AN APPROACH TO THE IDENTIFICATION OF *TRYPANOSOMA CONGOLENSE* CHROMOSOME-SPECIFIC HYBRIDIZATION MARKERS

BY

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A thesis submitted in partial fulfillment for the degree of Master of Science in Biochemistry at the University of Nairobi

1997

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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This work has been submitted to the University of Nairobi for examination with our approval as University supervisors.

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ACKNOWLEDGMENT

The work described in this thesis was carried out at the former International Laboratory For Research on Animal Diseases (ILRAD), now incorporated into the International Livestock Research Institiute (ILRI).
I would like to express my sincere gratitude to the ILRAD administration for giving me the opportunity to carry out my research project within the institute.

I wish to express my appreciation and sincere thanks to my ILRAD supervisor, Dr. Phelix Majiwa, for introducing me to scientific research and for his constant supervision, advise, discussions and encouragement during the course of this study. I was most spirited by his patience and encouragement during difficult times. The guidance and encouragement given to me by my University Supervisor Dr. P. W. Kinyanjui is greatly appreciated: I owe him many thanks.

My sincere appreciation and thanks also go to my lab colleagues, Dr. G. A. Ndoutamia and Mr. J. Osaso for always being ready to provide help and advice. The assistance given to me by Dr. A. Osanya, Mrs. M. Maina, Mr. S. Wasike and Mr. H. Gathuo is greatly acknowledged. Also many thanks go to Mr. D. Elsworth, Mr. Joe Mwaura and Mr. Francis Shikubari for assisting me in the graphic arts and photography. I would also like to thank Mrs. Lucy Thairo for typing some parts of this thesis.
Special thanks are due to my family and friends for their patience and encouragement.

DEDICATION

To my late sister Elizabeth Akinyi 'Toto', my parents, brothers and sisters.

ABSTRACT

Chromosome rearrangements that occur in trypanosomes appear to affect mainly the medium-sized chromosomes. A possibility exists that non-duplicative activation of telomeric VSG genes might involve chromosome rearrangements occurring several kilobase pairs upstream of the affected VSG gene, thus remaining undetectable by standard gel electrophoresis and southern blot hybridizations with VSG gene probes. Since trypanosomes do not condense their chromosomes at any stage of cell division, this hypothesis cannot be tested by cytological hybridization. Some of the size alterations in chromosomes occur when the trypanosomes are repeatedly passaged, cyclically transmitted through tsetse flies or grown in rodents in the presence of trypanocidal drugs. In some instances, the alterations in the sizes of some chromosomes may relate to a changed phenotype of the parasite. Pulsed field gradient gel electrophoresis (PFGE) of chromosome-sized DNA molecules of lower eukaryotic organisms is an effective way of characterizing such organisms. It has been used to characterize many different isolates of parasitic protozoa including *Plasmodium falciparum, Leishmania spp., Trypanosoma spp., Theileria spp.* and *Toxoplasma.* When a particular chromosome rearranges in such organisms, it is impossible to identify the rearranged chromosome in the absence of a marker specific for the chromosome.

Short oligonucleotide primers (10-mers) of arbitrary nucleotide sequences have been used in the polymerase chain reaction (PCR) to generate genomic fingerprints that facilitate the characterization and differentiation of various organisms and for physical mapping of loci which contain genes responsible for identifiable phenotypes.

It was proposed that the use of such primers to amplify purified individual chromosomes may generate fingerprints which are characteristic of each chromosome. To test this hypothesis, *T. congolense* clone IL1180 was used because of its sensitivity to trypanocides and clear pedigree. PFGE conditions were optimized which best separates the

four medium-sized chromosomes, whose sizes are: 340 kb, 360 kb, 400 kb and 500 kb. The four chromosomes thus separated were individually purified from agarose gel slices and then PCR-amplified with the random primers. The fingerprints of DNA fragments generated were resolved by standard agarose gel electrophoresis. The majority of primers used generated reproducible fingerprints, some of which were polymorphic for the different chromosomes used.

In this way, a fragment of DNA was identified which hybridizes only to one of the four chromosomes from which it was amplified by a random primer. Thus, taking this approach, it is feasible to generate chromosome-specific hybridization markers.

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ABBREVIATIONS

Abbreviations	Full name
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
bp	Base pair
CHEF	Contour-clamped homogenous electric field
cpm	Counts per minute
CTP	Cytosine triphosphate
DEAE-cellulose	Diethylaminoethyl-cellulose
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetra-acetic acid
GTP	Guanosine triphosphate
HEPES	N-2-hydroxymethylpiperazine-N'-2-ethane-sulphonic acid
hr	Hour
ILO	ILRAD oligonucleotide
kb	Kilobase(s)
Mb	Megabase(s)
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
M	Molar
^{32}P	Radioactive phosphorous

Sec	Seconds
µ	Micron
µg	Microgram
µl	Microlitre
µM	Micromolar
ng	Nanogram(s)
nm	Nanometer
OD	Optical density
PBS	Phosphate buffered saline
pg	Picogram(s)
RNA	Ribonucleic acid
rpm	Revolutions per minute
SDS	Sodium dodecly sulphate
Tris	Tris(hydroxy-methyl) aminomethane

CHAPTER 1

1.0 General introduction

1.1 Trypanosomes

1.1.1 The discovery

The term 'Trypanosome' comes from the Greek word meaning auger-body. It designates a group of parasitic protozoa responsible for widespread diseases in humans, livestock and wild animals in large parts of Africa and South America. The name *Trypanosoma* was first used by Gruby in 1843 to refer to haemoflagellates he discovered in the blood of frogs, and in 1878, Lewis proposed that trypanosomes were the cause of a parasitic disease in rats. Pathogenicity of trypanosomes in horses and camels was shown by Evans in 1880. Evans showed that trypanosomes caused 'surra' in horses and camels. Bruce in 1894 discovered *T. brucei* in the blood of cattle suffering from *nagana* and demonstrated it to be transmitted by tsetse flies in 1897. *T. gambiense* and *T. rhodesiense*, both of which cause human sleeping sickness, were first reported in 1902 and 1903, respectively (Hoare, 1972).

The name *T. congolense* was given by Broden to a small trypanosome he discovered in the blood of domestic ungulates from Congo in 1904. Later in the same year, Laveran and Mesnil used the name *T. dimorphon* for an apparent dimorphic trypanosome, while Laveran (1905) discovered a strain of similar but smaller trypanosome to which he gave the name *T. nanum*. With a mistaken idea of resolving the existing confusion in the nomenclature of these trypanosomes, Bruce *et al* (1910), proposed to unite all these species under a new name *T. pecorum*. For convenience. the species *T. dimorphon, T. nanum* and *T. percorum* are presently regarded as synonymous to *T. congolense*.

1.2 Classification of trypanosomes

Trypanosomes are protozoan parasites belonging to the genus *Trypanosoma*. The mammalian parasite can be divided into two 'sections': (1) the stercoraria, which

complete the development in the hindgut of the insect vector and are transmitted to the host via the faeces of invertebrate vector through skin abrasions or mucous membranes, and (2) the salivaria, which complete their development in the mouthparts of the insect vector and are transmitted via the bite of an infected vector (Hoare, 1972; Backer *et al.*, 1978).

The salivarian and the stercorarian sections of the *Trypanosoma* have been further divided into different subgenera according to their morphology and development in the mammalian host. The stercoraria have a free flagellum and a large non-terminal kinetoplast with a pointed posterior end of the body . Development in mammalian host usually involves amastigote or epimastigote stages and most, except *T. cruzi*, are nonpathogenic.

There are three subgenera of stercorarian trypanosomes: *Megatrypanum* with type species *T. theileri*, a parasite of cattle; *Herpertosoma*, with type species *T. lewis*, which infect rats; and *Schizotrypanum* with type species *T. cruzi*, a human pathogen.

The salivarian trypanosomes may or may not have a free flagellum; in these trypanosomes the kinetoplast is terminal or subterminal and the posterior end of the body is usually blunt. They develop as trypomastigotes within the mammalian hosts and are usually pathogenic.

There are four subgenera which may be regarded as unspecific (Backer *et al.*, 1978), although this has been disputed (Gibson *et al.*, 1980). The type species of subgenus *Duttonella* is *T. vivax*, *T. congolense* is the type species of subgenus *Nannomonas* while the subgenus *Trypanozoon* contains several subspecies of *T. brucei* including *T.b. gambiense*, *T.b. brucei, T.b rhodesiense*, *T.b. evansi*, *T.b equirum* and *T.b. equiperdum*.

The taxonomic classification briefly presented above continues to be the subject of debate. This is particularly true for the subspecies of *T. brucei* which are often classified as separate species. Morphologically, they are almost identical with only a gradation of differences within the subgenera.

The opposite problem is true of *T. cruzi* where the morphological, biochemical and clinical characteristics of different isolates are great enough to warrant that they be classified as different species. Individual strains within two species of *T.cruzi* have been shown to differ substantially in the morphology, physiology, pathogenicity, host and vector specificity and geographical distribution (Myler, 1993).

The ambiguity in taxonomic classification of trypanosomes presents a difficulty in any discussion in molecular variation within this group of organisms. It is particularly difficult to distinguish intra-specific (strain) variations or genetic diversity from inter-specific variations.

1.3 Trypanosome life cycle

Organisms within the genus *Trypanosoma* are digenetic parasites with life cycle alternating between a vertebrate host, where they are usually found in the blood or tissues and in the invertebrate hosts where they are found in the digestive tract (Myler, 1993).

1.3.1 Development in the invertebrate host

Trypanosomes get into the tsetse fly (*Glossina* spp) when the fly feeds on the blood of an infected vertebrate host. In the tsetse flies, the trypanosome undergoes elaborate cycles of development with characteristic division phases and morphological changes (Vickerman and Barry, 1982). Except for the species whose development is restricted to the proboscis (*T. vivax* and *T. uniforme*), trypanosomes ingested with the blood pass into the insect gut. Trypanosomes belonging to the stercorarian complete their life cycle confined to the alimentary tract. In contrast, those of the salivarian section migrate to the salivary glands and/or the proboscis during their life cycle of development. At the intestinal stage, all trypanosomes are trypomastigotes. In the insect gut, the trypanosome loses the surface coat and

differentiates into the procyclic trypomastigotes (Barry and Vickerman, 1979). The procyclic forms continue dividing for a few days and then migrate to the salivary glands and transform into epimastigotes. These in turn divide and transform into metacyclic forms (Tetly and Vickerman, 1985). The metacyclic forms express a small but dynamic set of variable surface glycoproteins and are infectious to the vertebrate host.

1.3.2 Development in the vertebrate host

Trypanosome infection of the vertebrate host begins with a bite of an infected tsetse fly and intradermal injection of metacyclic form of trypanosomes at the site of the bite where they differentiate into bloodstream form (Barry and Emery, 1984). These trypomastigotes divide rapidly by binary fission and express the bloodstream form VSGs. They begin to undergo antigenic variation and have the ability to generate an inexhaustible number of variant antigen types (VATs) in the blood of the host. VAT refers to a particular, antigenically unique, variable surface glycoprotein (VSG) expressed by a trypanosome (Vickerman, 1982). The bloodstream forms can cross the blood vessels into connective tissues and, at later stages, cross the coroid plexus into the brain and the cerebrospinal fluid (CSF). The bloodstream form trypanosomes are characterised by possession of a long flagellum, an elongated nucleus, a subterminal kinetoplast (Hoare, 1970) and a single unbranched mitchondrion with a few cristae.

As the parasitemia progresses, the bloodstream forms termed long slender trypomastigotes differentiate into the short stumpy trypomastigotes. These are characterised by a rounded nucleus, a kinetoplast that is closer to the posterior end, a much shorter flagellum (Wijers, 1959) and the presence of a dense lipid granule (Ormerod, 1958). They also posses a single mitochondrion with several cristae (Vickerman and Preston, 1970).

Between these two forms is the intermediate one which is thought to be a transition between the long slender and the short stumpy parasites.

The tsetse fly becomes infected when it bites an infected mammalian host and takes a bloodmeal with the short stumpy trypomastigotes and the cycle continues.

1.4 Trypanosomiasis

Trypanosomiasis is the disease which results from infection of a mammalian vertebrate with the haemoprotozoan parasite of the genus *Trypanosoma.* Trypanosomes can infect various vertebrate classes, ranging from fish to mammals, but have been well studied only in the latter, where they are of considerable veterinary and medical importance (Myler, 1993). The parasites can be transmitted cyclically by tsetse flies, non-cyclically by haematophagous diptera other than tsetse flies, or venerially. Trypanosomiases of economic importance affect humans and domestic animals.

1.4.1 Human trypanosomiasis

There are two major forms of trypanosomiases that occur in man. These are the African trypanosomiasis which is caused by salivarian trypanosomes and the South American trypanosomiasis (Chaga's disease) which is caused by stercorarian trypanosomes. The African human trypanosomiasis, also called sleeping sickness, is caused by trypanosomes of the subgenus *Trypanozoon.* It is a debilitating and, if untreated, fatal disease.

The type species responsible for human trypanosomiasis are *T.b. rhodesiense* and *T.b. gambiense* which cause the Rhodesian sleeping sickness in East Africa and the Gambian sleeping sickness in West Africa, respectively. *T.b. rhodesiense* causes acute sleeping sickness while *T.b. gambiense* causes chronic form of the disease. Both forms are diseases of the central nervous system (CNS) and other tissues. In both cases, there is an initial period of infection of the blood, which is followed by invasion

of the cerebrospinal fluid (CSF) and of the brain. The time course of the events in both cases vary tremendously. In the acute form, there is a rapid development of severe toxaemia and untreated patients can die within a few weeks or months of infection. However, in the chronic form of trypanosomiasis, the invasion of the CNS is delayed and death of untreated victims may not occur for several years; usually many months get spent on an emaciated condition with the patient displaying the typical 'sleeping' symptoms from which the disease derives its name.

1.4.2 Animal trypanosomiasis

Animal trypanosomiasis is caused by a bite of an infected tsetse fly of the *Glossina spp.* All *Glossina spp.* wherever they occur, are invariably infected with trypanosomes. Unlike human trypanosomiasis where, even in epidemic areas, the rate of infection of wild *Glossina* with trypanosomes infective to man rarely exceeds 0.1%, much higher infection rates of trypanosomes infective to livestock occur in all *Glossina spp.* (review by Button, 1955 and Jordan, 1974). In general, infection rates of 10-15% in the *G. morsitans* group is considered typical. Clearly, the risk of domestic animals contracting trypanosomiasis is enormously greater than the risk of humans contracting the disease.

In the cyclically transmitted species of trypanosomes, the metacyclic and the bloodstream forms are able to cause infection (Boyt, 1980). When an infective tsetse fly bites an animal, metacyclic trypanosomes are deposited in the dermis of the skin and a local inflammatory reaction termed a 'chancre' develops in 7-11 days (Roberts *et al.*, 1969; Luckins and Gray, 1979; Emery and Moloo, 1980). From the chancre the trypanosomes enter the draining lymphatics, and thereafter, the bloodstream. Both *T. vivax* and *T. congolense* tend to remain intravascularly (Losos and Ikede, 1972). *T.*

congolense appear to localise in small blood vessels, particularly in the capillaries of skeletal muscles where they attach to endothelium (Banks, 1978). However, under experimental conditions, Masake *et al.* (1984) found that two out of three Boran (*Bos indicus)* cattle infected with *T.b brucei* and *T. congolense* develop both clinical and histological evidence of cerebral trypanosomiasis. *T. congolense* was isolated from cerebrospinal fluid of one of these two animals.

It would therefore appear that *T. congolense* can invade the CNS in mixed infections. Although *T. vivax* is primarily an intravascular parasite, Whitelaw *et al.* (1988) demonstrated that this species can invade the CNS and aqueous humour of the eye in goats. They also demonstrated that these sites can constitute potential sources of relapse infection after treatment of infected goats with diminazine aceturate.

1.5 Importance of trypanosomiasis

Trypanosomiasis is considered the most important infectious disease holding back the development of livestock production in much of Africa. The impact of animal trypanosomiasis is to a large extent indirect, the threat of disease preventing the livestock owner from grazing or setting up homesteads within the fly belt.

An estimated 160 million cattle and 260 million sheep and goats are kept in this affected region of Africa and are therefore at risk of contracting trypanosomiasis. Additionally, about 12 million camels on the African continent are at risk of infection with *T. evansi*. It is estimated that approximately 25,000 cases of human sleeping sickness occur each year.

Trypanosomiasis has caused livestock concentration to shift to the arid Savannah regions of low agricultural potential, outside the main tsetse infested areas. The resultant pressure on this land has led to the widespread use of livestock rangelands for crop production and the subsequent movement of livestock into rangelands only marginally suitable for grazing, often also into closer contact with

tsetse. In some instances this has resulted in considerable degradation of the environment.

The precise socio-economic effects of trypanosomiasis on agricultural production systems and on livestock production in Africa in particular are difficult to quantify. This difficulty is mainly due to paucity of reliable data on such important factors as the distribution and number of humans, animals and insect vector population; insufficient knowledge of the effect of the disease on livestock production; and the difficulty in quantifying the value of livestock and their products in pastoral and mixed crop/livestock subsistence production systems (ILRAD Quarterly Report, 1993).

Inspite of it's acknowledged importance, therefore, the extent of trypanosomiasis problem has usually been expressed in crude estimations of the size of land area infested by tsetse fly and/or proportions of livestock population at risk. The total current annual cost of the disease, including both production loses and control costs are presently estimated to be more than 500 Million US dollars (ILRAD Quarterly Report, 1993).

1.6 Diagnosis

History of an affected animal, its geographical location and manifestation of certain clinical signs may suggest that the animal is infected with trypanosomes. However, since trypanosomiasis can often be confused with other wasting diseases and/or malnutrition (F. A. O, 1979), conclusive diagnosis should rely on detection of the parasites in the blood or other tissues of an infected host. Parasitological tests such as wet blood smear, Giesma-stained thin and thick blood smears, the micro-haematocrit centrifuge technique and the buffy coat phase contrast/dark ground technique can be used to confirm a clinical diagnosis by demonstrating the presence

of trypanosomes. However, depending on the purpose of the test, the sensitivity of such parasitological tests could be a limiting factor.

The choice as to which test to use for the diagnosis of trypanosomiasis depends on the level at which the test is to be carried out (farmer, veterinary diagnostic laboratory, research institute) and the purpose for which the results obtained will be used.

The above classical methods for diagnosis can be unreliable and subjective (Godfrey, 1977) since many trypanosomes differ from others only in the response elicited by a host on infection with a particular species. Therefore, it is desirable to have a more precise and efficient method of distinguishing closely related trypanosomes from each other.

Currently, the use of recombinant DNA probe hybridization (Petterson and Hyypia, 1985; and Tait, 1991) and monoclonal antibodies in diagnostic microbiology (Nantulya, 1989; Nantulya and Lindqvist, 1989) is revolutionizing the procedures, and will significantly influence the management of parasitic diseases. Some of the immunological tests in current usage are indirect immunoflourescent antibody test (IFAT), enzyme linked immunosorbent assay (ELISA) and card agglutination test for trypanosomes(CATT).

For diagnosis of microbial infection using recombinant DNA probe hybridization, a fragment of DNA specific to the parasite is radiolabelled and then used to detect it's complementary copy, which is immobilised on a solid support. Different approaches have been used to isolate highly repetitive DNA sequences which can identify species and subspecies of a number of different African trypanosomes (Majiwa *et al.*, 1985; Majiwa and Webster, 1987; Gibson *et al.*, 1988). These sequences can be used as probes for hybridization with samples recovered from vectors or hosts, where they reveal with high degree of specificity, the presence of parasites with such sequences in their genome (Majiwa and Otieno, 1990; Nyeko *et al.*, 1990).

The species specificity of the diagnostics can be extremely useful in providing additional information as to the optimal choice and dosage of trypanocidal drugs to be used in certain areas. The diagnostics can also assist tsetse control and eradication personnel in determining the particular species and strain of the parasite in different areas.

1.7 Control of trypanosomiasis

Avoiding high risk areas or evacuating an area when it becomes a high risk one is the simplest approach in disease control. This approach however, cannot be used in trypanosomiasis control because the alternate areas are marginal, arid, semi arid or wastelands. The main strategies in trypanosomiasis control are chemotherapy, vector control and the use of trypanotolerant breeds of livestock.

Chemotherapy and chemoprophylaxis are the most widely used approaches for control of trypanosomiasis (Boyt, 1980). Chemotherapy for trypanosomiasis in domestic animals currently relies on the use of the salts of three compounds namely, isometamidium, homidium and diminazine. These compounds are related and have been on use for at least three decades. When rationally applied, these drugs have effectively controlled the disease.

Vector control measures aim at reducing host-fly contact and thus break the cycle of transmission (Molyneux and Jefferies, 1986). Various methods are employed in tsetse control. These include 'indirect method' which involves tsetse habitat destruction, essentially deforestation and elimination of wild life (Ford *et al.*, 1970); 'direct methods' which are based on the use of chemical and biological techniques intended to either destroy the tsetse fly or eliminate their ability to breed (Finelle, 1983). These consist of insecticide application on tsetse fly habitat or the use of trapping techniques; and 'biological control' where organisms pathogenic to tsetse flies are used.

The use of trypanotolerant livestock in areas where the disease is prevalent has been considered as an option in control of trypanosomiasis. Trypanotolerant breeds of livestock include N'Dama, Muturu, Somba and Dahomey. These breeds have innate resistance which enables them to survive and maintain their productivity in tsetse infested areas where *Bos indicus* cattle would die (Finelle, 1973; Mortelmans and Kageruka, 1976; Murray *et al.*, 1983).

The use of trypanocidal drugs in trypanosomiasis control has not been very effective, mainly because they are expensive. The use of trypanocides is becoming complicated by the fact that the efficacy of the currently available drugs is diminishing fast in some areas due to the development of drug resistance by trypanosomes. A reason for this is very likely the fact that in many countries, the administration of drugs to livestock is carried out by the livestock owners rather than by the qualified veterinary practitioners.

Vector control method has been an approach of disease control in some countries. Both the use of, and method of applying, insecticide for tsetse control have been widely criticised on environmental grounds and this has led to introduction of simple insecticide-impregnated fly-attractive devices (target, screen, traps) and insecticide (pour-on formula) treated target animals.

1.8.0 Chromosomes

Trypanosomes, like all other eukaryotic organisms, have chromosomes. Isolation of intact nuclear DNA initially proved to be very difficult because procedures of freeing the DNA from cellular material generally involved exhaustively treating the cells with strong detergents and proteolytic enzymes (Gross-Belard *et al.*, 1973). In some cases double stranded molecules, the size of chromosomes, are released (Kavenoff and Zimm, 1973; Schwartz *et al.*, 1983). Such large DNA molecules are viscous and fragile and thus they cannot be manipulated without shearing (Burgi and Hershey, 1961; Levinthal and Davidson, 1961). It is now

possible to isolate intact chromosome-sized DNA molecules from a variety of prokaryotic and eukaryotic cells (Schwartz *et al.*, 1984), so that the study of DNA from these cells is carried out in a state as close to it's *in vivo* state as possible. The method involves embedding living cells whose DNA is required in molten agarose so that the cells are trapped *en bloc.* The pores in the agarose are large enough to allow free diffusion of proteins but not chromosomal DNA. This is followed by lysis of the cells using detergents like sodium dodecyl sulphate or Triton X-100. The proteins are subsequently subjected to enzymatic digestion over a period of time, and the digestion products together with RNA allowed to diffuse rapidly out of the agarose, leaving only chromosome-sized DNA. If cells are lysed in the presence of high concentration of EDTA which inhibits nuclease activity, the resulting DNA remains intact.

Analyses of DNA obtained in this way show that they are intact (Jackson and Cook, 1985), and chromosome-sized (Schwartz and Cantor, 1984)

1.8.1 Chromosomes of lower eukaryotic organisms

Pulsed-field gradient gel electrophoresis has been applied in the study of a variety of lower eukaryotic microorganisms including *Plasmodium, Leishmania, Toxoplasma, Crithidia, Entamoeba* et cetera.

In *Plasmodium falciparum*, examination of different isolates using PFG indicated that their chromosomes are polymorphic in size (Van der Ploeg *et al.*, 1985; Kemp *et al.*, 1985). The isolation of the *P. berghei* telomere repeat (Ponzi *et al.*, 1985) has proven very useful for the analysis of *Plasmodia* chromosomes, as have specific markers such as antigen-encoding genes, or anonymous probes (Kemp *et al* ., 1987; Langsley and Ponnundurai, 1988). These telomere probes, together with an improved PFG separation of *P. falciparum* and *P. chabaudi* chromosomes helped establish that 14 chromosomes in this organism can be resolved by PFG (Langsley *et al.*, 1987)

PFG analyses of genetic crosses of malaria parasites indicate that their chromosomes segregate independently, undergo unequal crossing over, show favoured linkage groups and have a high recombination frequency (Barale and Langsley, 1993).

Toxoplasma gondii chromosomes have been separated using TAFE into 9 distinct bands that defined a minimum of 10 physical linkage groups. The appparent apparent sizes of these chromosomes varied between 2 Mb and 6 Mb. Preliminary physical linkage groups have been defined by mapping 57 single or low copy number probes to specific chromosomes by hybridization. The majority of these probes consist of random DNA segments; however, a number of cDNAs encoding important structural and antigenic components were also mapped to specific linkage groups (Sibley and Boothroyd, 1992).

Chromosome analyses in *Leishmania* has allowed determination of the molecular karyotype of this organism (Van der Ploeg *et al.*, 1984) and revealed a high degree of inter- and intra-specific variation among strains of distant geographical origins, strains from the same endemic areas, including biochemically, epidimologically and clinically identical strains (Giannini *et al.*, 1986; Spithill and Samaras, 1987; Bishop and Miles, 1987; Pages *et al.*, 1989). Intra-strain variation has been reported in 3 clones of *L. amazonensis* (Comeau *et al.*, 1986), 2 clones of *L. major* (Spithill and Samaras, 1987) and 14 clones of *L. infantum* (Pages *et al.*, 1984).

This technique has also been used to differentiate the morphologically indistinguishable pathogenic and nonpathogenic strains of *Entamoeba histolytica* (Petter *et al.*, 1993).

1.8.2 Chromosomes of trypanosomes

The presence of chromosomes in trypanosomes was not clear for a long time because no condensed chromosomes could be visualised during nuclear division in these organisms (Vickerman and Preston, 1970). However, the formation of spindle

apparatus has been observed in *T. brucei* and found to differ from those of higher eukaryotes (Borst *et al., 1982*; Raikow, 1982; Tait, 1983). The compaction of the chromatin in the nucleus of trypanosomes is distinctly less pronounced as compared to that of rat liver nuclei (Hecker and Gander, 1985). In *T. brucei* and *T. cruzi* procyclic culture forms, the chromatin was shown to be organised in a nucleosome filament-like form. However, the nucleosomes were spaced irregularly and no condensation into a typical fibre could be observed under experimental conditions. The nuclear chromatin and the interactions between DNA and proteins were relatively weak and easily destabilised under experimental conditions, which are normally used for the isolation of chromatin from higher eukaryotes (Hecker and Gander, 1985; Hecker *et al.*, 1989; Bender *et al., 1982*). In addition, no histone H1 could be demonstrated (Hecker and Gander, 1985). Recent studies demonstrated H1-like histones in *T. cruzi* and *Crithidia fasiculata* (Toro and Galanti, 1988; Duschak and Cazzulo, 1990). These histones showed biochemical properties similar to that from *Tetrahymena fasiculata* (Johmann and Gorovsky, 1976).

Analysis of trypanosome chromosomes has revealed distinct variability in the molecular karyotype of these organisms. Using PFGE in chromosome fractionation, it has been observed that all trypanosome species posses a set of large chromosomes (0.7-6 Mb), that contain alleles of house-keeping genes. Recently it has been shown in a stock of *T. b. brucei* that the large chromosomes occur as 8-10 homologous pairs (Gottesdiener *et al.*, 1990). A similar number of large chromosomes appear to be present in other trypanosomes (Aymerich and Goldenberg, 1989; Van der Ploeg *et al.*, 1984). However Southern blot analyses (Southern, 1975) using probes for several housekeeping genes reveals that the same gene may be present on chromosomes of widely varying sizes in different stocks of *T. brucei* (Van der Ploeg *et al., 1984*; Engman *et al.*, 1987), and that genes linked to the same chromosome in one may not be so linked in another (Gibson and Garside, 1991),

The genome of African trypanosomes contain a variable number of 25-150 kb minichromosomes. The number of these chromosomes vary widely between species:

T.b. brucei, T.b. rhodesiense and *T. congolense* contain about 20-100, *T. gambiense* contain about 10, *T. equiperdum* contain one. *T. vivax* unlike other salivarian trypanosome species had for a long time been thuoght not to posses any minichromosomes (Gibson and Borst, 1986; Van der Ploeg *et al.*, 1984; Majiwa *et al.*, 1986), until Dickins and Gibson (1989), using species-specific DNA repeats cloned from *T. vivax* as probes, observed a band of hybridization in the minichromosomal region of *T. vivax* chromosomes separated by PFGE. These however, were not visible on ethidium bromide stained agarose gel, suggesting that *T. vivax* possesses only a few minichromosomes. However, these numbers appear to be variable even within species, since some stocks of *T.b. brucei* contain only a few minichromosomes (Van der Ploeg *et al.*, 1984). Minichromosomes also appear to be absent in *T. cruzi* (Aymerich and Goldenberg, 1989; Engman *et al.*, 1987; Gibson and Miles, 1986). The function of minichromosomes has not beeen established although it has been suggested that they may represent an adaptation to the molecular mechanism used for antigenic variation in African trypanosomes.

In addition to minichromosomes and megabase chromosomes, which contain house-keeping genes, most species of trypanosomes contain intermediate (medium) - sized chromosomes which vary in number and size (150-700 kb) among stocks of the same parasite species (Jasmer *et al.*, 1987). Studies carried out in *T. brucei* show that their chromosome-sized DNA can be resolved into four main categories by PFGE: minichromosomes, medium-sized chromosomes, chromosomes located at the compression zone and chromosomes that are non-specifically trapped at the wells (Van der Ploeg *et al.*, 1984; Van der Ploeg and Cornelissen, 1984). Cloning of VS genes and the ability to separate chromosome-sized DNA molecules has made it possible to follow the movement of VS genes among different chromosome-sized DNA molecules.

In *T. congolense*, it has been shown that trypanosome clones of a specific antigenic repertoire (serodeme) have overall similar chromosome patterns which are characteristic of that particular repertoire (Majiwa *et al.*, 1986). Masake *et al.* (1988)

analysed chromosome-sized DNA molecules of *T. congolense* clones by OFAGE to evaluate its application to serodeme identification. It was shown in this study that trypanosome clones belonging to one serodeme display similar overall chromosome profile with regard to number and size; the chromosome profile of trypanosome clones belonging to different antigenic repertoires differ significantly with regard to number and size of chromosomal bands; and that infrequently there can occur changes in the size of some medium-sized chromosomes but largely the chromosome profiles of cloned trypanosome population remained stable following transmission by tsetse flies.

Molecular karyotype of all developmental forms of a particular species of trypanosomes are identical (Brun and Schonenberger, 1979). In some cases, different species of trypanosomes have identical chromosome patterns but the overall pattern of chromosomes separated by PFGE correspond to the species and in some cases serodeme or antigenic repertoire of the parasite (Masake *et al.*, 1988). However, the molecular karyotype polymorphism occur in trypanosome clones not only from different serodemes but also from differently isolated field stocks (Gibson and Garside, 1991). The causes of the difference in these molecular karyotypes are not clear at present However, it has been suggested that sexual recombination may be involved in the generation of the observed polymorphisms. That the African trypanosomes mate and thus exchange genetic information has been demonstrated by Jenni *et al.*(1986).

1.9 Analyses of chromosomes

1.9.1 Pulsed-field gradient gel electrophoresis

Theory suggests that DNA is a free draining coil in electrophoresis (Hermans, 1955), hence solvent can penetrate the random coil with little or no molecular shielding. This predicts that the electrophoretic mobility of DNA should be independent of molecular weight which is what is observed when large DNA is

subjected to electrophoresis in a density gradient lacking any solid support (Olivera *et al.*, 1964).

To separate chromosome-sized DNA molecules, developments have been made in agarose gel electrophoresis systems. The systems utilise non-uniform, alternately pulsed electrical field and the technique is termed pulsed-field gradient gel electrophoresis (PFGE; Schwartz and Cantor, 1984). It is applicable to the separation of DNA molecules of all sizes but more particularly those of molecular weight higher than 23 kb. PFGE separates such large DNA molecules with resolution beyond the capacity of conventional electrophoresis. The electrophoretic mobility of DNA molecules in PFGE suggests that some orientation or condensation effect might be occurring (Schwartz and Cantor, 1984). The molecular weight resolution can thus be obtained by perturbing the orientation and extension of DNA molecules in the agarose gel matrices. Performing such perturbation in the gel is analogous to the viscoelastic technique (Klotz and Zimm, 1972), in which DNA molecules in free solution are stretched out of their random coil configuration and then allowed to relax back. The measured relaxation time is very sensitive to molecular weight but the technique does not actually separate molecules. To create a gel electrophoretic analogue of viscoelastic relaxation, the DNA molecule in agarose gel is subjected to alternately pulsed, perpendicularly oriented electrical fields. Both uniform and non-uniform electrical fields can be generated depending on whether one or all members of electrode array on a particular side are employed and the electrophoretic mobility of DNA will depend on pulse time (duration of applied field) and nature of applied field (uniform or non-uniform).

To separate chromosome-sized DNA molecules, an inhomogenous electric field is applied in one direction for a fixed period of time. Due to inhomogeneity of this field, a gradient of local mobility comparable to shear force is created across the DNA molecule, with the result that the DNA will move and re-orient parallel to the field. When this field is switched off and another one perpendicular to the first field is applied to the same DNA molecule, the DNA molecule must first relax before it can

reorient in response to the second field. It is then that translational motion can occur. The time taken to respond to the two fields applied alternately perpendicular to each other depends on molecular weight of the DNA molecule.

In a gel matrix, the small DNA molecules, which are able to reorient faster than large macromolecules, will experience greater net translational motion at any given pulse interval. On the other hand, the large DNA macromolecules will experience best translational motion when allowed optimal time interval in which to respond to each of the alternately applied electric fields. Thus, in order to achieve optimal resolution, the most important parameters to consider include the topology of the DNA (as this relates to the relaxation time of the DNA) and the duration of an applied field in a given direction (pulse interval)

There are various apparati for PFG electrophoresis, all of them using the basic principle of alternating direction of electric field. However, there are modifications in each one of them either in the homogeneity or inhomogeneity of the field and/or the arrangement of the array of electrodes. These include the Orthogonal field alternation gel electrophoresis (OFAGE; Carle and Olson, 1984) where electrodes are arranged between 105°-150° and the electric field is inhomogenous. In this system, there is good resolution of samples but usually the migration is distorted and comparison of sample sizes is difficult to perform. The other PFGE systems are the contour-clamped homogenous electric field (CHEF; Carle *et al., 1986*), field inversion gel electrophoresis (FIGE; Carle *et al.*, 1986; Turmel and Lalande, 1988), transverse alternating field electrophoresis (TAFE; Gardiner *et al.*, 1986), crossed field gel electrophoresis (CFGE) and pulsed homogenous orthogonal field gel electrophoresis (PHOGE; Lognonne, 1993).

The CHEF system uses a homogenous electric field alternating between two orientations 120° apart, whereas in FIGE, the electric field is periodically inverted without impairing resolution.

However, PFGE techniques are still not routine tools for separation of large DNA molecule. The determination of parameters for optimal separation of large DNA

molecules is done empirically for each new organism or to separate a wide range of DNA sizes in a single gel (Frederic, 1992).

1.10 Markers

1.10.1 Phenotypic markers

The study of genetics is based on the use of markers to investigate patterns of inheritance. A phenotypic marker such as colour or shape is relatively easy to identify. In some cases the phenotype may be difficult to score accurately because of modulation by environment or by modifier genes. Moreover, the utility of phenotypic markers is restricted because only a few markers are available in any single genetic cross; consequently, linkage can be detected in the genome (Williams *et al.*, 1993). Phenotypic markers have been used in genetics to map mutations. However, because of the drawbacks of phenotypic markers, it is desirable to develop new mapping methods that rely on molecular or physical markers.

1.10.2 Genetic markers

DNA markers circumvent the difficulties posed by phenotypic markers by detecting inheritance of allelic differences in DNA sequences.

Physical maps which comprise closely spaced DNA markers are useful for genome analysis (Rafalski and Tingey, 1991). DNA markers that are shown to be genetically linked to a trait of interest can be useful for gene cloning, medical diagnostics and for trait introgression in plant and animal breeding programs (Landegren *et al.*, 1988; Tanksley *et al.*, 1989).

In many organisms however, saturated genetic maps are not available and hence the DNA markers commonly used are polymorphic and show restriction fragment length polymorphism (RFLP; Bostetein *et al.*, 1980).

1.11. Polymerase chain reaction (PCR)

PCR is an efficient procedure for the enzymatic amplification of specific target nucleic acid sequences *in vitro* in the presence of appropriate nucleotide primers and a thermostable polymerase (Sambrook *et al, 1989).* Applications of PCR are many, including *in vitro* mutagenesis and engineering of DNA, genetic fingerprinting of forensic samples, assays for the presence of infectious agents, prenatal diagnosis of genetic diseases, analysis of allelic sequence variations, analysis of RNA transcript structure, genomic footprinting, direct nucleotide sequencing of genomic DNA and cDNA and detection of polymorphisms randomly distributed in the genome.

PCR involves a cycle of three temperature controlled steps. In the first step, the target double stranded DNA is denatured into single strands by heating . Synthetic complementary oligonucleotides are annealed to the denatured DNA strand in the second step and finally the primers are extended in the 5'-3' direction using thermostable *Thermus aquiticus* (*Taq*) DNA polymerase. Because the primer extension products synthesized in one cycle can serve as a template in the next, the number of DNA copies approximately doubles at every cycle, thus resulting in amplification.

1.11.1 Random amplified polymorphic DNA (RAPD) analysis

Detection of RFLP by southern blot hybridization is laborious and incompatible with high analytical throughput required for many applications (Beckman, 1988). Other polymorphism assays (Skolnick and Wallace, 1988) that are based on ploymerase chain reaction (PCR) require the target DNA sequence information for the design of amplification primers. The time and cost of obtaining this sequence information (Innis *et al*, 1990; Krawetz, 1989) is prohibitive for many large scale genetic mapping applications.

RAPD assay is based on DNA amplification but does not require knowledge of target DNA sequence. It is based upon an observation that a single oligonucleotide of randomly chosen DNA sequence, when mixed with genomic DNA and thermostable polymerase, and subjected to temperature cycling under conditions resembling those of the polymerase chain reaction (Williams *et al.*, 1990; Welsh *et al.*, 1991), will randomly prime the amplification of DNA at different places. Amplified segments are from regions of the template that contain two short DNA segments with some homology to the primer. The segments must be present on opposite DNA strands and be sufficiently close to each other to allow DNA amplification to occur. Short oligonucleotides primers when used at low annealing temperatures ensure that several sites randomly distributed in the template, give rise to amplification products. Amplification products are separated according to size on conventional agarose gels and visualized by ethidium bromide staining. The nature of fragments amplified is dependent on primer sequence and the sequence of the target DNA. Primers differing by a single nucleotide give rise to different amplified bands. This DNA amplification with random sequence primers is a sensitive method of revealing polymorphisms randomly distributed throughout any genome. A random primer usually amplifies several bands, each originating from a different genomic location. The exact molecular nature of RAPD polymorphisms has not been investigated by direct DNA sequencing, but is expected to include single nucleotide changes and insertions or deletions (Rafalski *et al.*, 1991).

1.12 Importance of this study

The economic impact of African trypanosomiasis direct or indirect on the lives of the people in the affected areas demand that serious action be taken in the understanding of their causative agents for proper management and eventual control of the disease. The genome of African trypanosomes like that of other lower

eukaryotic microorganisms is dynamic and undergoes alterations at frequencies dependent on the genotype of the trypanosomes.

In many cases, such alterations are mediated by repetitive DNA sequences and have been observed when they affect specific genes, mainly those that encode the VSG. The mutually exclusive activation of VSG genes involve a duplicative transposition of basic copy genes to a telomeric expression site to form an expression linked copy (ELC) (Hoeijmakers *et al.*, 1980; Pays *et al* 1981a, b, 1983; Bernards *et al.*, 1981, 1984; Majiwa *et al.*, 1982; Van der Ploeg *et al.*, 1982; Michels *et al.*, 1982, 1983, 1984; Borst and Cross, 1982; De Lange *et al.*, 1983; Laurent *et al.*, 1983). Telomeric VSG genes can also be activated by a different route that does not involve duplication or recombination in the vicinity of genes (Williams *et al.*, 1979, 1981; Borst *et al.*, 1981 Young *et al.*, 1982, 1983; Longacre *et al.*, 1983; Bernards *et al.*, 1984; Van der Ploeg *et al.*, 1984). The number of telomeres that can be activated either simultaneously or one at a time is not known. Since silent VSG genes can be activated by duplicative transposition to a transcriptionally active telomere, a possibility exists that non-duplicative activation of telomeric VSG genes might also involve chromosome re-arrangement occurring upstream of the affected genes, beyond detection by conventional gel electrophoresis and Southern blot analysis using VSG gene probes (Borst *et al.*, 1983). This hypothesis cannot be tested by cytological hybridization because trypanosomes never condense their chromosomes at any stage of cell division (Vickerman and Preston, 1970; Bernards *et al.*,1983). It cannot also be tested by chromosome walking because the presence of repetitive sequences which complicates chromosome walking (Bernards *et al.*, 1984). Some of the alterations are observed as gross changes in the sizes of some of the medium-sized chromosomes which are thought to be haploid in number (Gibson *et al.*, 1985). Some of the size alterations occur when the trypanosomes are repeatedly passaged, cyclically transmitted through tsetse fly or grown in rodents in the presence of trypanocidal drugs. In some instances, size alterations are concurrent with a changed phenotype in the parasite (e.g. changes in VSG which is the antigenic determinant)

When a particular chromosome undergoes size alteration for whatever reason, it is desirable to trace the chromosome to its new location within the trypanosome. Equally important is the capacity to identify allelic chromosomes in trypanosomes that belong to different antigenic repertoires. Both of these require a molecular marker specific for each of the chromosomes to be identified. Previously, dependence has been put on antigen encoding genes as chromosome-specific markers. However, these are present only in a few chromosomes (Van der Ploeg *et al.*, 1984).

To find a chromosome specific hybridization marker, several approaches can be followed. In this thesis, I have used the randomly amplified polymorphic DNA (RAPD) approach to search for hybridization markers specific for the four medium-sized chromosomes of *T. congolense* IL1180. The marker obtained in this way can be used to identify an allelic chromosome in other serodemes of *T. congolense*. Some of the chromosomes for which markers are needed are known to have translocated in some clones of *T. congolense* within the same antigenic repertoire. These markers can also be useful in identifying the chromosomes after exogenous DNA insertions during genetic manipulation of *T. congolense*.

1.13 Aim and objectives

1.13.1 Aim

The main aim of this study was to find out whether or not randomly amplified polymorphic DNA (RAPD) analysis can be used to generate chromosome-specific hybridization markers for the four medium-sized chromosomes of *Trypanosoma congolense* clone IL1180.

1.13.2 Objectives

Studies presented in this thesis were initiated to optimize the conditions for separation of the four medium-sized chromosomes of *T. congolense* clone IL1180 using PFG gel electrophoresis technique. This was to be followed by purification of DNA molecules representative of individual chromosomes from agarose gel then

PCR-amplify the purified chromosome-sized DNA molecules using random (10-mer) oligonucleotide primers and clone the PCR products that appear to be characteristic of each of the chromosomes.

Additionally, chromosomal location of some RAPD bands and cDNA clones from the Savanah-type *T. congolense* were to be verified.

CHAPTER 2

Materials and Methods

2.0 Trypanosome clones

The work described in this thesis was carried out using *T. congolense* clone IL1180 which was derived by cloning from STIB 212, an isolate from a lion at Serengeti, Tanzania (Nantulya *et al.*, 1984; Geigy and Kauffman, 1973; Masake *et al.*, 1983). Other *T congolense* clones used mainly for comparison included IL3000 (Fish *et al.*, 1989), X4 or ILNat 2.1 (Wellde *et al.*, 1974; Majiwa *et al.*, 1985), IL1616 (Nantulya *et al.*, 1984),

Based on the isoenzyme data, these clones are known to be the Savanah-type *T. congolense* (Majiwa *et al.*, 1985).

2.1 Trypanosome propagation

Cryopreserved glycerol stabilates of trypanosomes are normally stored in the ILRAD stabilate bank. The stabilates were allowed to thaw and diluted with cold (4°C) phosphate buffered saline glucose (PSG; 57 mM NaH_2PO_4, 3 mM Na_2HPO_4, 62.5 mM Nacl, 55 mM Glucose, pH 8). The number of viable trypanosomes were estimated by counting mobile organisms in a haemocytometer. Live parasites, numbering approximately $1x10^4$, were injected interperitoneally into , irradiated (600 rad) Spraque-Dawley rats obtained from the ILRAD breeding colony.

Parasitemia in each infected rat was monitored regularly by microscopic examination of tail blood placed on a slide. This was done at magnification of 250 x. When the number of trypanosomes visible in a field was over 100 trypanosomes , the rats were anaesthetised with diethyl ether and bled via cardiac puncture using 18 gauge, $1\,^1/_2$ inch needle and a twenty ml syringe. The blood was then collected into anticoagulant, sodium citrate (final concentration of 0.5% w/v).

2.2 Separation of trypanosomes from blood

The trypanosomes were separated from the blood elements by isopycnic percoll gradient centrifugation according to the method described by Grab and Bwayo (1982) followed by chromatography on a column of DEAE-cellulose (Lanham and Godfrey, 1970).

This was done by gently mixing trypanosome-infected whole blood (in sodium citrate) with an equal volume of percoll prepared by mixing 8.55 gm glucose, 2 gm sucrose and 100 ml of 100% percoll (density 1.129 gm /ml) then adjusting the pH to 7.4 by addition of 0.34 gm solid HEPES.

After mixing, the blood suspension was centrifuged at 15,000 RPM at 4°C for 30 minutes in a Beckman J21 centrifuge with a JA-17 rotor.

Under these conditions, the trypanosomes float as one layer near the top of the gradient while red blood cells sedimented at the bottom of the gradient. The buffy coat containing trypanosome was then collected into a 50 ml falcon tube and diluted with an equal volume of PSG buffer. This was then centrifuged in a Heraeus-Christ Minifuge at 2,500 RPM at 4°C for 25 minutes to pellet the trypanosomes. The pellet was then resuspended in 3 x volume of PSG buffer. The suspension was subsequently chromatographed through a column of DEAE-52 (Lanham and Godfery, 1970). The trypanosomes in the suspension were counted and their concentration adjusted to 2x 10^9 cells/ml PSG by pelleting and resuspended in an appropriate volume of PSG buffer.

2.3 Extraction and storage of DNA

DNA was extracted from trypanosome free of host blood using phenol equilibrated as indicated below.

Phenol (Bethesda Research Laboratories Inc. USA) was equilibrated by mixing with an equal volume of buffer (1.0 M Tris pH 8), followed by 0.1 M Tris pH 8 and 0.2% ß-Mercaptoethanol until the pH of the aqueous phase was >7.6. The phenol solution treated in this way can be stored at 4°C under equilibration buffer for a upto one month.

The equilibrated phenol was mixed with chloroform and isoamyl alcohol in the ratio, 25: 24: 1 (Phenol: Chloroform: Isoamyl alcohol). This was then mixed with the trypanosomes free of host blood and shaken gently to extract the DNA. The aqueous phase was separated by centrifugation at 12000 RPM for 5 minutes in an eppendorf microfuge, transferred into a new eppendorf tube and extracted once with 1-10 volumes of diethyl ether. A 3 M Sodium acetate solution was added to a final concentration of 0.2 M. Two times volume of cold (-20°C) absolute ethanol was added and DNA allowed to precipitate for 12 hours at -20°c. The DNA was pelleted by centrifugation at 15000 RPM in an eppendorf model 5414 microfuge, for 25 minutes at 4°C. It was then washed in 70% ethanol, vacuum dried and redissolved in sterile H_2O.

2.4 Preparation and storage of chromosome-sized DNA molecules

Chromosome-sized molecules from trypanosomes were prepared essentially as has been described by Van der Ploeg *et al.* (1984) and Majiwa *et al.* (1985a).

Briefly, equal volumes of trypanosome suspension (at a concentration of 2 x 10^9 cells/ml in PSG) and 1.5% (w/v) low melting point (LMP) agarose (Bethesda Research Laboratories Inc. USA) in PSG at 42^0C, were mixed and the suspension immediately poured into a 10 sample plug mold (BioRad, Richmond, CA, USA). This was immediately cooled on ice so that the agarose could set and embed the trypanosomes *en bloc*. The blocks of agarose with embedded trypanosomes were removed from the mold and incubated at 50^0 C for upto 36 hours in a lysis solution (2 ml lysis solution/cm^3 agarose block of embedded trypanosomes) containing sterile 1 M disodium EDTA, pH 8, 0.5% sodium dodecyl sulphate (SDS), 1 mg/ml Proteinase K and sterile water thereby making the concentration of 0.25 M EDTA. The beaker containing the above solution and the embedded DNA was then covered with a cling wrap.

The blocks of agarose were washed four times with 10 x volume sterile 10 mM EDTA, pH 8.0 for 25 minutes per wash at room temperature with gentle shaking

on a rotatest shaker (Model R 100 Luckham, Burgess Hill, Sussex, England) speed control set at 3. The blocks of agarose containing chromosome-sized DNA molecules were stored in 10 mM EDTA at 4°C for later use. The chromosome-sized DNA molecules made and stored in this way can stay intact upto 9 months, and perhaps even longer. They begin to degrade if the blocks of agarose are frozen.

2.5 Separation of trypanosome chromosomes

2.5.1 Pulsed-field gradient gel electrophoresis

2.5.1.1 Apparatus

Fractionation of chromosome-sized DNA molecules was performed by contour-clamped homogenous electric field using CHEF-DR II (Bio-Rad, Richmond, CA, USA) .

The systems consist of a 43 cm x 44 cm acrylic box with 24 horizontal electrodes arranged in a hexagon thus producing a field reorientation angle of 120°.

The ramping of the switch time from the beginning of the run to the end was performed by Pulsewave 760 switcher (Bio-Rad, Richmond, CA USA)

2.5.1.3 Casting the gel for electrophoresis

A 1.5 % agarose gel (Bethesda Research Laboratories, USA) in 0.5 x TBE (90 mM Tris-HCl, 90 mM Boric acid and 1 mM EDTA or 1.5 % SeaKem^{TM} GTG agarose (FMC Bioproducts, USA) in 1x TAE was cast to a depth of 5 mm on a 140 mm x 127 mm casting stand with removable endplates.

Sample wells were formed by using a 10 sample comb and a comb holder adjusted to 2 mm from above the casting stand surface using a levelling table. It was allowed to cool and set for one hour at room temperature.

For OFAGE, the gel was cast into a sealed gel former IBI model 44.

2.5.1.4 Loading the samples

Sample plugs were cut to fit the size of the wells and loaded while the gel was in the casting stand. The agarose plugs were placed onto the front of the walls of the sample wells using a spatula and then gently pressed into wells. Each sample well was then filled with molten 1.5% LMP agarose in 1x TBE and allowed to harden at room temperature for 10 minutes.

2.5.1.5 The pulsed-field gradient gel electrophoresis

The end plates were removed from the casting stand and the gel slid into the electrophoresis chamber containing 2.5 litres of buffer (either 0.5 x TBE or 1x TAE). The gel was secured, thus immobilized at the centre of the chamber using stoppers supplied with each apparatus.

The buffer temperature was maintained at 15^0 C by constant re-circulation at 600 ml/min through a tubing coiled in a temperature controlled water bath maintained at 4^0 C using a variable speed pump.

The electrophoresis was performed at a constant voltage of 150 volts for 60 hours with an initial pulse interval of 90 seconds and a final pulse interval of 180 seconds unless otherwise stated.

For the OFAGE system, the electrophoresis was carried out at 200 volts for 18 hours with a pulse frequency of 20 sec unless indicated otherwise.

2.5.1.6 Staining and photography

Fluorescent properties of ethidium bromide (EtBr) were exploited for the visualization of DNA in agarose gels (Sharp, 1973). This is because EtBr contains a planar group that intercalates between the stacked bases of DNA and upon excitation by UV, strong fluorescence emitted. EtBr can bind to both RNA and DNA under UV illumination.

During conventional gel electrophoresis, EtBr at a concentration of 0.5 μg/ml was incorporated both in the gel and the electrophoresis buffer. For electrophoretic separation of chromosomal DNA molecules by PFGE, the gel was stained by

immersion and gentle agitation in 500 ml of distiled water containing 0.5 µg/ml EtBr at room temperature for one hour. The gel was then photographed using UV transilluminator and a polaroid film.

The sizes of the chromosomes were estimated by comparison with chromosomes-size markers made from the budding yeast *Saccharomyces cerevisiae* catalogue number 170-3605 (Bio-Rad, Richmond, CA, USA) which has sizes ranging from 200 kb to 2 Mb.

2.6 Purification of individual chromosomes from agarose gels

The purification was done using the Genclean Kit II following the procedure recommended by the manufacturer (BIO 101 Inc, La, Jolla, CA)

To obtain DNA molecules representing an individual chromosome, PFGE was performed as described in section 2.5 above, using 1.5% SeaKem^{TM} -GTG agarose (FMC Bioproducts) in 1 x TAE. After electrophoresis, the gel was stained, destained and photographed as has been described above.

The individual chromosome bands visualized by UV illumination, in the dark room, were excised using sterile scalpel blade. The agarose gel slices were individually placed in 1.5 ml eppendorf tubes with three volumes of sterile water and kept at 4^0C overnight to allow outward diffusion of electrophoresis buffer. The water was decanted and 3 volumes of 6 M NaI solution added. It was then incubated at 55^0C for 5 minutes to dissolve the agarose. A 10 µl suspension of silica matrix in water (glass milk) was added to the dissolved agarose and gently mixed by inverting the tube several times. The suspension was placed on ice for 25 minutes with gentle mixing after every 5 minutes. This makes DNA bind to the glass beads. The silica particles were pelleted by centrifugation at 15,000 rpm in an eppendorf microfuge at 4^0C for 5 seconds. The aqueous phase was then decanted and saved for re-extration glass milk.

The silica pellet was then washed three times with a 50 x volume of a solution made by mixing 14 ml concentrated solution of NaCl, Tris and EDTA (New wash

concentrate; BIO 101, La Jolla, CA, Inc.) 280 ml of sterile water, 310 ml of absolute ethanol and stored at -20^0 C.

The DNA was eluted from the silica by resuspending the silica particles in 10 µl of sterile water followed by incubation at 55^0 C for 30 minutes. The absence of salt causes the DNA to dissociate from the beads. The suspension was then centrifuged at 12,000 RPM for 20 seconds on a bench-top eppendorf centrifuge 5415 C and the eluate containing the DNA transferred to a new tube. It was then extracted once with an equal volume of phenol as described in section 2.11. The yield of DNA recovered in this way was estimated by resolving an aliquot by electrophoresis in an agarose gel and comparing it with a known amount of DNA resolved in the same gel.

2.7 PCR amplification

2.7.1 Amplification using specific primers

Two pairs of oligonucleotides were used for the amplification of purified chromosome-sized DNA molecules. Primer ILO344: CGA GCG AGA ACG GGC AC and ILO345: GGG ACA AAC AAA TCC CGC were specific for the Savannah-type *T. congolense*, while primers ILO524: CGC GCC CGC T and ILO525: CGG ACG TCG C amplify approximately 2 kb fragment of Savannah type *T. congolense* .

PCR was performed in a 10 µl reaction containing approximately 50 pg of DNA from the purified individual chromosome-sized DNA molecules, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 150 µM each of the deoxynucleotide triphosphates, 5 µM each of the oligonucleotide primer, 2.5 ng of BSA and 1 unit of *Thermus aquiticus* (*Taq*) DNA polymerase (Promega, Madison, USA).

The reaction was overlaid with mineral oil (Sigma Chemical Co., St. Louis, USA) and the samples placed on a heating block in a thermal cycler (ASTEC, PC-700) programmed to perform 40 cycles of the following parameters: 94°C for 1 min to denature the template DNA, cooled to 55°C and kept at this temperature for 1 min to anneal the primers, and finally 72°C for DNA synthesis and kept at this temperature

for 1.5 min. At the end of the 40 cycles, the synthesis reaction was continued for 10 minutes at 72°C and then cooled to 4°C.

The entire PCR product was resolved by electrophoresis in a 1.5% agarose gel (BRL, Life Technologies Inc., USA), stained with EtBr and photographed under UV illumination as described in section 2.5.1.6.

2.7.2 Amplification using random primers

A list of oligonucleotide primers used for RAPDs, their sequences and amplification efficiencies is given in Table 3.

A RAPD reaction contained, in 50 µl final volume, 50 ng of DNA purified from chromosome-sized DNA molecules, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 3 mM $MgCl_2$, 0.05 % NP-40, 0.05 % Tween, 200 µM each deoxynucleotide triphosphate, 0.6 µM of primer and 5 units of *Taq* polymerase. The reaction was overlaid with mineral oil and samples placed in a thermal cycler programmed for 40 cycles of amplification at 94°C for 1.5 min, 36°C for 1.5 min, 72°C for 2 min, followed by a further extension of 10 min at 72°C. The reaction was then cooled to 4°C.

One tenth volume of each PCR product was resolved in a 1.5% agarose gel, stained with EtBr, photographed and blotted onto a filter paper for further analysis by hybridization. The remaining part of the PCR amplification was electrophoresed in the same way as above if it contained a chromosome-specific RAPD band. Any putative chromosome-specific RAPD bands were excised from the gel and purified for molecular cloning in appropriate vectors.

2.8 Conventional gel electrophoresis

PCR products or restriction enzyme digests of plasmid DNA were analysed by electrophoresis in agarose gels (1.5%) cast in 1x TBE (40 mM Tris-HCl, 40 mM

Boric acid and 1 mM EDTA). The gels were prepared by boiling the agarose (Bethesda Research Laboratories Inc, USA) in 1 x TBE. The boiled agarose was cooled to 50°C-55°C and poured into a sealed gel former (IBI model 44 horizontal electrophoresis apparatus). The loading buffer (2.5µl), containing 10 x TBE, 50 % glycerol, 0.25% (w/v) bromophenol blue, 0.25% Xylene Cyanol FF, was added to each sample before electrophoresis. Two millimetre thick combs were used to form the wells so that at least 40 µl of the sample could be loaded in each well. The gels were electrophoresed at a voltage of between 5-10 v/cm. The size markers used was a mixture of bacteriophage lambda DNA digested with Hind III and *Phi* x 174 DNA digested with Hae III, giving fragments in the range of about 23.1 Kb to 0.3 Kb.

2.9 Southern blot hybridization

This technique described by Southern (1975) is applicable to genomic DNA, chromosome-sized DNA molecules, cloned DNA and PCR amplified DNA, with various modifications. Processing and blotting of PCR products and chromosome-sized DNA molecules were performed differently.

PCR products and restriction enzyme digests of plasmid DNA were resolved in 1.5% agarose gels containing 0.5 µg/ml of EtBr. The gels were viewed with the aid of UV transilluminator and photographed. The gels were soaked, with gentle shaking, in denaturing solution (0.5 M NaoH, 0.5 M NaCl) for 30 min at room temperature, then transferred into neutralizing solution (1 M Tris-HCl pH 7.5, 1.5 M NaCl) for 30 minutes at the same temperature.

Gels containing chromosome-sized DNA molecules were soaked twice for 15 minutes in 0.25 M HCl at room temperature to depurinate DNA prior to alkaline denaturation (Wahl *et al., 1979*). The acid-induced cleavage increases the efficiency of transfer of DNA fragments. However, it should not be allowed to proceed for longer than 20 min since it will then cleave the DNA into fragments that are too short to bind effectively to nitrocelullose filter. Volumes of the soaking solutions were at least 2-3 times that of the gel.

The gels were placed upside down on a sheet of Whatman 3 MM filter paper resting on a 1.5-2.0 cm thick sponge in a shallow tray filled upto just below the top of the sponge with 20 x SSC (1 x SSC is 15 mM Sodium citrate, 50 mM NaCl). All the air bubbles in the sponge and between the sponge and the 3 MM paper were removed using a smooth glass rod. The sheet of nitrocellulose filter (Nitran; Schleiler and Schuell BA 95) cut to measure the size of the gel was wetted in sterile water and placed on top of the gel with the shiny side facing upwards and ensuring that no air bubbles were trapped between the filter and the gel. Whatman 3 MM filter was cut to the same size as the nitrocelullose filter and stacked 1 cm high, followed by absorbent paper towel to a height of 6 cm. The stack was firmly pressed down with 250 g of weights and the blotting allowed to proceed for 24 hours at room temperature. The rate of transfer of DNA is dependent on the DNA fragment size, the concentration of the gel and the concentration of the SSC. The towels and the 3 MM filters were removed after 24 hours. DNA transferred to nitrocelullose filter in this way can be permanently fixed on the filter by baking in an oven at 80°C, by placing on a UV lamp or in a UV oven. Fixing in this case was done by placing the filter in a UV oven (UV Stratalinker^{TM} 2400, Stratagene) for one minute.

2.9.1 DNA labelling by random priming

This method (Feinberg and Vogelstein, 1983) relies on the ability of random hexanucleotides to anneal to multiple sites along the length of any DNA template. The primer-template complex formed acts as substrate for the Klenow fragment of DNA polymerase I, which lacks the 5'-3' exonuclease activity and thus catalyses the synthesis of new DNA by incorporating nucleotide monophosphates at the free 3' hydroxyl group of the primer.

For the purpose of labelling, 25 ng of DNA was adjusted to 23 µl with sterile water and denatured by heating to 94-96°C in a boiling water bath then chilled on ice. The priming reaction contained 10 µl random hexanucleotides in an aqueous solution with nuclease - free BSA, 10 µl of primer buffer solution contain 0.1 M each of dATP,

dGTP, dTTP in 250 mM Tris-HCl, pH 7.8, 40 mM $MgCl_2$, 25 mM ß-Mercaptoethanol, 5 µl of dCTP in 50 µl. The labelling reaction was started by the addition of 2 units of Klenow fragment of DNA polymerase I and incubated at 37°C for 30 minutes. It was stopped by addition of 2 µl of 0.5 M EDTA, pH 8.0. The unincorporated nucleotides were separated from the labelled DNA by centrifugation at 1200 rpm for 3 min through a column of Sephadex G-50 (Sambrook *et al.*, 1989).

Amount of radioactivity incorporated was determined by counting 1 µl of the eluted, labelled DNA in a Beckman scintillation counter, using 10 ml of aquosol as the scintillant. The specific activity of radioactivity of a probe made in this way was as high as 1 x 10^9 cpm/ml

2.10 Hybridization and washing of the filters

The filters were rolled and placed in hybridization bottles with prehybridization solution (4 x SSC, 5 x Denharts', 0.1% SDS, 0.1% NaPPi) and incubated at 65°C in a Hybaid hybridization oven for at least 2 hours. The radiolabelled DNA probe was denatured by incubation in a boiling water bath for 5 min before adding to the hybridization solution. The probe concentration routinely used was 1 x 10^6 cpm/ml. The probe was added to the prehybridization solution and the filters incubated for 12 hours at 65°C in a Hybaid hybridization oven unless indicated otherwise. After hybridization, the probe was transferred into a Falcon tube and stored at -20°C for later use. The filters were washed with 10 ml of wash solution at high stringency (0.1 x SSC, 0.1% SDS at 65°C) unless indicated otherwise. The washing was done for a duration of 1 hour with a change of wash solution after 30 min. The filters were exposed to X-ray film at -80°C for 12-72 hours with an intensifying screen.

2.11 Purification of individual DNA fragments produced by PCR

A PCR fragment to be purified was excised from the agarose gels under UV illumination. The gel slice was transferred to a punctured sterile eppendorf microfuge tube that had been plugged with about 2-3 mm of sterile siliconized glass-wool. This was placed on top of another eppendorf tube in a two-tiered system and centrifuged at 4^0C for 10 min at 6000 RPM in a microfuge. The eluate containing DNA fragment was collected in the bottom tube and the recovery of the fragment verified by examining its fluorescence under UV illumination. The eluate was phenol extracted once as described in section 2.11.

This technique by He *et al* 1992 has an advantage of simplicity and speed. Maceration of gel slice is not required, thus reducing elution of contaminating agarose particles. The method is also ideal for rapid isolation of fragments for use as DNA probes.

2.12 Cloning a PCR product

In cloning a PCR product, it is important that the vector used alone does not give any transformants. Difficulties frequently encountered when cloning PCR products are due to the synthesis of 'ragged' ends on DNA amplified by *Taq* ploymerase (Williams, 1989). This is caused by the template independent terminal transferase activity of *Taq* polymerase which results in the addition of a single nucleotide at the 3' end of the PCR product fragment, hence cloning PCR product as blunt end is very inefficient (Clark, 1988; Mole *et al*., 1989).
Taq DNA polymerase has a stronger preference for dATP, therefore the nucleotide added at the 3' end is almost exclusively adenosine (Clark, 1988).

In this study, we exploited the template-independent activity of *Taq* polymerase to create a cloning scheme which has efficiency of sticky-end cloning but requires no schematic modification of PCR product. The vector used is designated T-Vector (Marchuk *et al*., 1990).

2.12.1 Construction of a T-vector

Plasmid was prepared from an overnight culture of bacteria (XL1-blue strain of *E. coli*) transformed with Bluescript^{TM} and grown in 2 x YT agarose plates with ampicillin at 50 µg/ml as described in section 2.13.3.

The DNA was dissolved in 50 µl of sterile water. Three µg of the plasmid DNA was then digested at 37°C for 2 hours with 20 units of *Eco* RV in a 10 µl reaction containing 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$,1 mM DTT, (pH 7.9 at 25°C) and 100 µg/ml BSA. The reaction mixture was then extracted with phenol/chloroform and dissolved in 200 µl of water.

Addition of thymine residue to the 3' end of the vector was done essentially as has been described by Holton and Graham (1990). This was done by incubating 10 µg of *EcoRV*-digested Bluescript at 70°C for 2 hours with 4 units of *Taq* polymerase in a reaction containing 50 mM Tris-HCl, pH 9.0 at 25°C, 0.1 % Triton X-100. This was followed by phenol/chloroform extraction as described above and the DNA redissolved in 50 µl of water,

The ability of the T-vector not to ligate upon itself was tested by incubating 2 µg of the vector overnight with 400 units of T_4 DNA ligase in a 20 µl reaction containing 50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$,10 mM DTT, 1 mM ATP, 25 µg/ml BSA. The entire ligation reaction was resolved in a 1 % agarose gel and a single band corresponding to a linear plasmid was observed.

2.12.2 Ligation

Ligation of the purified PCR fragments ranging in size from 0.3 kb to 2.3 kb was performed with T-vector prepared as has been described in section 2.12.1. The

concentration of the T-vector and the purified PCR fragment were adjusted by dilution to 40 µg/µl and 20 µg/µl respectively. A 10 µl ligation reaction was set up as follows: 2.0 µl of the T-vector DNA, 1 µl of purified PCR fragment, 50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, 25 µg/ml BSA and 2.5 units of bacteriophage T_4 DNA ligase (New England Bio-Labs). The reaction mixture was then incubated at 16°C for at least 12 hours.

2.12.3 Preparation of competent bacterial cells

E. coli strain SURE^{TM} (Stratagene, La Jolla, USA) was used to increase the efficiency of transformation with, and retention of, foreign DNA cloned in plasmid vector. Preparation of competent cells was done following the procedure described by Hanahan (1983).

Briefly a frozen stock of cells in glycerol stored at -70^0 C in screw-capped polypropylene tubes was scrapped with sterile pipette tip and inoculated into 50 ml of 2 x YT medium containing 12.5 µg tetracycline/ml and incubated at 37^{0}C with shaking at 225 RPM for at least 12 hours (overnight). Then 500 µl of the overnight culture was inoculated in 50 ml 2 x YT medium in 500 ml conical flasks and incubated with shaking until the cell density, monitored using a spectrophotometer, was in the range of 6-9 x 10^7 viable cells/ml ($OD_{600\ nm}$ =0.4-0.5). The culture was transferred to a sterile polypropylene tube and chilled on ice for 15 minutes; the cells were then pelleted by centrifugation at 2800 rpm in a Heraeus Christ minifuge RF at 4^{0}C for 10 minutes. The medium was drained completely off the pellet by inverting the tubes on paper towel (cleanex) then resuspended in 16.5 ml of ice cold FSB (FSB is 1 mM CH_3COOK, pH 7.4, 45 mM $MnCl_2.4\ H_2O$, 10 mM $CaCl_2.2\ H_2O$, 100 mM KCl, 3 mM Hexamine Cobalt Chloride, 10% (w/v) glycerol) by pipetting. The cell suspension was chilled on ice for 15 minutes, repelleted as above and resuspended in 3.6 ml of fresh FSB. The suspension was adjusted to 3.5% (v/v) by addition of 1.7 µl of DMSO. This was immediately swirled for 10 seconds then chilled on ice for 5 minutes. A 210 µl volume of the cells was aliquoted into pre-chilled sterile 1.5 ml

eppendorf tubes and immediately flash-frozen in liquid nitrogen. The cells thus made competent were stored in a freezer at -70^{0}C for later use.

2.12.4 Transformation of bacteria

Transformation of the competent bacterial cells was done as has been described by Sambrook *et al.* (1989). Competent cells from -70°C freezer were thawed on ice and then mixed gently by tapping the side of the tube. 100 µl of the cell suspension was aliquoted into pre-chilled 15 ml, polypropylene tubes. This was adjusted to 25 mM ß-mercaptoethanol by addition of 1.7 µl of a fresh 1:10 dilution of ß-mercaptoethanol (diluted in sterile water). The cells were swirled gently and allowed to sit on ice for 10 minutes with gentle swirling every 2 minutes.

One µl of the ligation mixture containing about 2 micrograms of DNA was added to the cells and swirled gently. This was placed on ice for 30 minutes, then transferred to a 42°C water bath for 45 seconds (heat shock) and returned on ice for 2 minutes. A 0.9 ml of 2 x YT pre-heated to 42°C, was added to the transformation mixture and the cells incubated at 37°C for 1 hours while shaking at 225 RPM. The cells were concentrated by centrifugation at 1000 RPM in a Heraeus minifuge RF at 4°C for 5 minutes and the pellet resuspended in 200 µl of 2 x YT. The cell suspension was then plated on NZYCM plates containing 50 µg ampicilin/ml and the plates incubated at 37°C for at least 12 hours in an inverted position.

2.13 Identification and maintenance of recombinant clones

2.13.1 Screening of bacterial colonies

Transformant colonies were grown for 12 to 18 hours. The colonies on a plate were overlaid with a dry nitrocellulose filter and each filter marked for alignment by stabbing with 23 gauge hypodermic needle dipped in indian ink. The filters were peeled away as soon as they were completely wetted and then placed with the side which had contact with the bacterial colonies in the upward position, for 5 min, on

two sheets of Whatman 3 MM paper soaked in denaturing solution (0.5 M NaOH, 1.5 M NaCl) then transferred to 3 MM paper soaked in 1 M Tris-HCl, pH 7.4, 3 M NaCl and left to neutralize for 10-20 minutes. They were then placed between clean sheets of 3 MM paper and pressed firmly to remove the residual bacterial debris. The DNA released from the bacteria was then cross-linked to the filter for 1 min using UV Stratalinker^{TM} 2400. The plates on which the transformed bacteria were initially grown were reincubated at 37°C until the colonies had re-grown (approximately 8 hours) and stored at 4°C. The filters were prehybridized for between 2-4 hours and hybridized overnight using purified PCR fragment as a probe (see section 2.8.1).

2.13.3 Isolation of plasmid DNA by alkaline lysis (miniprep)

Several methods, all of which exploit the relatively small size and covalently circular nature of plasmids, have been developed for the purification of plasmid DNA from bacteria. A modification of the procedures described by Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981) was used.

Bacteria containing the plasmid were grown overnight in 5 ml of 2 x YT medium containing 5 µg ampicilin/ml. A 3 ml aliquot of the overnight culture was transferred to a fresh sterile eppendorf tube and centrifuged at 12000 RPM for 30 sec in an eppendorf minifuge. The supernatant was drained and the bacterial pellet resuspended in 200 µl of a solution containing 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 100 µg RNase/ml. This was followed by lysis of the cells by the addition of 200 µl of lysis solution (0.2 M NaOH and 1% SDS). The tube contents were mixed by inverting the tube several times until the solution became clear. A 200 µl of neutralizing solution (2.55 N Potassium acetate) was then added, mixed as above and the bacterial lysate centrifuged at 12000 RPM for 5 min at room temperature. The supernatant was decanted to a fresh eppendorf tube and 1 ml of DNA purification resin (Promega -A714B) added and the content mixed briefly. The plasmid DNA binds to the resin. The mixture was then passed through a minicolumn by suction

pressure using a vacuum manifold (Promega Corporation, Madison, USA). The column was washed with 2 ml solution containing 0.2 M NaCl, 20 mM Tris-HCl, pH 7.5, 5 mM EDTA in 50% ethanol. Any residual wash solution in the minicolumn was eluted by centrifugation of the minicolumn at 12000 RPM for 20 seconds. Fifty µl of sterile water was then added to the column and left at room temperature for 10 min. The plasmid DNA was subsequently eluted by spinning the minicolumn in an eppendorf tube at 12000 RPM for 30 seconds in a benchtop eppendorf centrifuge. The liquid containing plasmid DNA was stored at -20°C for later use

2.13.4 Restriction Enzyme digestion

The plasmid DNA was subjected to double digestion with EcoR I and Hind III. These enzymes have restriction sites flanking the EcoR V site at which the PCR product was cloned. The double digest was set up in a 10 µl reaction containing 2 µg of plasmid DNA, 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$ 1 mM DTT, 100 µ/ml acetylated BSA, 5 µl sterile water, 1 µl EcoR I (10 weiss units) and 1 µl Hind III (10 weiss units). The reaction mixture was incubated at 37°C for 2 hours and the entire reaction volume resolved by electrophoresis in a 1.5% agarose gel and then photographed under UV transillumination.

CHAPTER 3

RESULTS

3.0 Some cDNAs hybridizes to several chromosomes

Individual genes are naturally located at discrete loci on single chromosomes. Some genes may have other copies located on more than one chromosome. Genes encoding VSGs are representative of this category. However, many genes exist as single copies each on a chromosome. It can be predicted that when a cDNA from such a gene is hybridized to separated chromosomes, it could hybridize exclusively to that chromosome. In an attempt to find chromosome-specific hybridization markers, it was necessary to determine first the chromosomal location of some cDNA clones derived from the Savannah-type *T. congolense*. These experiments were undertaken with the expectation that some of the cDNA clones could hybridize exclusively to one of the chromosomes for which markers were needed. In which case a search for specific markers would shift to the chromosomes to which none of the cDNA clones hybridized. However, as it can be seen in Table 1, none of the cDNA clones used hybridized exclusively to any of the four medium-sized chromosomes. Except for the p1616/5 cDNA which hybridized to both the medium-sized chromosomes and chromosomes located at the compression zone, all the other cDNA clones hybridized to chromosomes located within the compression zone (CZ). None of the cDNA clones used hybridized with the minichromosomes.

From these observations it was necessary to proceed with a search for hybridization markers specific for the four chromosomes.

3.1 The majority of cloned RAPD bands examined hybridize to chromosomal DNA molecules above 1 Mb

Some RAPD bands had been generated from PCR amplification of the Savanah-type *T. congolense* genomic DNA using arbitrary primers. Some of these bands which appeared informative either due to their specificity for any one particular

chromosome or intensity in EtBr- stained agarose gel were cloned in the appropriate vectors. However the chromosomal location of these bands had not been verified. In the continued search for chromosome-specific markers, it was necessary to determine the chromosomal location of some of the RAPD bands. It was anticipated that some of these could hybridize to any of the four medium-sized chromosomes. Chromosome-sized DNA molecules of *T. congolense* clones belonging to 3 different antigenic repertoires (ILNaR 1, ILNaR 2 and ILNaR 3) were electrophoresed using the contour-clamped homogenous electric field (CHEF). Figure 2 A shows a photograph of ethidium bromide-stained agarose gel containing the chromosomes. Although the number of chromosomes resolved vary both in number and size among clones from the different antigenic repertoires (Majiwa *et al.,* 1986), all the clones used here have minichromosomes (50- 150 kb), intermediate-sized chromosomes (200-700 kb), chromosomes located at the compression zone (large chromosomes > 1 Mb) and chromosomes that are trapped in the wells.

In order to determine chromosomal location of the cloned RAPD bands, the chromosome-sized DNA molecules (Figure 2 A) were transferred to a nitrocellulose filter paper and hybridized with radiolabelled cloned RAPD bands (Table 3). Analyses of autoradiograms represented by the one shown in Figure 2 B indicated that all the cloned RAPD bands examined were located on chromosomes in the region above 1 Mb.

From these observations, it is apparent that an alternate approach needed to be taken in the search for chromosome-specific markers.

Figure 1

p1616/5 cDNA hybridizes to several chromosomes.

Chromosomes prepared from trypanosome clones from three different antigenic repertoires were resolved in a 1.5% agarose gel by CHEF.

(B) A photograph of ethidium bromide stained gel containing chromosome-sized DNA of: 1, IL1180; 2, IL373; 3, X4T; 4, X4NT.

The chromosome-sized DNA molecules in the gel were blotted to a nitrocellulose filter paper and the blot hybridized with p1616/5 cDNA; the autoradiogram obtained is shown in panel A

Approximate sizes of the chromosome-sized DNA molecules were estimated by comparison with those of the *Saccharomyces cerevisiae*. These are indicated in megabase pairs in lane M in panel B.

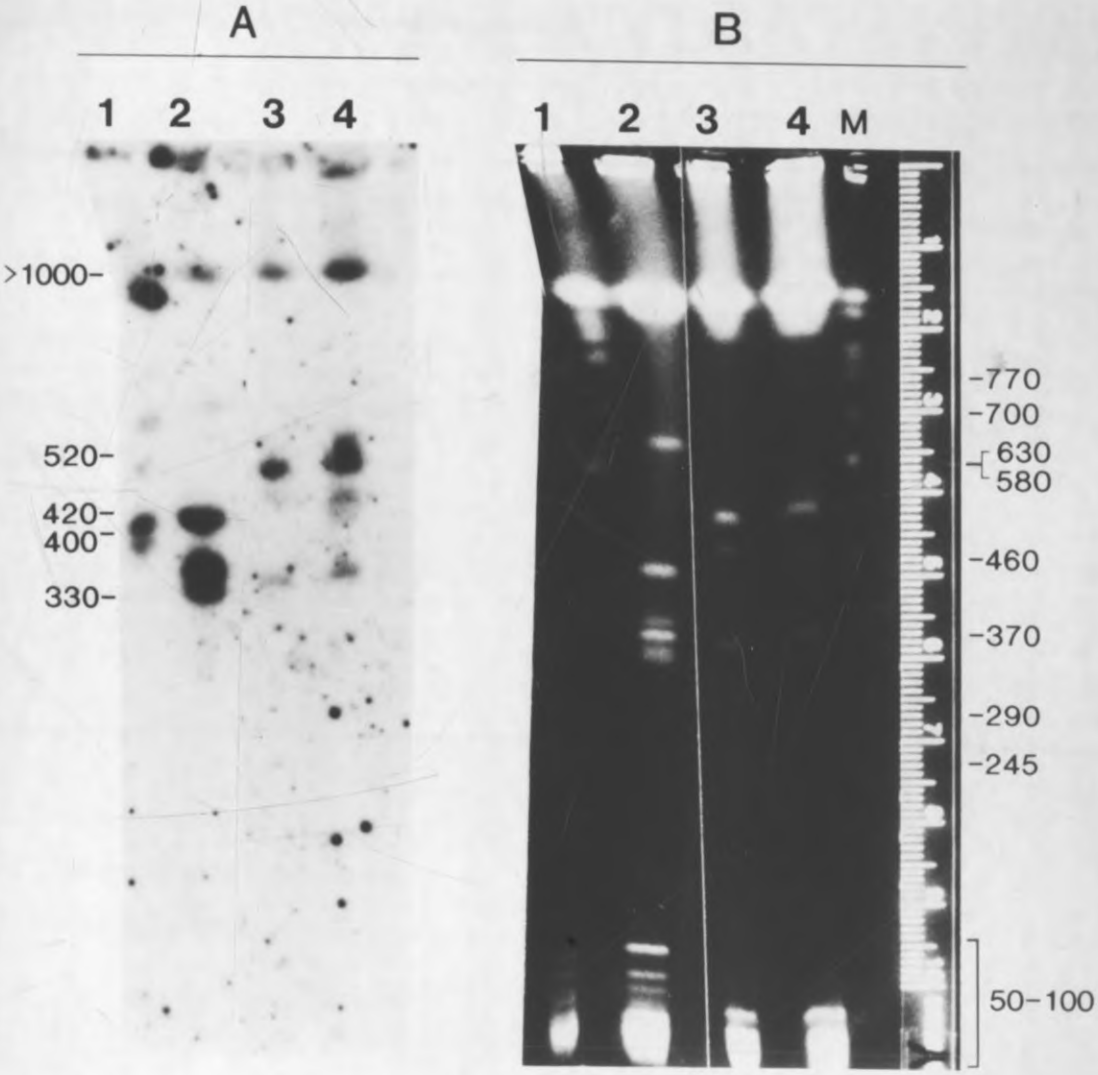

A
1
2
3
4
>1000-
520-
420-
400-
330-
B
1
2
3
4
M
-770
-700
630
580
-460
-370
-290
-245
50-100

Table 1

Hybridization of cDNA Clones to Chromosomes

cDNA	Insert Size	Source Trypanosome	Chromosomal Location*
p1616/5	1100	IL 1616 (ILNat 3.3)	CZ & MZ
pMLS	300	IL X 4 (ILNat 2.1)	CZ
p1616/1	900	IL 1616 (ILNat 3.3)	CZ
2143pCN7	770	IL 2143 (ILNat 3.2)	CZ
2143pCN2	ND	IL 2143 (ILNat 3.2)	CZ
pTF10	ND	IL 3000 (ILNat 2.)	CZ

*CZ, Compression Zone

MZ, Medium Sized Chromosomes

ND, Sizes not determined

Figure 2

The majority of cloned RAPD bands examined hybridize to chromosomal DNA molecules above 1 Mb.

Chromosome-sized DNA molecules from five trypanosome clones: IL3000, IL1180, IL373, IL308, ILX4T, in lanes 1 to 5 respectively were resolved in a 1.5% agarose gel by CHEF at an initial pulse time, 90 seconds and final pulse time, 180 seconds. A photograph of an ethidium bromide-stained gel is shown in panel A. The chromosome-sized DNA molecules in the gel were transferred to nitrocellulose filter paper and hybridized with pRAPD5/ILN 2/400.39. (B). An autoradiograph of the hybridization is shown. Approximate sizes of the chromosomes, estimated by comparison with those of *Saccharomyces cerevisiae*, are indicated in megabase pairs in lane 6.

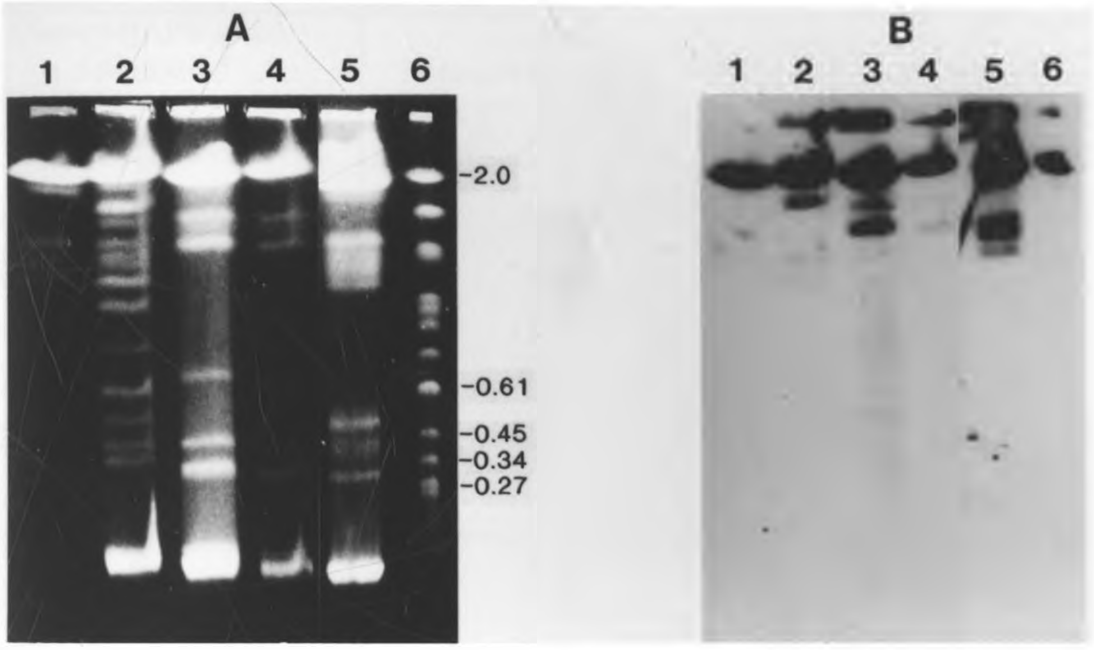

A
1 2 3 4 5 6
-2.0
-0.61
-0.45
-0.34
-0.27
B
1 2 3 4 5 6

Table 2

Some cloned RAPD bands which hybridized with *T. congolense* chromosomes located at the compression zone

Cloned RAPD band designation	Insert size (bp)	Source Trypanosome
pRAPD6/ILN2/165.2	165	IL 3000
pRAPD6/ILN2/250.7	250	IL 3000
pRAPD6/ILN2/400.9	400	IL 3000
pRAPD5/ILN2/1.2	1200	IL 3000
pRAPD5/ILN2/700.26	700	IL 3000
pRAPD5/ILN2/400.39	400	IL 3000

3.2 Electrophoretic separation of chromosome-sized DNA molecules

3.2.1 Effect of pulse interval on the separation of *T. congolense* chromosome-sized DNA molecules

In order to fractionate trypanosome chromosomes and to purify DNA from chromosome-sized DNA molecules representative of an individual chromosome, it is necessary to prepare intact chromosome-sized DNA molecules. However trypanosome chromosomes do not appear as discrete condensed structures (Vickerman and Preston, 1970). Therefore preparation of the chromosomes was performed as described in section 2.4. Chromosomes prepared in this way appear to be intact as can be judged from the observation that they migrate as distinct bands upon separation by PFG gel electrophoresis. Hybridization of blots of such chromosomes separated by PFGE with cDNA clones which localize to single unique chromosomes is the other evidence supporting the suggestion that these bands represent single, intact chromosomes. The sharpness of a band of chromosome-sized DNA molecules by PFG gel electrophoresis indicates the presence of a unique, high molecular weight DNA molecule. Thus the single bands observed and purified from gels in which chromosomes-sized DNA molecules have been separated most likely represent individual chromosomes.

Several conditions have been found to influence the separation by PFGE of large DNA molecules the size of a chromosome. These include pulse interval, duration of electrophoresis (Gemmil, 1991), temperature of the electrophoresis buffer (Mathew *et al.*, 1988a) and field geometrics (Clark *et al.*, 1989). All the conditions

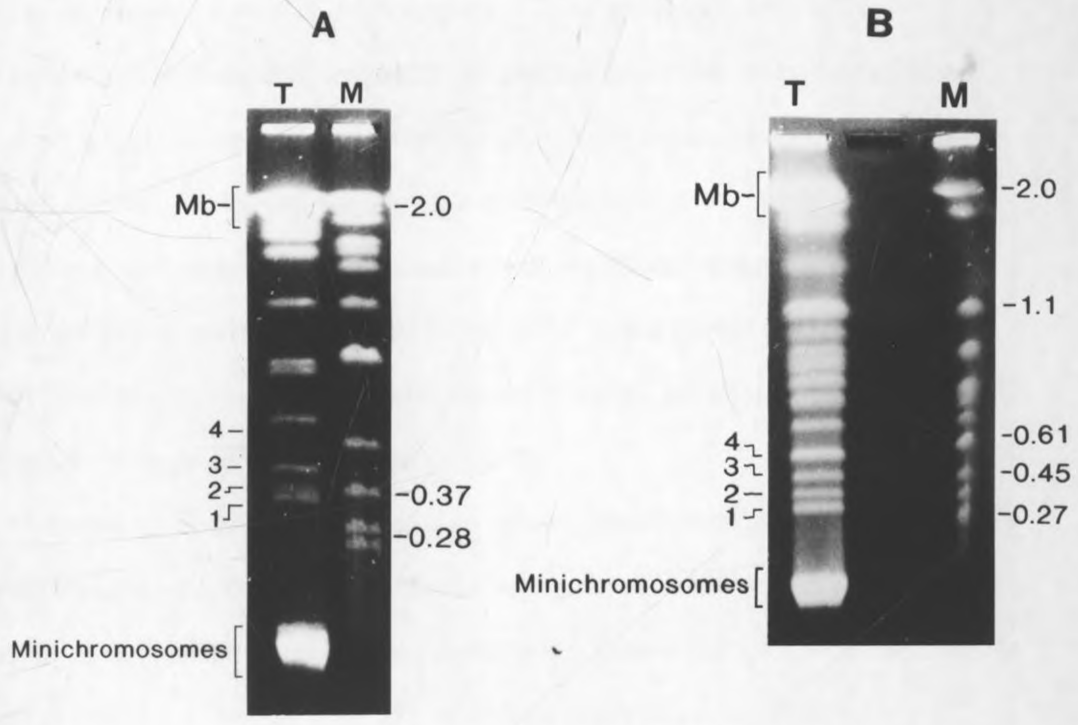
stated above were tested and it was apparent that pulse interval was the main factor which significantly affected the separation of large DNA molecules.

Chromosome-sized DNA molecules of *T. congolense* clone IL1180 were resolved using the Contour-clamped Homogenous Electric Field (CHEF) apparatus. The ethidium bromide-stained agarose gel shown in both panels of Figure 3 reveal that the pulse intervals used in both cases affect mainly the medium-sized chromosomes. The pulse interval used for the gel shown in panel A effectively separated 7 medium-sized chromosomes, whereas the pulse interval used for the gel shown in panel B effectively separated 13 chromosomes. However, regardless of the pulse interval used in both cases, the trypanosome chromosomes were still basically resolved into four size classes: minichromosomes (50-150 kb), medium-sized chromosomes (200-750 kb), chromosomes located in the compression zone (>1 Mb) and the chromosomes that are non-specifically trapped in the wells.

Figure 3

Effect of pulse interval on the separation of *T. congolense* chromosome-sized DNA molecules.

Chromosome-sized DNA molecules from *T. congolense* clone IL1180, lane T, were separated by pulsed-field gradient gel electrophoresis in a 1.5% agarose gel in a CHEF-DR II apparatus. The four medium-sized chromosomes are numbered 1 to 4 in both panels. Panels A and B are photographs of ethidium bromide-stained agarose gels of chromosome-sized DNA resolved at the different pulse intervals: (A). Initial pulse time, 60 seconds; final pulse time, 120 seconds. (B). Initial pulse time 120 seconds; final pulse time, 240 seconds. Other parameters were: electrophoresis time, 60 hours and 48 hours for panels A and B respectively; temperature, 15°C; voltage, 150 V. The sizes of the markers shown in lane M are indicated in megabase pairs.


A
T
M
Mb
2.0
4
3
2
1
0.37
0.28
Minichromosomes
B
T
M
Mb
2.0
1.1
4
3
2
1
0.61
0.45
0.27
Minichromosomes

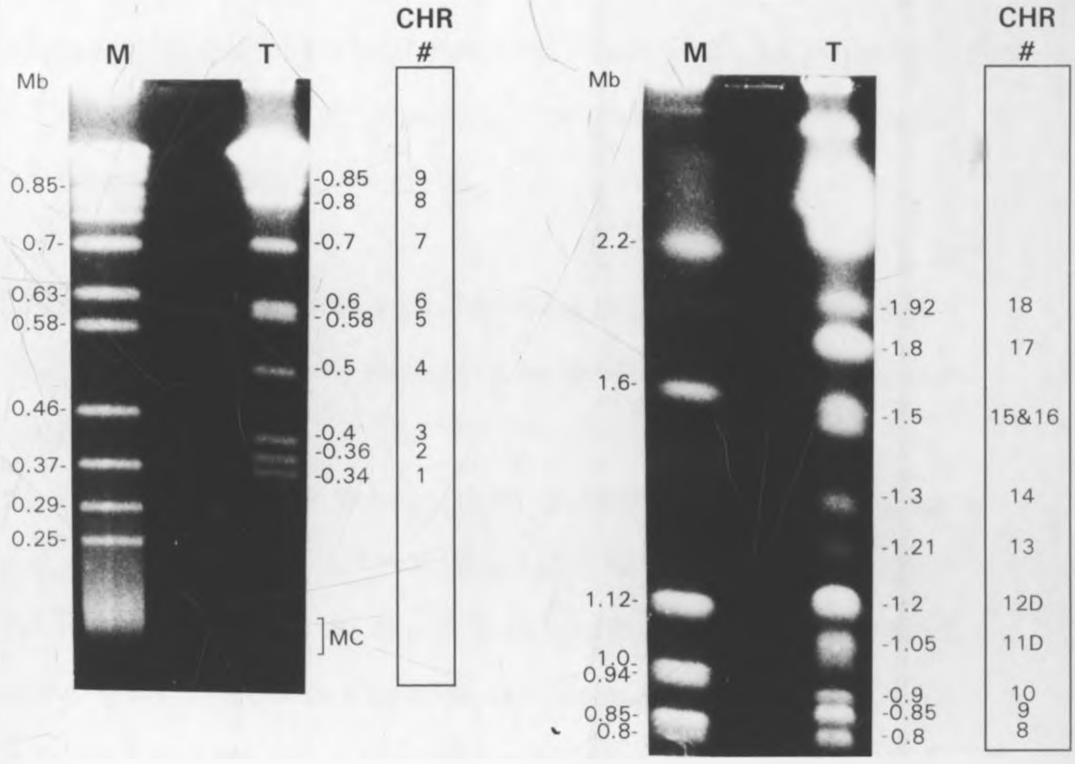
3.2.2 Separation of upto 18 chromosomes of *T. congolense* clone IL1180.

In molecular genetic studies of any organism, it is desirable to have a marker for each of the chromosomes of the organism . This can be best done if the chromosomes can be distinctly separated. An attempt was made to find conditions under which a high number of chromosomes of *T. congolense* clone IL1180 could be separated by PFGE. In Figure 4 is shown a photograph of an ethidium bromide-stained gel in which upto 18 chromosomes of this organism were separated. All the important conditions stated above were tested and it was apparent that pulse interval and duration of electrophoresis were the two main factors which significantly affected the separation of large DNA molecules (> 1 Mb).

Duration of electrophoresis and long pulse intervals were observed to separate upto approximately 2 Mb chromosomes of *T. congolense*. However, it could not be ascertained as to whether some of the chromosomes greater than one megabase which appeared as broad bands were doublets or discrete chromosomes. These are numbered 11D, 12D and chromosomes 15 & 16 in Figure 4 right panel. The minichromosomes and some of the medium-sized chromosomes have moved out of the gel in the right panel of Figure 4. This is attributable to the long electrophoresis time. However, in the left panel, the low intensity of the minichromosomes is attributable to the fact that it was an old preparation of chromosomes and the minichromosomes might have diffuesd out of the gel.

Figure 4

Separation of upto 18 chromosomes of *T. congolense* clone IL1180. The ethidium- bromide stained gel shown on the left panel was electrophoresed in a 1.5% agarose gel for 48 hours at an initial pulse time of 60 seconds and final pulse time of 120 seconds; voltage, 100 V. Shown on the right panel is a photograph of a 1% agarose gel electrophoresed for a total of 72 hours. The parameters used for the first 48 hours of electrophoresis were as follows: voltage, 150 V; initial pulse time, 120 seconds; final pulse time, 240 seconds. At the end of 48 hours, the buffer was replaced and electrophoresis re-started with the following parameters: initial pulse time, 240 seconds; final pulse time, 300 seconds; duration, 24 hours. All other parameters were unchanged. Lane T contain IL1180 chromosome-sized DNA molecules; lane M contain *Saccharomyces cerevisiae* DNA size markers catalogue number 170-3605 (Bio-Rad, Richmond, CA, USA) with sizes indicated in megabase pairs; CHR#. refers to chromosome number; Mb, megabase; MC, minichromosomes.


M
T
CHR
#
Mb
0.85-
0.7-
0.63-
0.58-
0.46-
0.37-
0.29-
0.25-
-0.85
-0.8
-0.7
-0.6
-0.58
-0.5
-0.4
-0.36
-0.34
MC
9
8
7
6
5
4
3
2
1
M
T
CHR
#
Mb
2.2-
1.6-
1.12-
1.0-
0.94-
0.85-
0.8-
-1.92
-1.8
-1.5
-1.3
-1.21
-1.2
-1.05
-0.9
-0.85
-0.8
18
17
15&16
14
13
12D
11D
10
9
8

3.3 Integrity of DNA recovered from separated chromosomes

In order to perform RAPD analyses on the individual medium-sized chromosomes, it was necessary to purify DNA from each one. This was done as described in section 2.6. To verify that DNA was successfully purified from the chromosomes, an aliquot of the extract from each chromosome was electrophoresed in an agarose gel. The data shown in Figure 5 demonstrates that intact DNA was recovered from each of the four chromosomes. However, due to their very high molecular weights, conventional gel electrophoresis could not separate the chromosomes according to their sizes (Lerman and Frisch, 1982). By including in the same gel DNA molecular size weight markers, it was possible to estimate that the purified DNA was greater than 23 kb in size.

3.4 PCR amplification of DNA recovered from individual chromosomes

3.4.1 The purified chromosome-sized DNA molecules are suitable substrates for *Taq* polymerase

The *Taq* polymerase, purified from thermophilic bacteria *Thermus aquaticus* (Chien *et al.*, 1976) can be inhibited by several factors including the target DNA. Impurities in an extract of DNA can impede the amplification reaction. Therefore, before subjecting the DNA purified from the chromosomes to RAPD assay, it was necessary to verify their suitability as substrates for amplification by *Taq* DNA polymerase. This was done by including oligonucleotide primers known to be specific for the Savannah-type *T. congolense* DNA in the PCR (Majiwa and Otieno, 1990). These oligonucleotide primers: IL344, CGA GCG AGA ACG GGC AC and IL345, GGG ACA AAC AAA TCC CGC, amplify a segment of highly repetitive DNA found so far only in the genome of Savannah-type *T. congolense*, a single unit of which is approximately 400 bp (Majiwa and Otieno, 1990). The products obtained after 40 cycles of amplification of DNA recovered from chromosomes were resolved in a 1.5% agarose gel. A photograph of ethidium bromide-stained gel is shown in

panel A of Figure 6. A 380 bp product was obtained as expected from the DNA of Savannah-type *T. congolense*.

Additionally PCR amplification was performed using a pair of primers which is expected to result in a product which is approximately 2.0 kb. The oligonucleotides used here amplify approximately 2.0 kb middle repetitive DNA of the Savannah-type *T. congolense*. The products obtained are shown in panel B of Figure 6. As expected, a 1.8 kb fragment was obtained from the DNA purified from each of the chromosomes. However, there is a band of approximately 1.1 kb on lane 3 of panel B. This is attributable to non specific priming. From these observations, it appears that the DNA recovered from these chromosomes can be used in other PCR amplification related experiments.

Figure 5

Integrity of the DNA recovered from separated chromosomes. Each of the four medium-sized chromosomes of IL1180 was individually purified from agarose gels after PFG gel electrophoresis. Lanes 1 to 4 contain chromosomes 1 to 4 respectively. A 4 µl aliquot of DNA recovered from each chromosome was electrophoresed in a lane of a 1.0% agarose gel, stained with ethidium bromide, destained in distiled water and photographed under UV illumination.

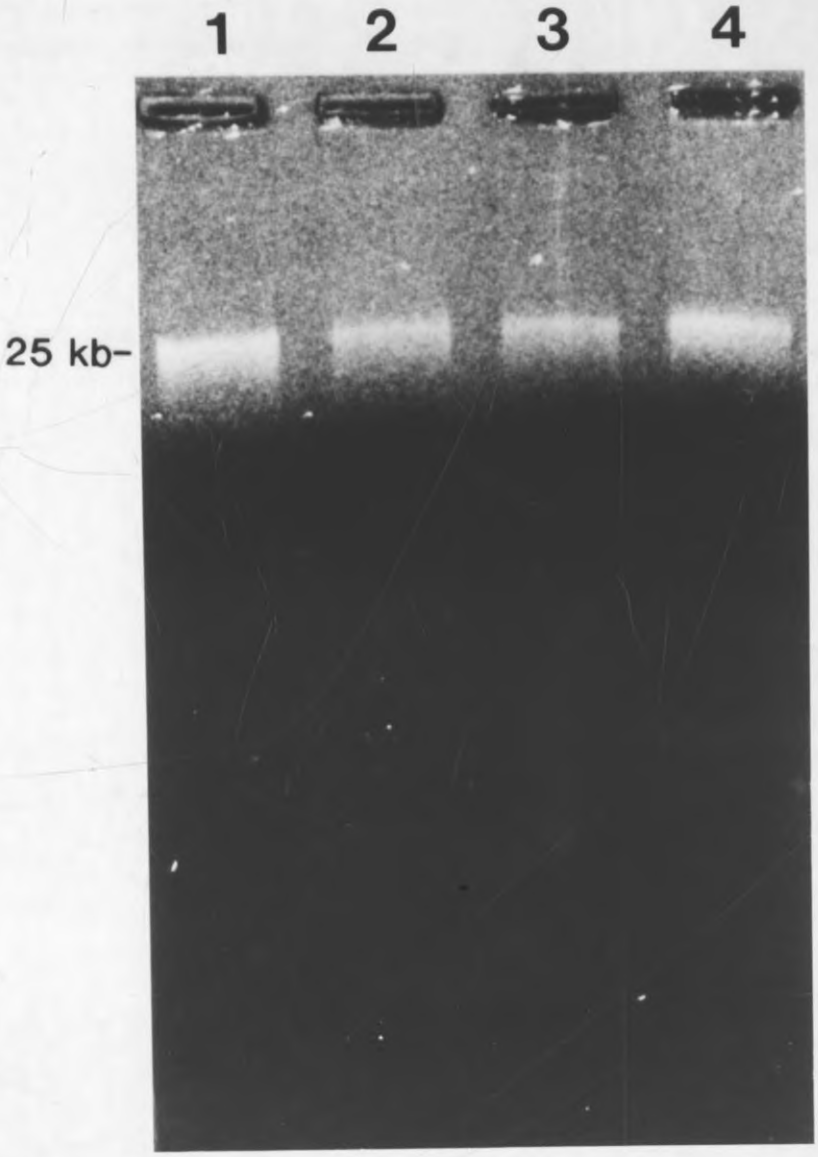

1
2
3
4
25 kb–

Figure 6

The purified chromosome-sized DNA molecules are suitable substrates for *Taq* polymerase.

Approximately fifty pg of DNA from different chromosomes were individually subjected to PCR amplification using 200 ng of each of a pair of oligonucleotide primers, ILO344: CGA GCG AGA ACG GGC AC and ILO345: GGG ACA AAC AAA TCC CGC, specific for the Savannah-type *T. congolense* genomic DNA. Lanes 1 to 4 contain products of amplification of chromosomes 1 to 4 respectively; lane 5 contains amplification products of IL1180 genomic DNA; lane M contains DNA size markers, phi X174 DNA digested with Hae III. (B). A pair of oligonucleotides that amplifies approximately 2 kb fragment of the Savannah-type *T. congolense* DNA was used for amplification of each of the chromosomes as in (A) above.

The PCR products were separated by electrophoresis in a 1.5% agarose gel, stained with ethidium bromide and photographed under UV illumination. The sizes of the fragments are indicated in kilobase pairs.

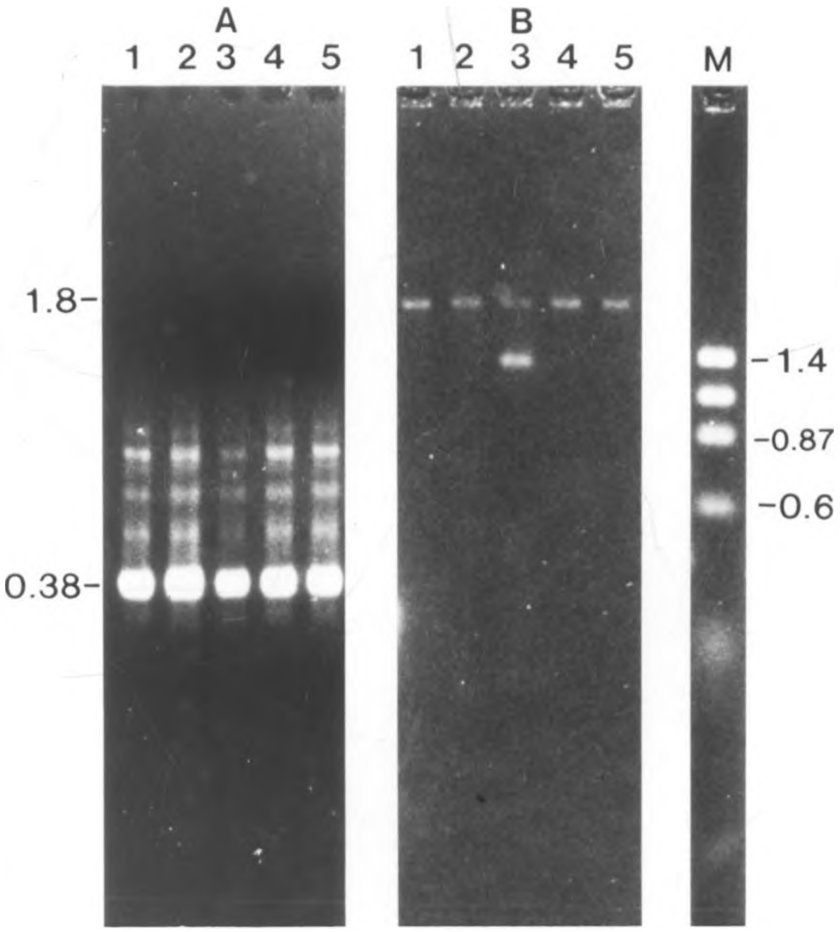
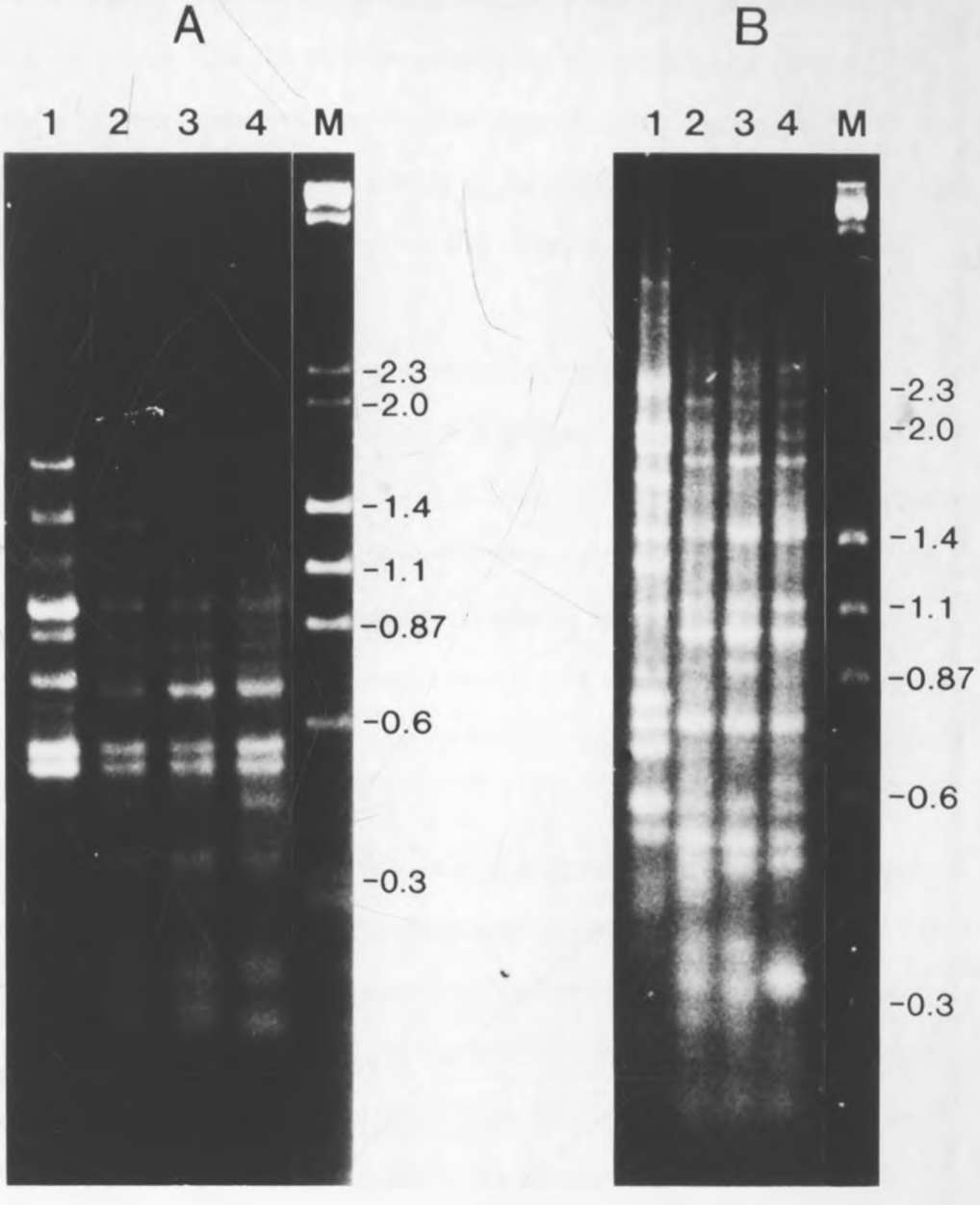

A
1 2 3 4 5
B
1 2 3 4 5
M
1.8-
0.38-
-1.4
-0.87
-0.6

Figure 7

Effect of $MgCl_2$ concentration on the RAPD patterns produced using an oligonucleotide primer. Purified chromosome-sized DNA molecules from each chromosome were amplified using an oligonucleotide primer (ILO1106) at two different $MgCl_2$ concentrations. The amplification products were separated by electrophoresis in a 1.5% agarose gel, stained with ethidium bromide and photographed under UV illumination.

Lanes 1 to 4 in both panels contain amplification products of chromosomes 1 to 4, respectively. Panel A contains amplification products obtained using $MgCl_2$ at a concentration of 1.5 mM. Panel B contains amplification products obtained using $MgCl_2$ at a concentration of 3.0 mM.

Lane M contains DNA size markers, *Phi* X 174 DNA digested with Hae III and lambda DNA digested with Hind III.


A
1 2 3 4 M
-2.3
-2.0
-1.4
-1.1
-0.87
-0.6
-0.3
B
1 2 3 4 M
-2.3
-2.0
-1.4
-1.1
-0.87
-0.6
-0.3

3.5 Putative chromosome-specific RAPD bands

The use of random primers in PCR under conditions of low stringency makes it possible to amplify a number of loci randomly distributed throughout the genome (Rafalski *et al.*, 1991). This results in the production of a complex mixture of DNA fragments that appear as patterns when separated according to size by gel electrophoresis. Characteristically, a portion of the amplified loci is polymorphic and can be used as markers for phylogenetic studies or population biology (Neto *et al*, 1993).

I was interested in investigating whether this method can be used to distinguish different chromosomes within an organism. A series of experiments was performed using 23 arbitrary primers, listed in Table 3, to find those that could reveal polymorphisms among the four medium-sized chromosomes of *T. congolense* clone IL1180. The number of amplified fragments produced by a primer varied from 0 to 60. The amplified sequences ranged in size from 0.1 kb to 3 kb. Some of the primers used in these assays revealed a significant number of polymorphic bands, allowing easy distinction of each chromosome.

In Figure 8 is shown a photograph of a representative agarose gel displaying the patterns obtained with three different oligonucleotide primers. It is apparent that each primer produces a different pattern of bands when used on each chromosome. Many fragments appear to be common to two or more of the chromosomes; however, some bands appear to be more intense than others. This implies either a better target sequence accessibility for the primer and *Taq* polymerase or that the amplified sequence corresponds to repeated DNA. Some of the primers tested gave reproducible fingerprints; this was evident from the observation that chromosome-sized DNA molecules purified from agarose gels at different times and the same DNA amplified at different times produced identical patterns with differences, if any, being confined to intensities of a few bands.

3.6 Amplification efficiences of different oligonucleotide primers

In an attempt to find chromosome- specific hybridization markers, 23 different 10-mers were tried to find which ones had best amplification efficiency on the chromosome-sized DNA molecules purified from the four medium-sized chromosomes. The primers were from the ILRAD oligonucleotide unit. The sequences of these primers were selected randomly with the requirement that their (G + C) content be 60%- 80%, and that they have no self- complementary ends.

The results shown in Table 3 give the sequence code, the 10-mer sequence 5′-3′ and the amplification efficiency of each primer tested. For each of the primers used, a control tube containing all the components except the target DNA was included to detect potential contamination. However, none of the control tubes showed any amplification (Figure not shown). The results shown in Table 3 confirmed that different primers have different amplification efficiencies to different purified chromosome-sized DNA molecules.

3.6.1 Effect of Magnesium concentration on RAPD products

Magnesium concentration in the reaction mixture of PCR is very important as it affects primer annealing, strand dissociation temperatures of both template and PCR product specificity, formation of primer-dimer artifacts and enzyme activity (Innis *et al.*1990). Taq DNA polymerase requires free magnesium on top of that bound by the template DNA, primers and dNTPs. Therefore PCR mixtures should contain 0.5. to 3 mM Mg^{++} over the total dNTP concentration.

As shown in Figure 7, a higher Mg^{2+} concentration produced more RAPD bands, because it enhances the stability of primer/template interaction. Therefore in the subsequent PCRs, a high concentration of Mg^{++} was used.

Figure 8

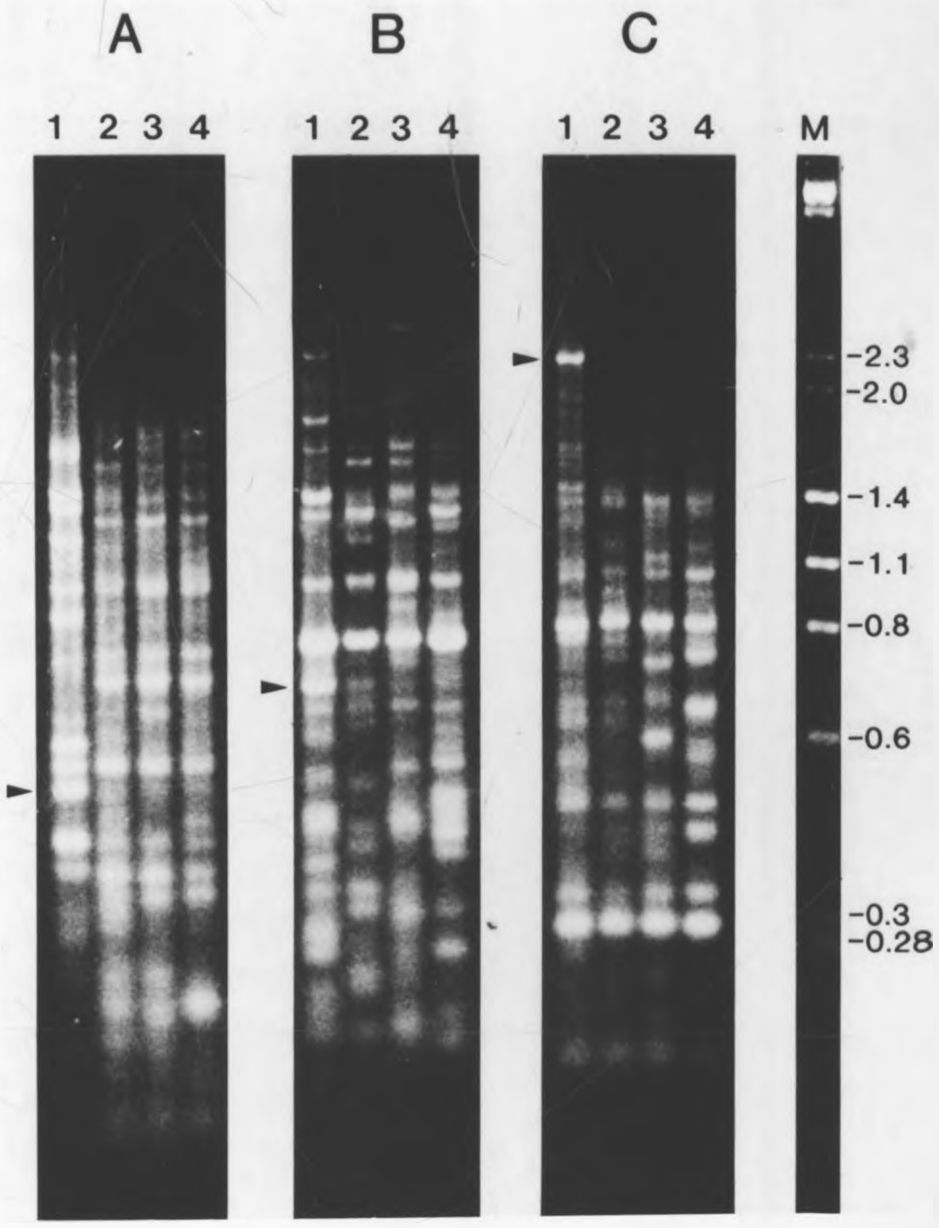
Putative chromosome-specific RAPD bands.

DNA from purified individual chromosomes of *T. congolense* IL1180 were amplified using single oligonucleotide (10-mer) primers. After amplification the products were resolved by electrophoresis in a 1.5% agarose gel, stained with ethidium bromide and photographed under UV illumination.

Panels A, B and C contain amplification products of DNA purified from the four chromosomes, using primers ILO1106, ILO901 and ILO904 respectively.

Lane M contains DNA size markers, which is a mixture of bacteriophage lambda DNA digested with Hind III and phi X 174 digested with Hae III.

Arrow heads and arrows point at some of the bands which appear to be specific to a chromosome when RAPD patterns of the four medium-sized chromosomes are compared.


A
B
C
1 2 3 4
1 2 3 4
1 2 3 4
M
-2.3
-2.0
-1.4
-1.1
-0.8
-0.6
-0.3
-0.28

Sequence (5' to 3') of Oligonucleotide primers screened for production of Chromosome-specific RAPD markers

ILO Number	Sequence (5' to 3')	Amplification Efficiency*
509	TGG TCA GTG A	+
525	CGG ACG TCG C	+
867	CGT TCC CCG C	+++
872	CCC GCC ATC T	+++
876	GGG ACG TCT C	+++
901	GGG GAC TAA G	++
904	CAA CCA CAA C	++
1078	TCC CCT TCG C	+
1106	CAG CCG CTC G	
508	CGG CCC CTG T	-
1107	GGG CCC CAG C	+
1109	GTC GGG GCG C	+
1077	TCC CAG GGG C	++
909A	CAG CCT CGT C	+++
909B	CCG TCC CAA C	+++
1052	CCG TCC ACC C	-
1063	CGG TCC CGC A	+
1068	CCG GTC GGG G	+
1079	GGG CGC GAG A	++
1066	GGC CTC GCA C	+
1067	GTG CCG CGC T	++
875	GTC CGT GAG C	-
841	GCG GCT GCC A	+

*, No amplification product with the DNA used
+, Poor amplification (a smear or diffused bands)
++. Good amplification (few distinct or intense bands)
+++, Excellent amplification (many distinct or intense bands)

3.7 Recombinant plasmids containing PCR products

After identification of chromosome specific PCR product, the next step was to clone these fragments in appropriate vectors. The cloning of these fragments was done so that the entire sequence of the fragment can be used as a hybridization probe in the subsequent experiments. A T-vector was used because it does not require any prior modification of the PCR product.

Plasmid DNA was prepared from bacterial colonies which showed positive signals of hybridization when screened by differential colony hybridization, (see section 2.13.3). The plasmids were digested with both *Eco* RI and *Hind* III to release the insert, if any. These enzymes have restriction sites flanking the *Eco* RV site at which the PCR product was cloned. As shown in Fig 10, most of the plasmids digested in this way released an insert; however, only a few of the inserts corresponded in size to the original PCR product ligated to the vector (Figure 10). This might have been caused by excision and subsequent purification of more than one fragment from PCR products resolved in agarose gels. The specificity of these inserts was verified by hybridization using radiolabelled PCR fragments as probes and in this way positive hybridization signals were observed (data not shown). There were some recombinant plasmids which on digestion released two fragments. This may have been caused by the presence of a restriction enzyme site for either of the enzymes used in the digestion.

Figure 9

Identification of bacterial colonies harbouring recombinant plasmid containing PCR products.

Bacterial colonies transformed with PCR fragments ligated to a T-vector (and grown in 2 x YT plates with ampicilin) were lifted from the plates using nitrocellulose filters. The filters were hybridized with radiolabelled purified PCR band and washed with 0.1 x SSC, 0.1% SDS, at 65°C, for 2 x 30 minutes then exposed to X-ray film with an intensifying screen at -70°C for 12 hours. An autoradiograph obtained is shown. The arrows mark the position of some of the positive, recombinant colonies, the arrowheads mark non recombinant colonies and the open triangles point at orientation marks made on the filter paper using indian ink.

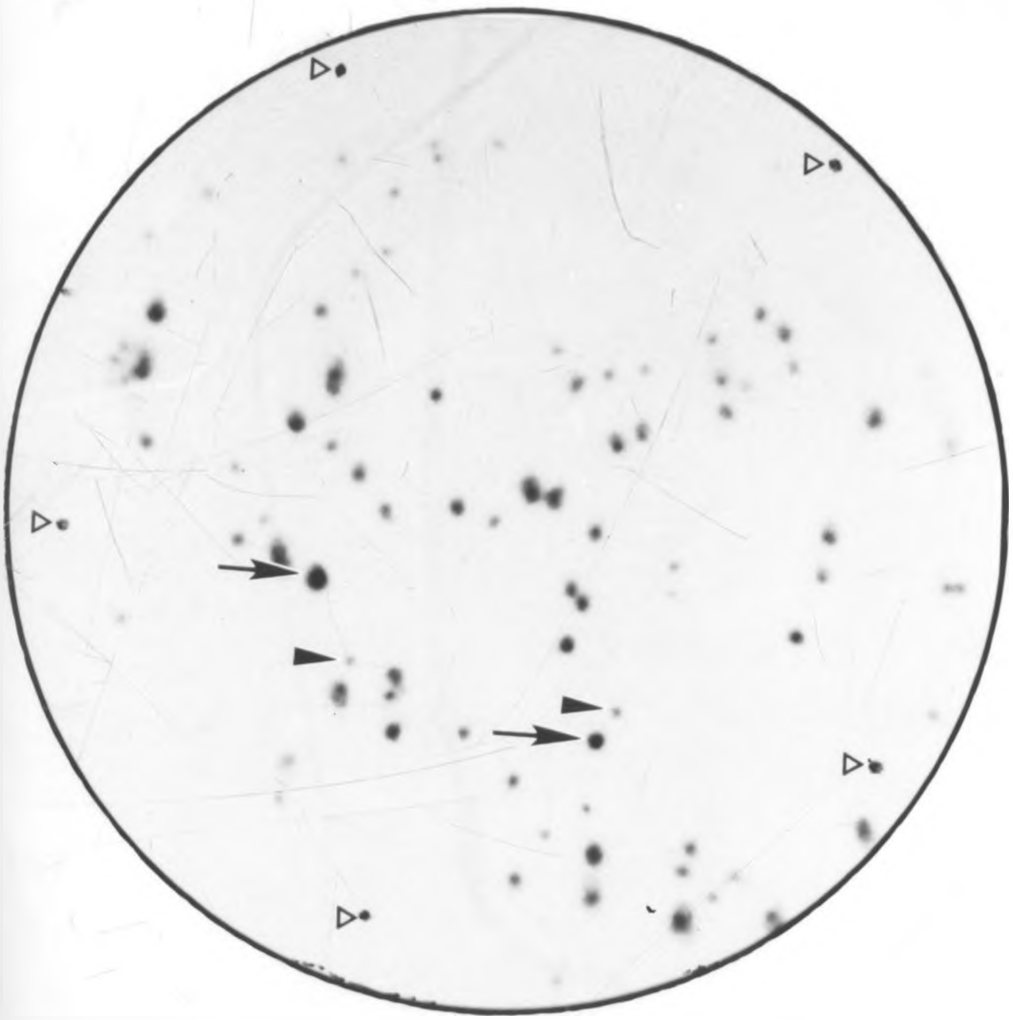
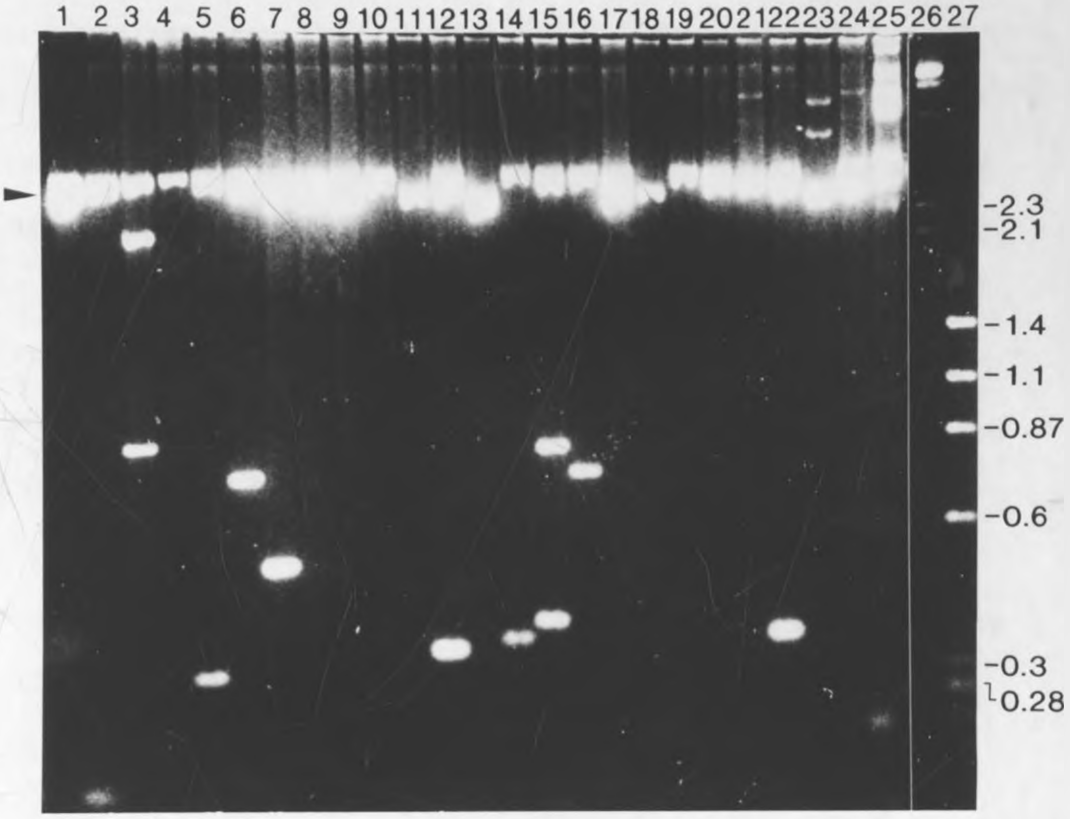


Figure 10

Recombinant plasmid clones containing PCR fragments. The DNA prepared from each recombinant plasmid was digested with Hind III and *Eco*RI to release the insert. The sizes of some of the inserts are shown. The arrowheads point at the vector. Size markers are indicated in lanes 26 and 27.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
2.3
2.1
1.4
1.1
0.87
0.6
0.3
0.28

3.8 Sequence specificity of RAPD bands

Random amplified polymorphic DNA has been used to find specific markers in different organisms. In *Schistosoma,* a polymorphic fragment of DNA was identified only in the genomic DNA extracted from a female parasite. This polymorphism was revealed by PCR amplification of the genomic DNA extracts using a single arbitrary (10-mer) primer (Barral *et al* , 1993). To determine whether this sequence was present also in DNA of the male schistosome, the RAPD band appearing as specific to the male worm was purified, radiolabelled and hybridized to a blot of DNA amplified using the same primer from DNA of both sexes. Although hybridization was observed in products amplified from DNA of both sexes, the signal was significantly lower in the patterns obtained from the DNA of the male worm (Barral *et al., 1993*). In this study the RAPD amplification of trypanosome chromosomes was performed using twenty three different single oligonucleotide primers out of which primers: ILO872, ILO867 and ILO509 revealed distinct polymorphisms for the purified chromosomal DNA molecules.

In Figure 11, a polymorphic fragment of about 1.5 kb (shown with an arrowhead) was observed only in the RAPD product from chromosome 2 primed with ILO867. To determine whether this fragment was indeed present only in the RAPD product of chromosome 2, it was purified, cloned, radiolabelled and hybridized to a blot of the gel containing RAPD products from all the four purified chromosomal DNA molecules using the same primer ILO867 (Figure 12 A). Panels B and C of this Figure show the autoradiograms obtained from the hybridization. It is apparent that sequences homologous to this RAPD fragment are present in the RAPD products of the other chromosomes. However, the level of homology is low with the products since the signal of hybridization disappear when post hybridization stringency is increased (Figure 12 C). From these observations, I infer that a RAPD band may

appear to be present exclusively in PCR products from only one chromosome when actually it has different levels of homologies with other RAPD products of other chromosomes.

Figure 11

Specificity of a RAPD band produced from chromosome 2. A single oligonucleotide primer, ILO867, was used to amplify DNA from each of the four chromosomes. The products obtained were separated according to size by electrophoresis in a 1.5% agarose gel. A photograph of an ethidium bromide-stained gel is shown in panel A. A putative chromosome-specific band in lane 2 is shown with an arrowhead at approximately 1.5 kb (and referred to as RAPD2/867/1.5 hereinafter). In all panels, lanes 1, 2, 3 and 4 contain PCR products from chromosomes 1, 2, 3 and 4 respectively. (B) DNA in the gel, a photograph of which is shown in panel A, was transferred to a nitrocellulose filter paper and hybridized with the cloned fragment RAPD2/867/1.5, the filter was washed 2 x 30 minutes with 1 x SSC, 0.1% SDS at 65°C; an autoradiograph of this filter is shown. The autoradiograph in panel C shows the same blot washed 2 x 30 minutes with 0.1 x SSC, 0.1% SDS at 65°C. Both the autoradiographs were obtained by exposing the film for a duration of 12 hours.

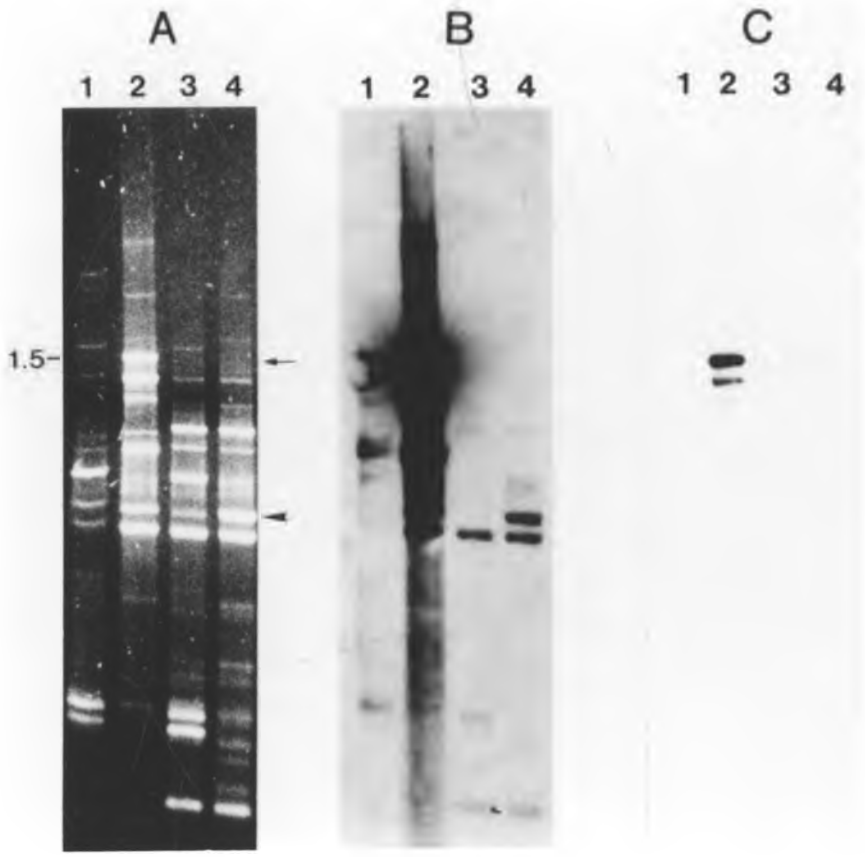

A
1 2 3 4
1.5-
B
1 2 3 4
C
1 2 3 4

Figure 12

Specificity of a RAPD band produced from chromosome 3.

PCR products obtained from amplification of chromosomes with oligonucleotide primer ILO509 were separated according to size by electrophoresis in a 1.5% agarose gel . A photograph of an ethidium bromide-stained gel is shown in panel A. Putative chromosome-specific RAPD band in lane 3 is shown at about 700 bp (and designated RAPD3/509/700 herein after). In all panels, lanes 1, 2, 3 and 4 contain PCR products amplified from chromosomes 1, 2, 3 and 4 respectively. Lane M contains DNA molecular weight markers which is a mixture of lambda DNA digested with Hind III and phiX174 DNA digested with Hae III. (B) DNA in the gel in panel A was transferred to a nitrocellulose paper and hybridized with radiolabelled RAPD 3/509/700. The filter was washed 2 x 30 minutes with 1 x SSC, 0.1% SDS at 65°C to obtain the autoradiograph shown. The autoradiograph in panel C shows the same blot washed with 0.1 x SSC, 0.1% SDS for 2 x 30 minutes at 65°C. Both the autoradiographs were exposed to the film for a period of 12 hours.

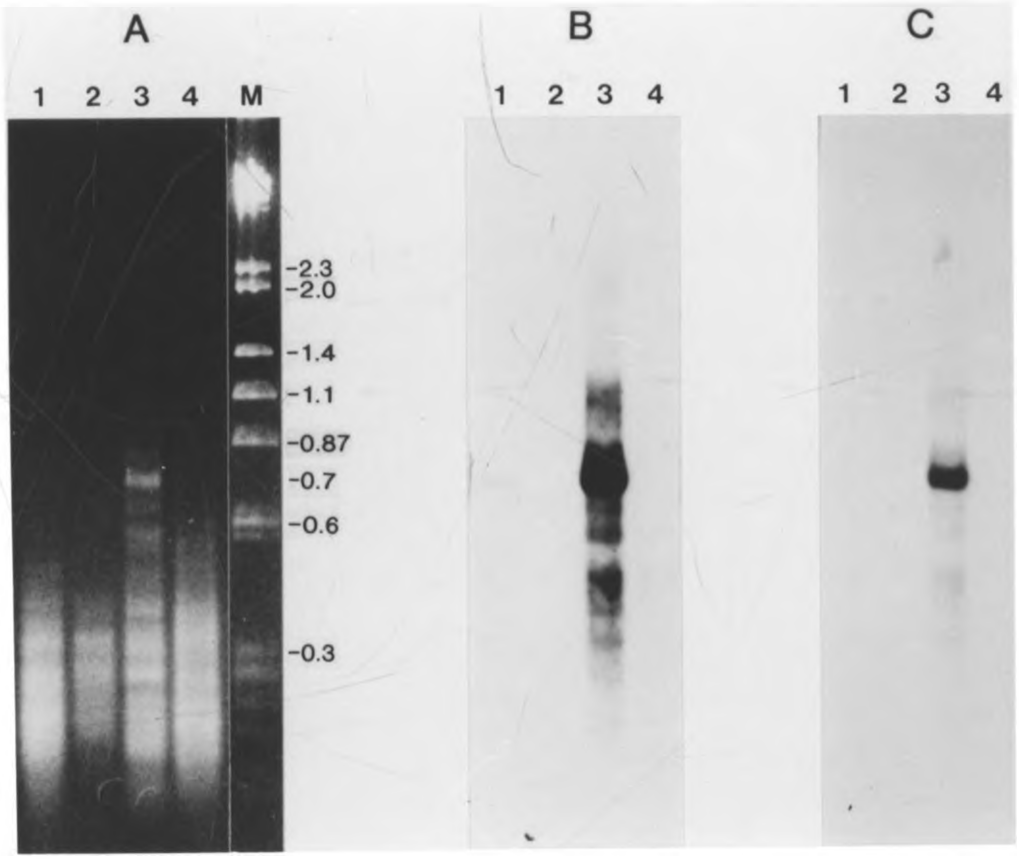
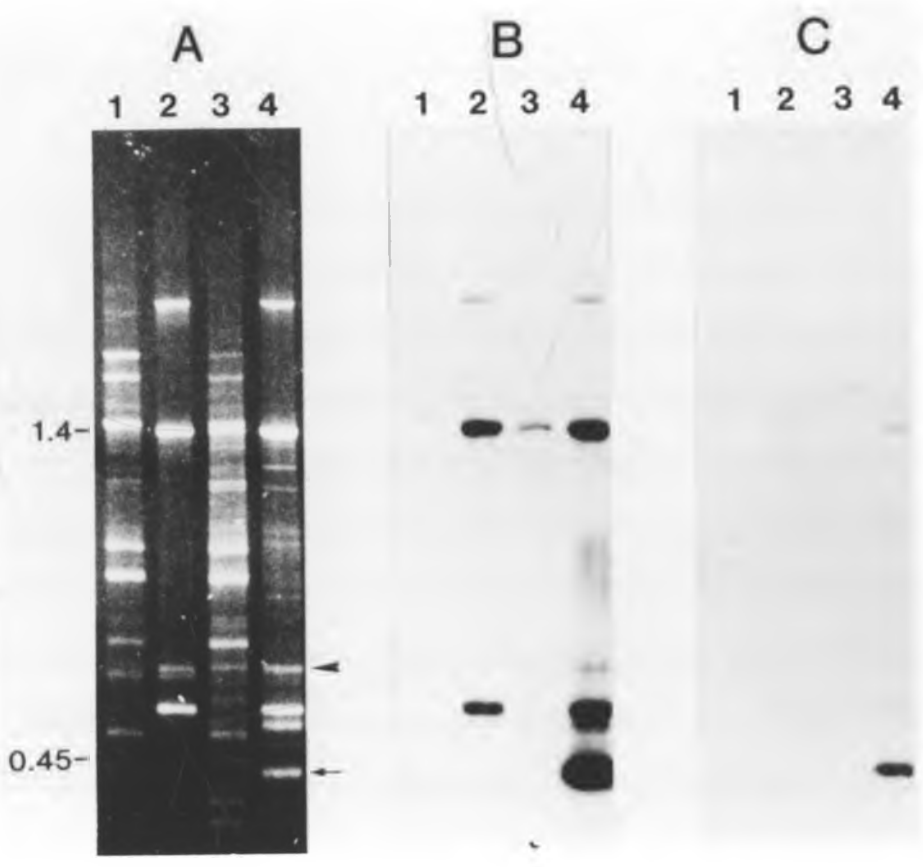

A
1 2 3 4 M
-2.3
-2.0
-1.4
-1.1
-0.87
-0.7
-0.6
-0.3
B
1 2 3 4
C
1 2 3 4

Figure 13

Specificity of a cloned RAPD band produced from chromosome 4.

Products obtained from amplification of each of the chromosomes with a single oligonucleotide primer ILO872 were separated according to size by electrophoresis in a 1.5% agarose gel . In panel A is shown a photograph of an ethidium bromide-stained gel. Putative chromosome-specific RAPD band in lane 4 is indicated with an arrow at about 450 bp (and referred to as RAPD4/872/450 hereinafter). Lanes 1, 2, 3 and 4 contain products of amplification from chromosomes 1, 2, 3 and 4 respectively. The arrowhead points at some of the bands that co-migrate. (B) DNA in the gel (panel A) was transferred to a nitrocellulose filter paper and hybridized with a of RAPD4/872/450. The filter was washed 2 x 30 minutes with 1 x SSC, 0.1% SDS at 65°C to obtain the autoradiograph shown. Panel C shows the same blot washed with 0.1 x SSC, 0.1% SDS for 2 x 30 minutes at 65°C. Both the blots were exposed to the film for 12 hours.


A
1 2 3 4
1.4–
0.45–
B
1 2 3 4
C
1 2 3 4

3.9 A cloned RAPD band hybridizes to one of the medium-sized chromosomes

Having cloned some of the RAPD bands which appeared to be specific to each of the four medium-sized chromosomes, the next step was to determine whether any of them could hybridize specifically with the chromosomes in which they were identified. Therefore, chromosome-sized DNA molecules from 3 trypanosome clones IL1180, IL3000 and IL373, each one from a different antigenic repertoire were separated by PFG gel electrophoresis (Figure 14 A). The DNA molecules in the agarose gel shown were transferred to a nitrocellulose filter and hybridized with a cloned RAPD band designated, RAPD4/872/450. The autoradiograph obtained (Figure 14 B) shows that RAPD4/872/450 hybridizes only to chromosome 4 out of the four medium- sized chromosomes of IL1180. Thus the sequence contained in the RAPD band is specific to chromosome 4. However, there is hybridization to the minichromosomes and chromosomes located in the compression zone. This is possibly because of the presence sequences homologous to those in RAPD4/872/450 at the minichromosomes and chromosomes located at the compression zone either in single or low copy numbers. In clones IL3000 and IL373, this fragment hybridizes mainly at the compression zone and weakly to the minichromosomes. Other RAPD bands which appeared to be specific to the other chromosomes were used in similar experiments but the hybridization observed was not specific to any of the four chromosomes. The hybridization occurred with chromosomes at the compression zone or to all the chromosomes.

3.10 Some RAPD bands which appear to be specific to one chromosome hybridize to several other chromosomes

The chromosomes of *T. congolense* clone IL1180 were separated by CHEF (Figure 15 A). DNA from this agarose gel was transferred to a nitrocellulose filter paper and hybridized with cloned radiolabelled PCR product RAPD3/509/700. The autoradiogram obtained (Figure 15 B) shows that the sequence contained in the RAPD band is homologous to other loci located in many other chromosomes including the four medium-sized chromosomes. It is possible that no RAPD band of similar size or sequence was amplified from the other chromosomes because of inefficient primer binding site due to an insertion or a deletion of one or more bases. This is in agreement with the observation (Rafalski *et al.*, 1991) that patterns produced by RAPDs is dependent on the sequence of the target DNA and an insertion or deletion of one or more bases can alter significantly the patterns of the PCR products.

Figure 14

Hybridization of a putative chromosome-specific band to one of the four medium-sized chromosomes.

A photograph of an ethidium bromide-stained agarose gel of chromosome-sized DNA molecules fractionated by CHEF is shown in panel A. The lanes contain samples as follows: 1,IL1180; 2,IL3000; 3,IL373. The chromosome-sized DNA molecules in this gel were transferred to nitrocelullose filter paper and hybridized with RAPD 4/872/450. The filters were washed at low stringency (2 x SSC, 0.1% SDS, 65°C, 2 x 30 min) and exposed to an X-ray film for 48 hours with an intensifying screen at -70°C to obtain the autoradiograph shown in panel B.

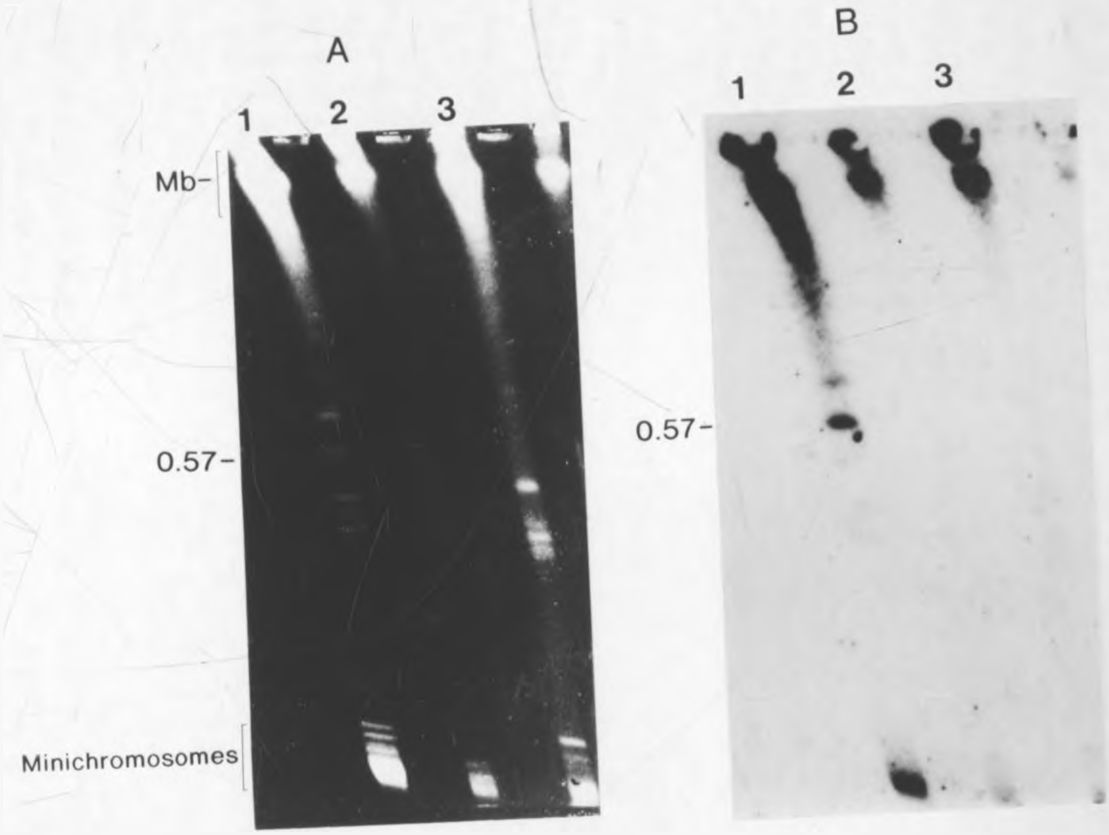
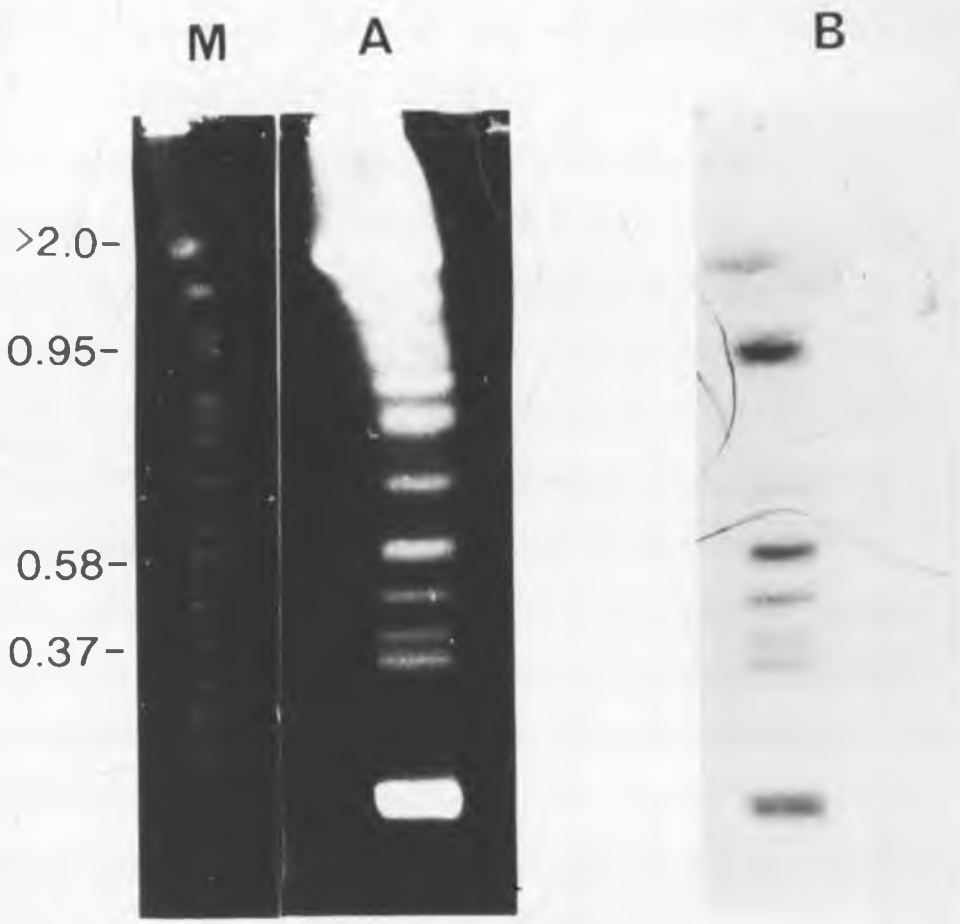

A
1
2
3
Mb–
0.57–
Minichromosomes
B
1
2
3
0.57–

Figure 15

Some RAPD bands which appear to be specific to one chromosome hybridize to other chromosomes. IL1180 chromosomes were fractionated by CHEF at an initial pulse time, 90 seconds and final pulse time 180 seconds for 48 hours at a voltage of 150 V. Panel A shows an ethidium bromide-stained agarose gel with sizes of the markers in lane M indicated in megabase pairs. DNA from the gel was transferred to nitrocellulose filter paper and hybridized with radiolabelled PCR fragment, RAPD 3/509/700. (B). The blot was washed at high stringency then exposed to an X-ray film with an intensifying screen at -70°C for 12 hours to obtain the autoradiograph shown.


M
A
B
>2.0-
0.95-
0.58-
0.37-

CHAPTER 4

DISCUSSION

Studies on the molecular mechanisms of processes and phenomena in trypanosomes are to a large extent hampered by the lack of suitable markers of individual chromosomes of these organisms. It has been observed that the molecular karyotype of these organisms, like that of many other lower eukaryotic micro-organisms, is very dynamic. As has been indicated earlier, lack of cytogenetic procedures complicates the study of chromosome inheritance and polymorphisms in trypanosomes. Long range restriction fragment length polymorphisms (RFLP) can provide an initial guide to the differences between homologous chromosomes but systematic analyses of chromosomal breakpoints and location can be best approached by developing sets of suitably linked markers of individual chromosomes. The definite proof of linkage of these kinds of markers has to come from either the 'reconstruction' of the chromosome from overlapping DNA fragments or Southern blot hybridization of PFGE-separated chromosomes using the markers as probes. Here, the construction of a physical map using cloned DNA is more suitable than, for example, restriction enzyme site mapping.

Different approaches can be employed in the search for chromosome-specific markers and in the physical mapping of trypanosome genome. A particular example is the use of chromosome-specific libraries. This involves partial restriction enzyme digestion of DNA in a chromosome band excised from an agarose gel and cloning these fragments into vectors which can contain large DNA fragments. These vectors include cosmids, bacteriophage P1 and yeast artificial chromosomes (YACs). The alternate strategy to this is selecting PFGE band-specific subsets of cosmid clones of trypanosome DNA. These cosmid libraries of trypanosome DNA are then screened by differential colony hybridization using an individual chromosome-sized DNA molecule as probe. In anticipation of homologous sequences among trypanosome

DNA hybridizing to the cosmid colonies, the filters are prehybridized with sheared genomic trypanosome DNA. Here, it is expected that the highly repetitive genomic DNA in the prehybridization mixture will hybridize more rapidly to homologous DNA in the cosmid clones, thus reducing the possibility of these sequences hybridizing with the radioactive chromosomal probe. This procedure has the disadvantage of the possibility of low copy and even single copy genomic DNA of the trypanosome hybridizing to the homologous DNA in the cosmid clone, occurrence of which results in the absence of signal with the chromosomal probe.

In this study, I have, investigated alternative approaches to the identification of chromosome-specific hybridization markers of *T. congolense*, one of the trypanosomes which places the greatest economic burden on the continent of Africa (Hoare, 1970).

One approach I took was to define chromosomal location of some cDNA clones from genes encoding various VSGs. These genes are naturally believed to be located on chromosomes. However, some genes may be located on only one chromosome. These cDNAs cloned from the VSG genes were expected to hybridize to more than one chromosome. However, some of the cDNA clones were expected to hybridize exclusively to any one of the four medium-sized chromosomes for which markers are needed. In the event of this occurring, the cDNA clone responsible would be both a genetic and physical marker for which-ever chromosome it exclusively hybridized. Since none of the cDNA clones used hybridized exclusively to any of the *T. congolense* chromosomes, medium-sized or otherwise, I proceeded with the search for chromosome-specific markers by determining the chromosomal location of some RAPD bands cloned from the Savannah type *T. congolense*. None of these hybridized specifically to any of the medium-sized chromosomes. It was apparent that an alternate approach needed to be taken in the search for chromosome-specific hybridization markers. The alternate approach which I took was the use of PCR in the amplification of DNA using arbitrary (random) primers (Rafalski *et al.*, 1991).

Comparison of RAPDs and other kinds of markers, specifically RFLP, reveals that the RFLP analysis is usually performed with specific cloned probes, which can be either a cDNA or a genomic clone. The identity of the probe is immaterial for RFLP analyses. The DNA blotting and hybridization require several micrograms of DNA, which has been digested with a restriction endonuclease. Radioactive probes are usually used, with each probe detecting one or more genetic loci that share sequence homology. Each allele at a locus is identified as a mobility variant of an endonuclease restriction fragment. More than one allele can be detected at a locus so that RFLP assay can be used to identify co-dominant traits. Thus among progeny segregating a phenotype linked to a specific RFLP pattern, each homozygote can be distinguished from the heterozygote

In contrast, the RAPD assay does not require prior sequence information nor cloned DNA probes. A single primer of arbitrary nucleotide sequence can be used with any target DNA. The amount of target DNA used per assay is much lower than that used in RFLP assay. Amplified bands are not necessarily digested with a restriction endonuclease but can be observed directly after separation in ethidium bromide-stained agarose gel and recorded photographically or electronically. Each primer usually amplifies several different loci that are generally unrelated except by short primer recognition sites. Usually, RAPD assay detects the presence of only one allele at a locus, which is the allele that gave rise to the amplification. The absence of an amplified band represents all other alleles that fail to prime with the primer used.

Studies described in this thesis confirm that short primers of arbitrary nucleotide sequences can indeed be used to reproducibly amplify segments of any DNA purified from individual chromosome-sized DNA molecules of trypanosomes. Polymorphisms among the amplification products are detected frequently, and in some cases these are useful as markers. The patterns of amplified fragments depend on the primer sequence and the sequence of the target DNA. Usually any one random primer amplifies several bands each originating from a different chromosomal location. The exact nature of RAPD polymorphisms has not been investigated but is

expected to include single nucleotide changes and insertions or deletions. These polymorphisms can be detected by examination of ethidium bromide-stained agarose gels (Willies *et al.*, 1990). RAPD assays can detect single base changes in a target DNA. Even a single nucleotide change in a primer sequence can cause a change in the pattern of amplified DNA bands. By inference, a single base change in the target DNA may also prevent amplification by introducing a mismatch at just one end of the DNA segment.

Figures 10, 11, and 12 show the patterns produced by the different primers. However poor in amplification a primer is, polymorphisms can still be detected using the same to prime on DNA purified from different chromosomes. This is particularly evident in Figure 12 where the products of amplification appear as smears although a band designated RAPD 3/509/700, is clearly characteristic of PCR products for chromosome 3. It is also apparent that with some primers, more than one polymorphic band can be revealed for DNA from a single chromosome; similarly a primer can reveal polymorphisms in more than one chromosome.

The template used for amplification in Figure 12 appears to be degraded. This may explain why the amplification products appear as a smear on an ethidium bromide-stained gel. However, this is not necessarily the case since the same sample used with a different primer produced more fragments of higher molecular weight DNA and more distinct bands (see for example figure 11). That the templates used here were degraded can be disqualified further on the grounds that amplification products of chromosomes 1, 2 and 4 did not hybridize with RAPD 3/509/700, (see Figure 12).

The RAPD data have been used for the construction of phylogenetic trees of different strains of organisms. In these analyses, the assumption made is that bands of the same size (co-migrating bands) are of the same sequence and the reverse is true (Chapcro *et al.*,1992) However, this does not seem to be the case as is evident in the data presented in Figures 11 and 13. RAPD bands that are identical in size are apparent in the products from 2 or more chromosomes. Some of such products are

indicated with an arrowhead in each of the figures. These are probably indicative of either a common sequence or distinct sequences amplified as fragments of identical size. That some of these bands have common sequences is supported by the observation that upon hybridization of a blot of the gel whose photograph is shown in Figure 13 A with RAPD4/872/450, hybridization occurs to bands of approximately 1.4 kb present in the RAPD products of chromosomes 2, 3, 4. That some of the co-migrating bands have different sequences amplified as fragments identical in size is evident in Figure 13 where there is hybridization only with the sequence amplified from chromosome 4 (shown with an arrowhead in panel A) even though a band of an identical size was present in products of chromosomes 2, 3 and 4.

The technique of RAPD analyses is likely to be widely applied to the study of genetics of many organisms because of its technical simplicity, speed, reproducibility, high resolution and considerable reduction in the amount of DNA required for routine analysis. As with other powerful and sensitive procedures, a number of precautions need to be taken; these must include sterilization of reagents, reaction tubes and pipette tips, and the use of established standard conditions. Using this technique, makers which can be used to distinguish chromosomes can be revealed quickly and without the need for sequence information. The only requirement is that the individual chromosomes be purified.

Large DNA molecules migrate at a rate independent of size in conventional gel electrophoresis and thus they are not resolved according to size by this technique (Lerman and Frisch, 1982). Several theories have been proposed to explain the mechanism of DNA separation in agarose gels (Noolandi *et al.*, 1992; Viovy *et al.*, 1992). Computer simulation (Duke and Viovy, 1992) and direct microscopic observations of DNA (Bustamante *et al.*, 1990) have made it possible to study the mechanism of DNA movement in agarose gels.

In PFGE, migration of DNA through the gel is a two step process: the first of these comprise orientation and deformation of the molecules to align with the applied electric field followed by migration. Small molecules rapidly orient themselves to the

applied electric field and spend most of their time in migration, whereas large molecules reorient relatively slowly in response to directions of the applied electric field.

Electrode arrangements in PFGE described by Schwartz and Cantor (1984) create a double inhomogenous electric field which generates an electric field gradient. In this arrangement, the angle of reorientation and velocity of DNA molecules vary depending upon their position in the gel. The inhomogenous field gives a good resolution of the DNA molecules but the migration is distorted (Carle *et al.*, 1986). However, with the various developments in PFGE, it appears that the homogenous electric field provides the best conditions to separate the chromosome-sized DNA molecules. FIGE and CHEF (Carle *et al.*, 1986) configurations are the most popular methods for this purpose (Lognonne, 1993).

Field geometries (different field angles) have been shown to have little effect on the range of molecular sizes resolved but has a significant effect on the distance of migration (Clark *et al.*, 1989). The type of agarose, composition and temperature of running buffers modify the ability of PFGE to separate large DNA molecules (Mathew *et al.*, 1988a). For example, the conformation of DNA is affected by the ionic strength of running buffer (Mathew *et al* 1988b)

Pulse time, duration of applied electric field, has been shown to significantly affect the separation of DNA molecules. By successively increasing the pulse time, larger DNA molecules can be separated more efficiently than the smaller ones. For any given pulse time, the resolution of separated molecules is not uniform over the entire separation range (Gemmil, 1991). Empirically, it is apparent that the longer pulse time (Fig 3 b) gives a better resolution and separation of medium-sized chromosomes and chromosomes between 1 and 2 Mb, than the shorter pulse time (Figure 3 A).

For interpretation of the separation of large DNA molecules, it is important to have suitable size markers. The most widely used chromosome-sized DNA size markers are the chromosomes of *S. pombe* which are only three (3.5 Mb, 4.7 Mb and

5.7 Mb), chromosomes of *Saccharomyces cerevisiae*, which range in size from 0.2 Mb to 2.2, Mb and concatemers of phage lambda which can range in size from 48.5 kb-485 kb (Lognonne, 1993). In this study, chromosomes of *S. cerevisiae* were used as size markers since the medium-sized chromosomes of *T. congolense* are of sizes within this range (Majiwa *et al.*, 1985a)

I separated chromosomes of *T. congolense* by CHEF and confirmed that they fall into four main size categories: the minichromosomes of about 50 kb - 150 kb; the medium-sized chromosomes, 200 kb - 750 kb; the large chromosomes, > 1 Mb and chromosomes that are non-specifically trapped in the wells. This is in agreement with earlier observations by Van der Ploeg *et al.*(1984). Most of the *T. (N.) congolense* clones whose chromosomes were examined had minichromosomes of uniform sizes and chromosomes located at the compression zone (> 1 Mb) (Majiwa *et al.*, 1985a). However, all the trypanosome clones examined were found to have different numbers and sizes of medium-sized chromosomes in the size-range between 200 kb and 750 kb. Chromosome rearrangements that occur in trypanosomes are relatively easier to follow when they affect the medium-sized chromosomes since these are more easily separated as distinct chromosomes, unlike the minichromosomes and chromosomes located at the compression zone where many chromosomes appear as one band. Majiwa *et al. (1985a)* analysed the molecular karyotype of *T. congolense* and observed an apparent deletion of one of the medium-sized chromosomes and a concomitant increase in the size of the other medium-sized chromosomes. This indicated that there had occurred translocation of a chromosome segment from one to the other. The reason for this translocation is not clear. However, possibility exists that the ability of trypanosome chromosomes to undergo translocation may be either a general property of trypanosomes or that this occurs when the trypanosomes undergo the process of mating and gene exchange (Jenni *et al.*, 1986).

Purification of DNA molecules from agarose gels after electrophoretic separation is of major importance if these molecules have to be used later. A variety

of techniques by which DNA fragments are released from agarose gels chemically or by electroelution have been described (Sambrook *et al.*, 1989; Sealey *et al.*, 1989). Some of the methods require expensive reagents and apparatus, are time-consuming or lead to a low yield recovery of the DNA. To isolate a putative chromosome-specific RAPD band for subsequent cloning and analysis, we used an improved version of the method described by Heery *et al. (1990)*. The improvement was done by optimizing the time and force of centrifugation required to elute the DNA fragment from agarose gels (He *et al.*, 1992). Under these conditions, the gel slice is retained intact and contaminants, which may inhibit enzymes used in subsequent DNA manipulation, are retained in the gel. This elution of DNA produced no change in their electrophoretic mobility, confirming that physical modification of the DNA fragment has not occurred. This DNA was used directly for cloning without any further purification.

Even though PCR can be used to produce a large amount of DNA from a complex source, cloning of PCR products has not proven to be simple. This problem is caused by the template-independent terminal transferase activity and strong preference for dATP of *Taq* DNA polymerase. These result in the addition of one adenosine nucleotide at the 3' end of each strand of DNA produced by PCR (Clark, 1988; Mole *et al*, 1989). Therefore, cloning a PCR product requires either enzymatic modification of the product to remove the 3' overhang using an enzyme with 3' to 5' exonuclease activity so that the cloning is done as a blunt end (Hemsley *et al.*, 1989) or creating a cloning vector which has a sticky end and hence require no additional enzymatic modification of PCR product. In the cloning scheme described in this thesis, I used the latter alternative. A blunt-ended bluescript vector was prepared by Eco RV digestion and then tailed with dideoxythymidine triphosphate (ddTTP) using a terminal transferase. The use of ddTTP ensured the addition of only one thymine residue. The vector ends thus generated have a single 3' overhanging thymine residue which lacks a 3'-hydroxyl (OH) group and is therefore incapable of forming a phosphdiester bond and thus cannot ligate to itself or other molecules like it. Such a

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APPENDIX

BUFFERS AND SOLUTIONS

Phosphate / Saline /Glucose (PSG) pH 8.0
57 mM Na_2HPO_4
2.6 mM $NaH_2PO_4(H_2O)_2$
42.8 mM NaCl
55.5 mM glucose

BACTRIAL MEDIA AND PLATES

2 x YT (per litre)
16 g bactotyptome
10 g yeast extract
5 g NaC1

Ampicillin and tetracycline (Penbritin, Dawa Pharmaceuticals Ltd, Nairobi, Kenya) were dissolved at a concentration of 100 mg/ml each and stored at -20° C. Ampicillin was added to the media at a concentration of 50 µg/ml while tetracyclin was used at a concentration of 12.5 µg/ml

NZCYM Medium for Plates (per litre)
10 g NZ amine
5 g NaCl
1 g casamino acids
5 g Bacto-yeast extract
1.71 g $MgSO_4$ $7H_2O$
12.06 g Agar

All the plates contained 15 g/l of agar and were poured in 15 x 15 large plastic petri dishes with about 60ml of the agar per plate. When required, ampicillin and tetracyclin were added to the autoclaved agar after it had cooled to 60°C before pouring. All the plates were dried by incubation at 37°C, with the lids slightly open, overnight.

SOB Medium (pH 7.0)
2% (w/v) Bacto-typtone
0.5 % (w/v) Bacto-yeast extract
10 mM NaCl
2.5 mM KCl
10 mM $MgCl_2$
10 mM $MgSO_4$

Frozen Storage Buffer (FBS) pH 6.2
100 mM KCl
45 mM $MnCl_2$
10 mM $CaCl_2$
3 mM HA $CoCl_3$
10 mM potassium acetate

10 % glycerol

SM Phage Buffer
5.8 NaCl
2 g $MgSO_4$
50 ml lM Tris-HCl pH 7.5
5 ml 2 % gelatin

BUFFERS AND SOLUTIONS FOR DNA ANALYSIS

TNE Buffer
100 M NaCl
10 mM Tris-HCl pH 8.0
1 mM EDTA pH 8.0

T.E. Solution
10 mM Tris (pH 7.4)
1 mM EDTA(pH 8.0)

Cell resuspension buffer
50 mM glucose
25 mM Tris-HCI pH 8.0
10 mM EDTA pH 8.0

Cell Lysis buffer
0.2 N NaOH
1% SDS

50 x Tris-Acetate EDTA Buffer (TAE) - per litre
242 g Tris base
57.1 ml glacial acetic acid
100 ml 0.5 M EDTA pH 8.0

DNA Loading Buffer
0.25% bromophenol blue
0.25 % xylene cyanol
15 % Ficoll type 400

PCR PRODUCT PURIFICATION BUFFER

Direct Purification Buffer
10 mM Tris HCl pH 8.8
50 mM KCI
1.5 mM MgC12
0.1% Triton X-100

DNA BLOTTING SOLUTIONS

DNA Denaturing Solution
1.5 M NaCI
0.5 M NaOH

20 x SSC
0.3 M sodium citrate
1 M sodium chloride

Acid Depurinating Solution
0.25 N HCI (9.5 ml concentrated
HCI in 1000 ml water)

DNA Neutralising Buffer
2.55 M Potassium acetate, pH 7.5

Pre-hybridisation Solution
6 x SSC
0.5% SDS
5 x Denhardt's solution
0.1% sodium pyrophosphate

50 x Denhardt's Solution
5% Ficoll
5% polyvinlpyrrolidon
5% BSA

10 x Klenow Buffer
0.5 M Tris-HCl (pH 7.6)
0.1 M MgC12

Column wash solution
200 mM NaCl
20 mM Tris-HCl
5 mM EDTA
Diluted 1:1 with 95% ethanol