983d). Gene conversions of single copy telomeric VSG genes can lead tc their loss from the genome being replaced by the incoming gene copy (Pays et al,1983b; Laurent et al,1984a). On the other hand activation of a new telomere can lead to conservation of the inactitvated ELC which then becomes a new telomeric member of the gene family (Young et al,1983b; Pays et al,1983b; Buck et al,1984; Laurent et al,1984a). These changes which occur frequently at telomeres could lead to rapid diversification of antigen repertoires. Given that VSG genes are rapidly evolving it may be more appropriate then when testing the relatedness of strains to study polymorphisms in more highly

conserved sequences. On the other hand, had gene similarities been found between the strains tested in this study this would have been indicative of some relationship between stocks from Kenya and those from Zambia.

Gibson et al (1980), from isoenzyme analysis found that there was a striking, though not perfect, division between East and West African stocks and further that for T.b.rhodesiense stocks there was a division between those isolated from North-east Africa and those isolated from the South-east regions of Africa . This is in agreement with Ormerod's division of T.b.rhodesiense stocks into Northern and Southern strains (Ormerod,1961;1963;1967). From the electrophoretic evidence there is a dissimilarity between the Northern and Southern stocks making it unlikely that T.b.rhodesiense found in Uganda resulted from a northernly spread of an increasingly virulent strain as has been suggested (Ormerod,1961).

Over the past 20-30 years sleeping sickness has become a major health problem in Zambia. The Kafue River area has suffered small epidemics in the past (Ormerod,1961) and at Kasempa between 1960-68 several cases were reported. At the head of the Luangwa Valley a major outbreak was reported 1971-74 at Isoka and further down the valley at Luwembe a smaller outbreak occurred at the same time (see Figure 9 and Gibson et al,1980). Gibson et al (1980) investigated seventeen Trypanozoon stocks isolated from humans in Zambia in 1972-79 and eleven stocks isolated from domestic animals in the Luangwa Valley in 1971-74. Among the human stocks variation was found in only two enzymes. The other ten enzymes were identical in all stocks and some of these enzyme patterns were typical of those found in East African Trypanozoon

stocks as opposed to West African stocks. The stocks could be divided into five different zymodemes thus providing evidence for the existence of at least a limited number of zymodemes circulating in the same focus.

More variation was found in the stocks isolated from animals than those isolated from humans. Five of these were either identical to, or differed by only one enzyme, from stocks isolated from humans in Zambia, whereas the remaining six stocks were very different from those stocks isolated in Zambia, or indeed elsewhere. The authors provided evidence to suggest that two different zymodemes of T.b.rhodesiense were responsible for the two different epidemics in the Luangwa Valley, the one in Luwembe, the other in Isoka. In that analysis evidence is also presented which indicates that human infective trypanosomes isolated in 1958-76 from the endemic areas of Busoga (Uganda) and West-central Kenya belonged to a single zymodeme distinct from other zymodemes found in East Africa.

Gibson and Gashumba (1983) studied stocks of T.b.rhodesiense isolated from Uganda during the recent epidemic (1976-82), which is thought to have arisen from the old endemic foci on the shores of Lake Victoria and spread north into new areas. Six zymodemes were recognised. The authors suggested this finding is in agreement with the proposal by Ford (1979) that epidemics are a consequence of changing ecological conditions rather than the appearance of a particularly virulent strain. One of the zymodemes defined in this study was also found in Busoga and West-central Kenya in 1959-76 and the authors proposed this as a likely candidate for the original zymodeme which arrived on the shores of Lake Victoria in the 1940's. A second zymodeme was also

found in the South, in Zambia and Botswana and also to the North in Ethiopia and it is suggested its appearance in Uganda is a result of refugee and military movements. The origin of the remaining four zymodemes remains uncertain. The presence of different trypanosome zymodemes found circulating in the one epidemic area or the one endemic focus supports the view that T.b.rhodesiense found in different foci may have arisen de novo from T.b.brucei.

As mentioned previously T.b.rhodesiense is thought to have spread North from its proposed origin in the Zambezi basin through Zambia, Tanzania and on into Uganda and Kenya (Ormerod,1961). However the electrophoretic evidence presented (Gibson et al,1980) does not support this view. The major zymodemes found in South-east Africa (Zambia, Tanzania,Rwanda and Botswanna) are distinct from those found in Uganda and Kenya. It would seem likely that human infective trypanosomes from North and South East Africa have separate origins. Also results from restriction enzyme analysis of maxi-circle kDNA indicated that there was a difference between Northern and Southern stocks. The evidence presented here would support this view as no similarity between Zambian and Kenyan stocks was demonstrated with regard to antigen repertoires.

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