IDENTIFICATION AND CHARACTERISATION OF AN EXTRACHROMOSOMAL ELEMENT FROM A MULTIDRUG-RESISTANT ISOLATE OF *TRYPANOSOMA BRUCEI BRUCEI*

A thesis submitted for the degree of Doctor of Philosophy

By

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ABSTRACT

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Drug resistance together with difficulties involved in the development of new trypanocides are a major problem in the present control of African trypanosomiasis. DNA based diagnostics for drug resistance would overcome problems in the identification of drug-resistant populations and contribute to effective control measures. However, this requires a detailed knowledge of the mode of action and the mechanisms by which trypanosomes can overcome the toxic effects of trypanocides. In this study, a search for molecular differences between a multidrug-resistant isolate of *Trypanosoma brucei brucei*, CP 547, and a reference drug-sensitive population, ILTat 1.4, led to the identification of a 6.6 kbp extrachromosomal element in the multidrug-resistant population. In light of the involvement of extrachromosomal elements in drug resistance in *Leishmania* spp. and cancer cells, the identification of the 6.6 kbp element warranted its characterisation.

Several different approaches were attempted before a sequence which hybridised to the 6.6 kbp element was eventually isolated. This sequence is represented by a 108 bp repeat sequence which forms long arrays of tandem repeats. Since *Nla* III is the sole restriction enzyme that cuts within the repeat, it has been referred to as an *Nla* III repeat. The repeat is flanked by a 5 bp spacer sequence. However, a unique 5 bp direct repeat flanking two complete, and one partial copy of the *Nla* III repeat may signify the transposition of these sequences. Hybridisation with the *Nla* III repeat revealed the presence of 'higher' hybridising elements which also appear to be predominantly composed of long tandem arrays of the *Nla* III repeat. Through exploitation of the polymerase chain reaction using arbitrary primers (AP-PCR), additional sequences were identified which are associated with some of the 6.6 kbp and 'higher' hybridising elements.

The 6.6 kbp element and some of the 'higher' hybridising elements display features of circular DNA molecules. The 6.6 kbp element also displays some level of size and sequence heterogeneity within different populations of the same trypanosome isolate. The copy number of the 6.6 kbp element is also not stable and appears to be directly affected by the application of selective drug pressure, but a direct association between the presence of the element and the expression of multidrug resistance could not be determined.

The *Nla* III repeat family represents a newly identified repetitive family specific to members of the *Trypanozoon* subgenus. This repeat family, representing about 5% of the parasite genome, is dispersed through all size classes of chromosomes, in addition to its presence on the extrachromosomal elements.

Transcriptional studies of the *Nla* III repeats have revealed that their transcription is developmentally regulated, since heterogeneous transcripts ranging from greater than 10 kb to smaller than 300 bp are present in the actively dividing long slender bloodstream and insect stage procyclic forms of the parasite but not non-dividing, stumpy bloodstream forms. Lastly, the *Nla* III repeat lacks an open reading frame and transcripts do not appear to have a spliced leader sequence at the 5' end. Furthermore, there is almost an equal representation of polyadenylated and non-polyadenlyated transcripts.

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DEDICATION

TO MY PARENTS-IN LAW MR. BASHESHAR. N. JAMNADASS AND MRS. SHEELA JAMNADASS; TO MY FATHER, MR. RAM SHARDA AND MOTHER, (LATE) MRS. LAJYA SHARDA; AND TO MY HUSBAND, RAJAN, AND MY SONS, GAURAV AND ARJUN.

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Abbreviations:

The following abbreviations are used throughout this thesis:

AP-PCR:	Arbitrary primed polymerase chain reaction
bp:	Base pair
BME:	ß-mercaptoethanol
BSA:	Bovine serum albumin
CP:	Chemotryp Project
DEAE:	Diethyl aminoethyl
DMSO:	Dimethyl sulphoxide
dNTP:	Deoxynucleotide triphosphates
DTT:	Dithiothreitol
EDTA:	Ethylene diaminetetraacetic acid
HEPES:	4-(2hydroxyethyl)-1-piperzine N'-2-ethanesulphonic acid
ILRAD:	International Laboratory for Research on Animal Diseases
ILO:	ILRAD oligonucleotide
ILTat:	ILRAD *Trypanozoon* antigenic type
IPTG:	Isopropyl ß-thiogalactopyronoside
kbp:	Kilobase pair
NaPPi:	Sodium pyrophosphate
oligo:	oligonucleotide
PCR:	Polymerase chain reaction
PEG:	Polyethylene Glycol
PFGE:	Pulsed field gel electrophoresis
PMSF:	Phenylmethylsulphonyl fluoride
TEMED:	N,N,N,N'-tetramethylethylene diamine
Tris:	Tris-(hydroxmethyl)aminomethane
VSG:	Variable surface glycoprotein
X-gal:	5-bromo-4-chloro-3-indolyl-p-D-galactoside

CHAPTER 1

INTRODUCTION

1.1 Introduction to salivarian trypanosomes

1.1.1 General

Members of the genus *Trypanosoma,* within the order Kinetoplastida, are all parasitic organisms. Amongst them are the salivarian trypanosomes, some of which live within the bloodstream and tissue spaces of their mammalian hosts (Mulligan, 1970). These parasites have developed a remarkable mechanism of antigenic variation enabling them to escape destruction by the host's immune system (Brigden *et al.*, 1976; Boothroyd, 1985; Donelson and Rice Ficht, 1985; Borst, 1986; Pays and Steinert, 1988; Cross, 1990; Donelson, 1989; Hajduk *et al.*, 1992).

Gruby (1943), a Hungarian, is credited with providing the generic name, *Trypanosoma*, to parasites of amphibians. However, Timothy Lewis' discovery of a trypanosome in the blood of rats in India (Lewis, 1878), termed *T. lewisi*, helped the veterinary surgeon Griffith Evans (1880) to recognise the parasite which he discovered in the blood of Indian horses and camels suffering from the disease Surra. Evans' work gave the first indication that these organisms were capable of causing disease. Subsequent investigations contributed to the realisation that there are many different species of trypanosomes, some that are pathogenic and others which are not. The identification of all the species is by no means complete as new species are still being identified (Majiwa *et al.*, 1993) The success of the genus *Trypanosoma* in general is apparent from the findings that they have adapted to a variety of hosts ranging from mammals, reptiles and birds to plants.

African trypanosomes have received most of the attention since they cause potentially fatal diseases in both man and animals. In some instances, the reservoir for

African trypanosomes are the wild game animals of Africa, but these parasites do not always appear to have a deleterious effect on these animals. However, man and his domestic animals do succumb to trypanosome infections, although there are livestock (*Bos taurus* breeds, e.g. the N'Dama, Baoule', Muturu etc) which are more resistant to the pathogenic effects of trypanosomiasis than the *Bos indicus* Zebu cattle. The typical course of a pathogenic infection is a relapsing parasitaemia and eventual death, caused by the combined effects of anaemia and loss of ability to sustain recurrent infections due to haemopoetic and lymphoid exhaustion.

1.1.2 Biology

The taxonomic classification of the African trypanosomes based on Hoare (1970, 1972) is outlined in Table 1. The classification of trypanosomes is based on morphological and biological criteria (Hoare 1964). Morphologically, characterisation is based on the parasite size, motility, whether there is a free flagellum, and the size of the flagellum. Biological characterisation is based on the site of development of the parasite in the vector, the host specificity, and the clinical course of the disease produced. Recently, immunological and biochemical techniques have been used to characterise trypanosomes. These studies used monoclonal antibodies to detect antigenic characteristics. Other techniques were used to detect isoenzyme polymorphisms, repetitive DNA sequences, polymorphism of restriction fragment lengths, polymerase chain reaction (PCR) products and karyotypes (Godfrey, 1987; Majiwa *et al.*, 1986a; Moser *et al.*, 1989; Paindavoine *et al.*, 1989).

The family Trypanosomatidae is divided into two major sections (Table 1); salivaria and stercoraria. This division is based on whether the developmental cycle of the parasite in the vector takes place in the anterior part of the alimentary tract and transmission is by inoculation, or whether it takes place in the posterior region of the gut and the transmission is by faecal contamination.

TABLE 1:

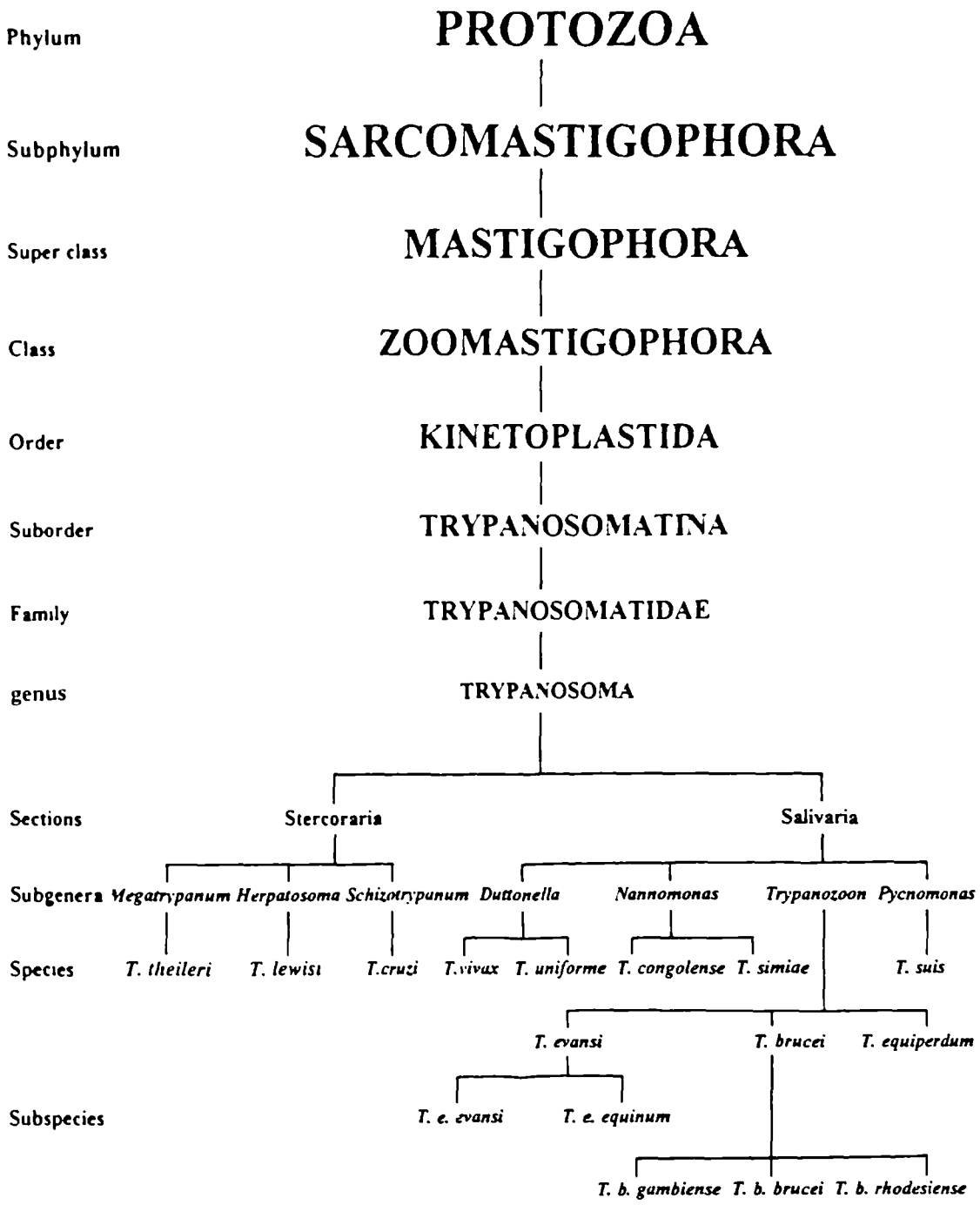
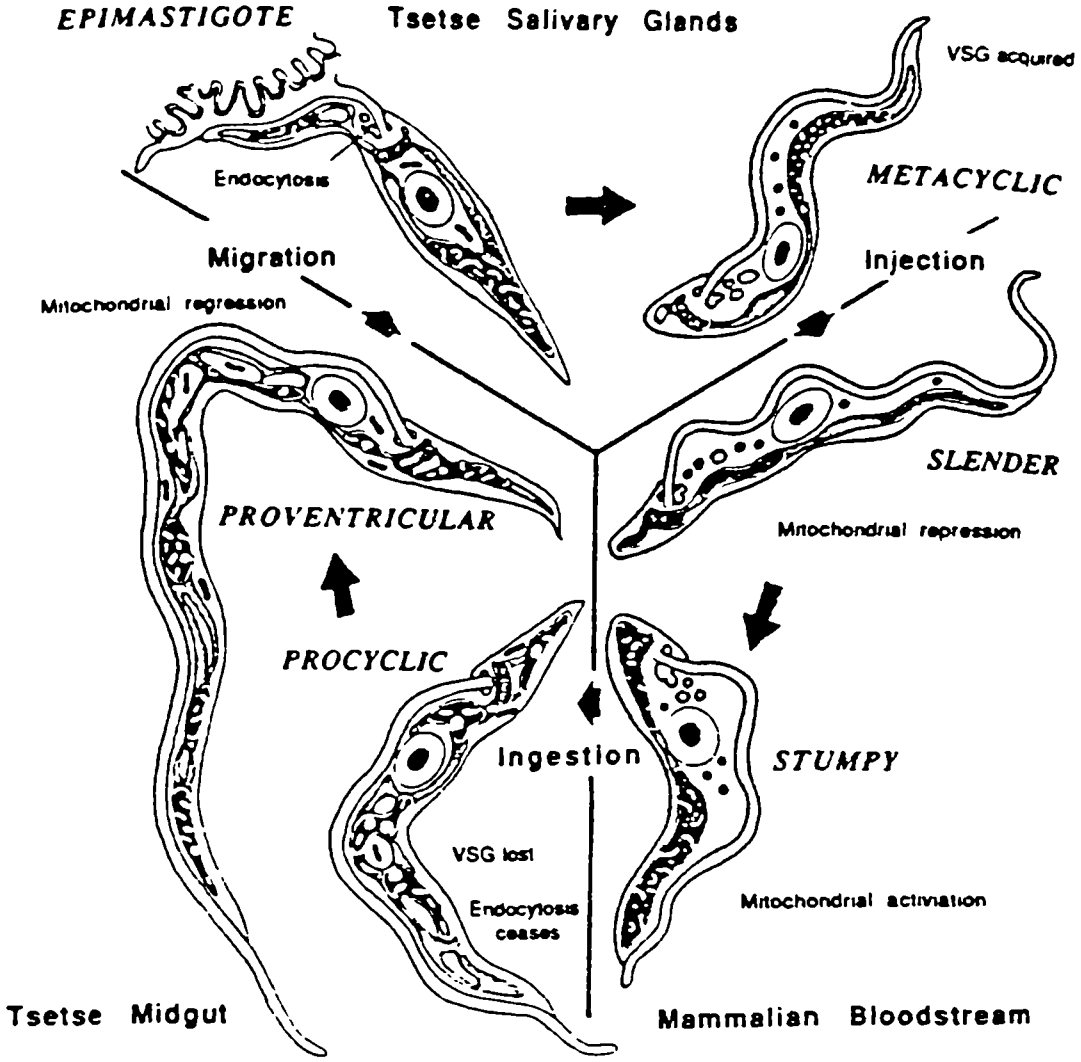

Phylum
PROTOZOA
Subphylum
SARCOMASTIGOPHORA
Super class
MASTIGOPHORA
Class
ZOOMASTIGOPHORA
Order
KINETOPLASTIDA
Suborder
TRYPANOSOMATINA
Family
TRYPANOSOMATIDAE
genus
TRYPANOSOMA
Sections
Stercoraria
Salivaria
Subgenera
Megatrypanum
Herpatosoma
Schizotrypanum
Duttonella
Nannomonas
Trypanozoon
Pycnomonas
Species
T. theileri
T. lewisi
T.cruzi
T.vivax
T. uniforme
T. congolense
T. simiae
T. suis
T. evansi
T. brucei
T. equiperdum
Subspecies
T. e. evansi
T. e. equinum
T. b. gambiense
T. b. brucei
T. b. rhodesiense

Figure 1

The life cycle of *Trypanosoma brucei* (simplified from Vickerman, 1985). The parasite proliferates in the mammalian host in the "long slender" form, which displays an extraordinary capacity for changing its surface antigen coat. Long slender bloodstream forms give rise to the less active "stumpy" trypanosomes, which are believed to initiate the infection of the tsetse fly. After ingestion by the insect, the parasites transform into large "procyclic" forms that multiply in the midgut of the fly. Mature procyclics subsequently migrate towards the salivary glands and differentiate into the non-dividing "metacyclics" form. At this stage, the parasite has resumed the synthesis of variable surface antigens and has recovered its infectivity to the mammalian host.


EPIMASTIGOTE
Tsetse Salivary Glands
VSG acquired
Endocytosis
METACYCLIC
Migration
Injection
Mitochondrial regression
SLENDER
PROVENTRICULAR
Mitochondrial repression
PROCYCLIC
Ingestion
STUMPY
VSG lost
Endocytosis ceases
Mitochondrial activation
Tsetse Midgut
Mammalian Bloodstream

The principal pathogens of livestock in sub-Saharan Africa belong to three different sub-genera (Fiennes, 1950; Mulligan, 1970): *Trypanosoma brucei brucei*, *T. evansi* and *T. equiperdum* (subgenus *Trypanozoon*) (Plimmer and Bradford, 1899; Bruce *et al.*, 1914), *T. congolense* and *T. simiae* (subgenus *Nannomonas)* (Broden, 1904) and *T. vivax* (subgenus *Duttonella*) (Ziemann, 1905). When examining the blood of livestock from sub-Saharan Africa, other non-pathogenic species such as *T. theileri*, *T. melophagium* (subgenus *Megatrypanum*) (Hoare, 1964), which are stercorarian, and *T. suis* (subgenus *Pycnomonas*) (Hoare, 1964) are also encountered.

The principal agent responsible for transmission of trypanosomes occurring in livestock confined to Africa is the tsetse fly, belonging to the genus *Glossina* (Mulligan, 1970). Trypanosomes are digenetic, undergoing distinct developmental stages in the two hosts; the tsetse fly and the definitive host. Among these pathogenic trypanosomes, two species, *Trypanosoma evansi*, *T. equiperdum* and sometimes *T. vivax*, are not transmitted by the tsetse fly, but by non-cyclical, mechanical transmission (Gardiner and Mahmoud, 1992). The agents responsible for mechanical transmission are flies belonging to the family Tabanidae (Dirie *et al.*, 1989).

The salivarian species found in domestic livestock have a similar cyclical pattern of development in the vector: trypanosomes ingested with blood transform into epimastigote forms, which eventually give rise to the infective metacyclics (metatrypanosomes).

Unlike many parasites, trypanosomes have a relatively simple life cycle (Vickerman, 1974). The life cycle of *T. brucei* is drawn schematically in Figure 1. In the mammalian bloodstream, *T. b. brucei* is pleomorphic, consisting of long slender, dividing forms, intermediate forms, and non-dividing short-stumpy forms. All these forms have the variable surface glycoprotein (VSG) surface coat. The slender forms rely on aerobic glycolysis, excreting pyruvate as the sole end product, for energy generation. When a tsetse fly bites an infected mammal, trypanosomes ingested with a blood meal by the fly differentiate and multiply in the tsetse midgut as noninfectious procyclic forms. The glycoprotein surface coat is replaced by the procyclin coat, and the procyclic forms

change from anaeroic to aerobic growth. Procyclic forms migrate to the salivary glands where, attached to the epithelial cells, they multiply as a distinct morphological stage, the epimastigote. After cessation of division, the epimastigote starts to express the VSG coat once more (Tetley *et al.*, 1987), and this expression leads to detachment and maturation as non-dividing, infective, metacyclic trypomastigotes. The infected tsetse fly, on biting a mammalian host, passes mature metacyclics in saliva to the host, resulting in the initiation of an infection. Shortly after being deposited in the skin, trypanosomes begin to multiply by binary fission of the long slender forms, and undergo a variety of additional morphological and metabolic changes (Vickerman, 1985).

Trypanosoma congolense can produce a pathogenic form of African trypanosomiasis in ruminants but is non-infective for humans (Mulligan, 1970). This species goes through a similar life cycle like *T. brucei*. However, epimastigotes attach and form metacyclics in the proboscis and not in the salivary glands. The *Duttonella* subgenus is represented by monomorphic trypanosomes which are parasitic chiefly in wild and domestic ruminants. These parasites have the least complex insect cycle. They develop solely in the mouthparts of the *Glossina*; the parasites ingested with the blood meal attach to the wall of the proboscis and transform to elongated trypomastigotes and then into epimastigotes which eventually give rise to infective metacyclic forms. All these trypanosome species are found throughout the tsetse infested areas with mixed infections being common in the field (Stephen, 1970). A summary of the characteristics of the trypanosomes of veterinary importance is given in Table 2 (Food and Agricultural Organisation [FAO], 1986).

1.2 Economic importance of trypanosomiasis in domestic livestock in Africa.

1.2.1 General

In Africa, the disease caused by the major animal pathogenic trypanosomes can be acute or chronic, depending on the susceptibility of the mammalian host species, and is generally characterised by intermittent fever, wasting, anaemia and infertility. In

Table 2: Morphological characteristics of trypanosomes (FAO, 1986)

Subgenus	Group	Species	site of development in the *Glossina*	Free flagellum	Kinetoplast	Undulating membrane	Size (µm) and motility in wet film and dark ground microscopy examination
Duttonella	vivax	*T. vivax* *T. uniforme*	Proboscis only	Present	Large terminal	Not prominent	20-26, large, extremely active, traverse the whole filed very quickly, pausing occasionally.
Nannomonas	congolense	*T. congolense* *T. simiae*	midgut and proboscis	Absent	medium subterminal or marginal	Not prominent	9-18, small, sluggish active, adheres to red blood cells by anterior end
Trypanozoon	brucei	*T. brucei* * *T. rhodesiense* * *T. gambiense* ** *T. evansi* ***T. equiperdum*	midgut and salivary gland None None	Present in all but stumpy forms	small subterminal centre	prominent	12-35, large, rapid movement in confined areas

Key:
- * Causal agents of human sleeping sickness
- ** No-cyclical transmission, carried by biting flies
- *** Transmitted during sexual contact

susceptible species, if left untreated, the disease is usually fatal. There are, however, certain indigenous breeds of cattle in West and Central Africa which can better tolerate exposure to trypanosomiasis (Murray *et al*,. 1982). In these cattle, even though mortality is reduced, infection is often accompanied by losses in productivity in areas of high tsetse challenge (Stephen, 1986).

There are as many forms of trypanosomiasis as there are specific trypanosomes (Table 3). Each species of trypanosome may infect several species of animals with different effects and degrees of severity resulting in the clinical signs varying in different mammalian species. Moreover, the clinical picture is often complicated further by the presence of mixed infections within one host.

According to a 1992 report by Winrock international, 65-70% of the world's livestock population resides in developing countries, yet they provide only 30% of the world's meat output. The estimated production of animal protein from livestock farming in Africa per 1,000 hectares is almost 1/77 of that in Europe and 1/8 of that in Latin America (FAO, 1975). This condition was expected to deteriorate even further as Africa's human population increased by twice the rate of food production between 1980 and 1985. Presently, Africa has the highest population growth rate in the world, which is estimated to be expanding at 3.2% per annum. There is, therefore, a continually increasing need for significant improvements in food productivity in the developing world.

Trypanosomiasis has had profound adverse effects on agriculture in Africa. According to the Food and Agricultural Organisation of the United Nations, (FAO) (1975) and the International Livestock Centre for Africa (ILCA) (1985), trypanosomiasis has been the most significant factor limiting extension of the cattle producing areas of Africa. It has been estimated that 31 species and subspecies of the tsetse fly are distributed over 37 countries lying between latitudes 15°C North to 25°C South across the African continent. Their habitat includes approximately 7 million square kilometres of savannah and light woodland with good rainfall, suitable for agricultural development (Finelle, 1974). Currently, of a total population of approximately 160 million cattle in

Table 3: The occurrence of pathogenic African trypanosomes of veterinary importance (FAO, 1986).

Trypanosome species	Domestic animal affected	Reservoir hosts	Experimental hosts
T. congolense	Cattle Horses Dogs Sheep Goats Pigs	Wild game species	Rats Mice Rabbits Guinea-pigs
T. simiae	Pigs	Warthog Bushpigs	Rabbits Monkeys
T. vivax Stocks	Cattle Sheep Goats	Wild game species	None (mice for West African
T. brucei brucei	Horses Dogs Sheep Goats Cattle Pigs	wild game species	Rats Mice Guinea-pigs Rabbits
T. b. evansi	Camels Cattle Water Buffalo Horses Dogs		Rats Mice Guinea-pigs Pigs Rabbits
T. b. equiperdum	Horses	None	None

Africa, only about 50 million are located in the tsetse-infested subhumid/humid zone. Of these, only 5% are the more resistant *Bos taurus* breeds and their crosses. Where cattle are exposed to trypanosomiasis, severe losses occur in livestock productivity due to poor growth, weight loss, lower milk yield, reduced traction capability, infertility and abortion (McDowell, 1977; FAO/WHO 1982). Thus in 1963, Africas annual loss in meat production due to trypanosomiasis was estimated to be US $5 billion (Gyening, 1990).

The agricultural production of Africa cannot even today sustain the existing population. This is shown by the facts that in 1983/1985, it was estimated that 35% of the population of sub-Saharan Africa was malnourished and 26% severely malnourished (The African Trypanotolerant Livestock Network [ATLN], 1987). This is a reflection of poor economic growth and low levels of agricultural productivity. *Recent projections* indicate that in sub-Saharan Africa the deficits for meat and milk production will reach -1.7% and -0.8% per annum, respectively, by the year 2,000. Meeting these deficits through imports would cost an estimated US$15 billion annually (Mc Namara, 1990; Winrock, 1992), which Africa cannot afford.

1.2.2 Control of trypanosomiasis

Presently, there are three main approaches to the control of animal trypanosomiasis:

i) Combating the vector by control or eradication of tsetse fly populations.

ii) Reducing the affects of the disease through the use of trypanotolerant cattle.

iii) Combating the disease in the mammalian host through chemotherapy and/or chemoprophylaxis.

1.2.2a Tsetse fly control

Control of tsetse flies is based on the use of insecticides, the restriction of their hosts and habitats, and extensive utilisation of traps. The oldest and traditional method of control has been selective bush clearing. With the expanding human populations within Africa, there has been a growing need for arable land for either settlement or cultivation.

Clearance of vegetation creates an adverse environment for tsetse. In addition, workers have also been encouraged to destroy game to decrease the reservoir for trypanosomes (Jordan, 1974). Both methods are clearly non-sustainable and environmentally dangerous approaches, and are now discouraged.

Modern strategies for tsetse control can be either chemical or non-chemical. Chemical control is divided into two distinct categories: (i) the use of residual insecticides which kill tsetse by contact and are active over a period of weeks or months and (ii) the use of non-persistent insecticides with no detectable residual effect but which require repeated application. Due to the toxic nature and the environmental damage caused by the three commonly used residual insecticides, dichlorodiphenyl trichloethane (DDT), dieldrin and endosulfan (toxic to fish), non-residual insecticides such as the synthetic pyrethroids (Spielberger *et al.*, 1979) are now generally used. Application of these insecticides is by aerial-spraying programs and dipping and spraying cattle. With advances in the production of ultra-low volume aerosols (ULV), very low doses of insecticide are applied using aircraft and helicopters (MacLannan *et al.*, 1967; Takken *et al.*, 1986).

The selective application of insecticides in tsetse control programmes unfortunately suffers from the eventual major drawback of reinvasion of areas allegedly freed from tsetse (Hadaway, 1977). In addition, the insecticides can produce environmental pollution and unwanted ecological effects (Koeman *et al.*, 1971). Furthermore, the application of insecticides is not suited to all ecological zones. Areas of high rainfall and dense vegetation tend to limit the feasibility and efficacy of insecticide application (Jordan, 1974).

Of the non-chemical approaches, use has been made of traps and screens. In the past these were used for tsetse population surveys. With improvements in terms of both simplicity and greater catching efficiency, and with the incorporation of chemical attractants and insecticides, traps and screens can now be used to complement other control methods such as aerial spraying (Vale, 1980, 1988; Jordan, 1985).

A sterile insect release technique (SIT) has also been investigated. This method has been employed with limited success in areas of Tanzania and Nigeria (Williamson *et al.*, 1983). Failure to completely eradicate the fly has been attributed to immigration of flies from surrounding areas. Enormous costs are involved in implementing the SIT, making it prohibitive in most of Africa, and furthermore, the density of the target population must be low for SIT to be effective.

1.2.2b Trypanotolerance (Genetic resistance):

Trypanotolerance, the heritable ability of some bovidae to remain productive in the face of trypanosome challenge, offers a sustainable means of reducing the impact of trypanosomiasis on cattle productivity (ATLN, 1987) .

Investigations have shown that increased resistance of wild animals to the ill-effects of trypanosome infections may be related to their innate ability to control the level of parasitaemia (Murray *et al.*, 1982). Comparative studies have shown the possible involvement of the immune system in the control of parasitaemia; phagocyctic cells from wild animals are superior to those of susceptible cattle breeds in engulfment and subsequent destruction (phagocytosis) of antibody-sensitised trypanosomes (Rurangirwa *et al.*, 1986). This ability is also possessed by the trypanotolerant cattle (Murray *et al.*, 1982). It has been shown both in the field and experimentally that trypanotolerant cattle are capable of resisting the pathogenic effects of trypanosomiasis to varying extents (Murray *et al.*, 1982; Nantulya *et al.*, 1986); they remain productive despite the infection, and often spontaneously recover from infections (Nantulya *et al.*, 1984, 1986).

A search for genetic markers for the trypanotolerant trait, which might be used quantitatively in the breeding of trypanotolerant livestock, is in progress at ILRAD (Teale, 1993). Identification of the genes responsible (providing these are not too many) should help in breeding programmes to select for more productive trypanotolerant cattle. The possibility of transfecting the genes for trypanotolerance into more productive cattle breeds is a possibility on the basis of the success in transfer of the chloramphenicol acetyltransferase structural gene into the bovine genome (Biery *et al.*, 1988).

1.2.2c Chemotherapy of African trypanosomiasis:

In areas where the tsetse populations have posed difficulties in being eliminated, chemotherapy may be the preferred means for controlling trypanosomiasis in domestic livestock at the present time.

The first application of chemotherapy to bovine trypanosomiasis was brought about by observations of Plimmer and Thompson (1908), that mice infected with *T. b. brucei* or *T. evansi* were cured by administration of tartar emetic (potassium antimonyl tartrate). Early in the 1940s, several other types of veterinary trypanocidal drugs, belonging to the chemical classes of acid naphthylamines, diamidines, quinaldines and phenanthridines, were put to use.

Chemotherapy of trypanosomiasis in domestic livestock is at present dependent on a relatively small number of synthetic compounds: homidium (Ethidium$^{(R)}$, Novidium$^{(R)}$), a phenanthridine; isometamidium (Samorin$^{(R)}$, Trypamidium$^{(R)}$), a phenanthridine aromatic amidine; diminazene (Berenil$^{(R)}$, Veriben$^{(R)}$), an aromatic diamidine; quinapyramine (Trypacide$^{(R)}$), a quinoline pyrimidine; suramin (Naganol$^{(R)}$), a naphthylamine sulphonic acid derivative; and a relatively new drug melarsomine (Cymelarsan$^{(R)}$). Melarsomine (Raynaud *et al.*, 1989) is an arsenical which is used against only *T. evansi* infections in camels. Besides melarsomine, all these compounds have been on the market for a minimum of 30 years, primarily because of the costs involved in developing a new drug for a relatively small trypanocide market (Goodwin, 1978); $80 million is required to make a new drug commercially available (Williamson, 1980a, b) deters pharmaceutical companies since returns expected on new trypanocidal drugs would be low due to problems of limited resources and foreign exchange in developing countries. However, there is an urgent need for new trypanocidal drugs as the extensive use of available drugs for the control of bovine trypanosomiasis has resulted in the appearance of drug resistance in *T. congolense*, *T. vivax* and *T. brucei* in many parts of Africa (Peregrine, 1994). Furthermore, because of the close chemical relationships between trypanocides (Leach and Roberts, 1981), the development of resistance to individual compounds often appears to be associated with cross-resistance to others

(Whiteside, 1961; Williamson, 1970). In some areas, drug resistance has become so prevalent that some drugs have been withdrawn from general use (e.g., homidium in northern Nigeria [Aliu, 1981]). However, despite these problems, chemoprophylaxis and chemotherapy can be effective and economically justified if managed in an efficient manner (Blaser *et al.*, 1977; Leach and Roberts, 1981).

Data regarding the generic name, compound class, trade name, route of administration, spectrum of activity and the species of animal for which the veterinary trypanocides are used is shown in Table 4 (from Peregrine, 1994).

Phenanthridines

Homidium and isometamidium

The phenanthridinium salts phenidium and dimidium were demonstrated as having high levels of tryanocidal activity in 1938 (Browning *et al.*, 1938). Subsequently, an improved substitute, homidium, was introduced in 1952 (Watkins and Woolfe, 1952) followed by the introduction of isometamidium in 1960 (Berg, 1960). These drugs have been used for the treatment and prophylaxis of infections of *T. congolense, T. vivax,* and other species of trypanosomes in domestic ruminants (Wragg *et al.*, 1958) throughout Africa, and have profoundly benefited the African cattle industry (Williamson, 1970). However, like other trypanocides, resistance of various strains of *T. vivax* and *T. congolense* to isometamidium has been reported across Africa (ATLN, 1987). In some cases the strains that were resistant to isometamidium were also resistant to other trypanocides such as homidium, diminazene and quinapyramine (Röttcher and Schillinger, 1985; Schönefeld *et al.*, 1987). However, some investigations have failed to establish the presence of isometamidium resistance, even in areas where the drug has been used for about 20 years (Trail *et al.*, 1985).

Mode of action

Newton *et al.* (1964) studied the effects of homidium on non-parasitic kinetoplastids such as *Crithidia oncopelti*. Homidium was found to irreversibly inhibit multiplication of these kinetoplastids. Evidence suggests that homidium probably

Table 4: ***(From Peregrine, 1994)***
Chemotherapeutic and chemoprophylactic compounds used for animal trypanosomiasis

Compound	Trade name	Treatment regimen		Use	Activity in the field	Animal
		Dose (mg kg^{-1})	Route			
Diminazene aceturate	Berenil®, Veriben®, Ganaseg®	3.5-7.0	i.m.	T	*T. congolense*, *T. vivax*, (*T. brucei*), (*T. evansi*)	Cattle, small ruminants, [dogs], [equidae]
Homidium chloride Homidium bromide	Novidium®, Ethidium®	1.0	i.m.	T/P[a]	*T. congolense*, *T. vivax*	Cattle, small ruminants, pigs, [equidae]
Isometamidium chloride	Samorin®, Trypamidium®	0.25-0.50 0.50-1.0	i.m. i.m.	T P	*T. congolense*, *T. vivax*, *T. brucei*, *T. evansi*	Cattle, small ruminants, equidae, camels
Quinapyramine dimethylsulphate	Trypacide sulphate®	3.0-5.0	s.c.	T	*T. congolense*, *T. vivax*, *T. brucei*, *T. evansi*, *T. equinum*, *T. simiae*	Camels, equidae, pigs, dogs
Quinapyramine dimethylsulphate: chloride (3:2 w/w)	Trypacide Pro-salt®	3.0-5.0[b]	s.c.	P		
Suramin	Naganol®	7.0-10.0[c]	i.v.	T(P)	*T. evansi*	Camels, equidae
Melarsomine	Cymelarsan®	0.25	s.c./i.m.	T	*T. evansi*	Camels

i.m., intramuscular; s.c., subcutaneous; i.v., intravenous; T, therapeutic agent; P, prophylactic agent; ( ), limited activity; [ ], small therapeutic index.
[a]Prophylaxis observed in areas of low tsetse challenge.
[b]Dosage of sulphate.
[c]Grams per animal.

prevents DNA replication by intercalating between adjacent base pairs and destroying the double helix, thereby preventing DNA polymerase from functioning (Newton, 1974). Similarly, isometamidium has been shown to be capable of binding to DNA (Kinabo and Bogan, 1987), and inhibiting RNA polymerase (Richardson, 1973) and DNA polymerase (Marcus *et al.*, 1982), resulting in the inhibition of incorporation of nucleic acid precursors into DNA and RNA (Lantz and van Dyke, 1972). These findings suggest that the primary action of isometamidium is blockade of nucleic acid synthesis. Additional studies have indicated that isometamidium also interacts with other intracellular molecules (Bacchi *et al.*, 1980; Bacchi, 1981; Shapiro and England, 1990) suggesting that its trypanocidal activity may be due to a combination of inhibitory action on several target molecules. Recent investigations (Zilberstein *et al.*, 1993) indicate that isometamidium is transported across the trypanosome plasma membrane via a protein carrier.

Diminazene aceturate

Diminazene aceturate was introduced as a trypanocide for domestic livestock in 1955 (Jensch, 1955) and subsequently became the most commonly used therapeutic agent for trypanosomiasis in domestic livestock (Williamson, 1976). The relative success of diminazene has been due to a number of factors: a higher therapeutic index than other trypanocides in most livestock species (Fairclough, 1963a, b; Williamson, 1970), activity against trypanosomes that are resistant to other trypanocides used in cattle (Williamson, 1970), and a low incidence of diminazene resistance despite extensive usage of the compound (Williamson, 1976). Although the incidence of resistance to diminazene is much lower in comparison to other trypanocides, significant levels of resistance have been reported across Africa (Mamman *et al.*, 1993). Whether such resistance is a result of cross-resistance induced by other drugs, such as quinapyramine, is however, not clear (Leach and Roberts, 1981).

Mode of action

Diminazene binds to trypanosomal kDNA (MacAdam and Williamson, 1972; Newton and Burnett, 1972). The binding occurs via specific interaction with sites rich in adenine-thymine base pairs (Newton, 1972; Lane *et al.*, 1991). The molecule binds with higher affinity to 5' -AATT- 3' than to 5' -TTAA- 3' regions of DNA (Hu *et al.*, 1992). It has been shown that binding to the double stranded DNA occurs via the minor groove (Gresh and Pullman, 1984; Hu *et al.*, 1992) via electrostatic and hydrogen bonding at specific sites (Gresh and Pullman, 1984; Lane *et al.*, 1991). Through this interaction in trypanosomes, diminazene inhibits kDNA replication (Brack and Delain, 1975). Shapiro and Englund (1990) have shown that diminazene specifically inhibits mitochondrial type II topoisomerase in viable trypanosomes. Thus, inhibition of DNA replication may occur via this interaction. Although diminazene selectively fragments kinetoplast DNA, it is not established whether it is the primary target for diminazene *in vivo* (Newton, 1980).

When exposed to diminazene, diverse changes are observed in the structure and function of trypanosomatids. These alterations include aggregation and loss of ribosomes (Wallis, 1966), modification of lysosomes and cytoplasmic membrane (MacAdam and Williamson, 1972), inhibition of basic amino acid transport (Gutteridge, 1966), inhibition of DNA and RNA polymerases (Waring, 1965; Marcus *et al.*, 1982), and interference with polyamine uptake, synthesis and function (Bacchi, 1981; Bitonti *et al.*, 1986).

To date, there is no published information on the molecular basis of resistance to diminazene in trypanosomes. In studies with the closely related diamidine, pentamidine, Damper and Patton (1976a, b) concluded that the drug appears to be actively transported into trypanosomes via a carrier-mediated process that is substrate specific, concentrative and energy coupled. They also concluded that alteration in pentamidine transport is the primary mechanism responsible for pentamidine resistance in trypanosomes (Damper and Patton, 1976a). Drug efflux and drug metabolism were not shown to play a role in drug resistance (Damper and Patton 1976b).

Quinapyramine

Quinapyramine was first described by Curd and Davey (1949, 1950). Two salts of quinapyramine are used as trypanocides; the chloride and the dimethylsulphate. Both salts have a broad spectrum of activity, being active against *T. evansi, T. equiperdum, T. b. brucei* (Curd and Davey, 1950; Leach, 1961), *T. congolense* and *T. vivax* (Davey, 1950). This drug was shown to be more active against trypanosomes affecting livestock such as *T. congolense* and *T. vivax*, than other species (Davey, 1957).

Between 1950 and 1970, quinapyramine was widely used in Africa as a therapeutic and prophylactic agent in cattle (Fiennes, 1953; Ruchel, 1975). However, this appeared to lead to the rapid development of quinapyramine resistance in trypanosomes of domestic livestock (Wilson, 1949; Unsworth, 1954), which often appeared to be associated with concomitant cross-resistance to isometamidium, homidium and diminazene (Whiteside, 1960; Mwambu and Mayende, 1971a, b). In 1976 quinapyramine ceased to be manufactured for the control of trypanosomiasis in cattle (Holmes and Scott, 1982) due to the ease with which resistance appeared to develop apparent concomitant-cross resistance to other trypanocides (Leach and Roberts, 1981) and because of drug toxicity (Davey, 1957). However, the compound was reintroduced onto some African markets in the 1980s for use in camels only.

Mode of Action

Although quinapyramine has been marketed as a trypanocide for approximately 40 years, very little is known about its mechanism of action. In experimental studies with the trypanosomatid flagellate *Crithidia oncopelti*, quinapyramine appeared to inhibit growth by aggregating and inactivating ribosomes (Newton, 1962, 1963, 1966). The compound has also been shown to inhibit synthesis of RNA from exogenous purines, but not from purines synthesized *de novo* (Waring, 1965; Newton, 1966). In *T. b. brucei*, quinapyramine appears to interfere with polyamine metabolism (Bacchi *et al*, 1979, 1981), although the molecular basis of this inhibition is unclear.

Suramin

Suramin was first produced by Bayer in 1916 and is still used extensively for the treatment of trypanosomiasis in horses and camels (Apted, 1980). Suramin has been shown to be ineffective against *T. congolense* and *T. vivax* infections of cattle (Ruchel, 1975), but is efficacious against infections with trypanosomes of the subgenus *Trypanozoon* (Leach and Roberts, 1981).

Mode of Action

Suramin complexes with serum proteins and enters trypanosomes by endocytosis (Fairlamb and Bowman, 1977, 1980). In *in vitro* studies, several enzymes of *T. b. brucei* appear to be very sensitive to inhibition by suramin. Two characterised enzymes involved in the glycolytic pathway, glycerol-phosphate oxidase and NAD-linked glycerol-3-phosphate dehydrogenase, have been shown to be particularly sensitive to suramin (Fairlamb and Bowman, 1977; Fairlamb, 1981). Two enzymes involved in pyrimidine biosynthesis in *T. b. brucei* are also inhibited by suramin; dihydrofolate reductase (Jaffe *et al.*, 1972) and thymidine kinase (Chello and Jaffe, 1972). Suramin may also act by interfering with either ribosomal or lysosomal function, thus resulting in retardation of protein synthesis (Williamson and MacAdam, 1965). Finally, the compound is extremely anionic and binds readily to plasma proteins, a property which is thought to account for the drug's prophylactic activity (Dewey and Wormall, 1946).

Melarsomine

Melarsomine is an arsenical, patented in 1985, and is effective against members of the subgenus *Trypanozoon*, where it is limited for use against *T. evansi* infections in camels (Raynaud *et al.*, 1989).

Mode of action

In view of the short duration of trypanocidal levels in camel plasma, melarsomine is regarded as a therapeutic product (Zelleke *et al.*, 1989). The drug inhibits the metabolism of the cell by inhibition of trypanothione reductase, an enzyme which is

central to the regulation of the thiol/disulphide redox balance in the parasite and which is absent in the mammalian host (Fairlamb *et al.*, 1989).

1.2.3 Problem of trypanocidal drug resistance

Drug resistance in trypanosomes can be defined as the ability of a trypanosome strain to survive, despite the administration of recommended drug doses (Röttcher and Schillinger, 1985). From its earliest days, the development of chemotherapy for bovine trypanosomiasis has been accompanied by the development of drug resistance (Williamson, 1979). Resistance to almost all the available drugs has now been described and has been observed in all species of pathogenic *Trypanosoma* (Leach and Roberts, 1981; Pinder and Authie 1984; Röttcher and Schillinger, 1985; Schönefeld *et al.*, 1987; Mbwambo *et al.*, 1988). Recent reports have shown that drug resistance in African trypanosomes is an increasing and significant problem over Africa (Table 5, Peregrine, 1994). It is therefore important to define the various forms of drug resistance which can occur since the success of chemotherapy might be determined by the factors that are responsible for such drug resistance. Resistance can occur as follows:

1.2.3a Acquired drug resistance

The term acquired resistance is used to describe cases where a resistant strain or cell line emerges from a population that was previously drug sensitive. Conditions under which acquired drug resistance develops in the field are all thought to be derived principally from underdosing; that is, the concentration of the trypanocide in the animal's body is below levels that are required to eliminate an infection, thus facilitating the selection of a drug-resistant sub-population (Whiteside, 1962; Leach and Roberts, 1981). Sub-therapeutic drug levels may occur because of incorrect dosage, irregular treatment with prophylactics, or stopping the usage of a prophylactic whilst animals are still at risk (Davey, 1950, 1957; Whiteside, 1960).

Table 5: *(From Peregrine, 1994)* **Reports of resistance to standard recommended doses of diminazene, homidium, isometamidium and quinapyramine in cattle.**

Country	Trypanosome species	Resistance to	Reference
Nigeria	*T. congolense*	h,i	Jones-Davies and Folkers, 1966
Nigeria	*T. vivax*	d	Jones-Davies, 1967
Nigeria	*T. congolense*	d,h,i	Na'Isa, 1967
Nigeria	*T. congolense*	d	MacLennan and Jones-Davies, 1967
Chad	*T. vivax*	d	Graber, 1968
Nigeria	*T. congolense*	d	Jones-Davies, 1968
Nigeria	*T. congolense* *T. vivax*	d,h,i,q d,h,q	Gray and Roberts, 1971 " " " "
Uganda	*T. vivax*	d	Mwambu and Mayende, 1971
Sudan	*T. congolense*	h,i	Abdel Gadir et al., 1972
Zimbabwe	*T. congolense*	i	Lewis and Thomson, 1974
Kenya	*T. congolense*	d,h,i,q	Gitatha, 1979
Nigeria	*T. vivax*	d,h,i	Ilemobade, 1979
Sudan	*T. congolense* *T. brucei/T. vivax*	h h	Abdel Gadir et al., 1981 " " " " "
Côte d'Ivoire	*T. congolense* *T. vivax*	h,i h,i	Küpper and Wolters, 1983 " " " "
Tanzania	*T. congolense*	d	Njau et al., 1983
Burkina Faso	*T. congolense*	i	Pinder and Authie, 1984
Kenya	*T. vivax*	d,h,i,q	Röttcher and Schillinger, 1985
Kenya	*T. vivax*	d,h,i,q	Schönefeld et al., 1987
Somalia	*T. vivax*	h,i,q	Schönefeld et al., 1987
Tanzania	*T. congolense*	d	Mbwambo et al., 1988
Somalia	*T. congolense*	d,i	Ainanshe et al., 1992
Burkina Faso	*T. congolense*	d,h,i,q	Clausen *et al.*, 1992
Sudan	*T. congolense* *T. brucei* *T. vivax*	d,h,i d,h,i h	Mohamed-Ahmed et al., 1992 " " " " " " " "
Ethiopia	*T. congolense*	d,h,i	Codjia et al., 1993

d = diminazene; h = homidium; i = isometamidium; q = quinapyramine

1.2.3b Cross resistance

Cross-resistance occurs when, in addition to developing resistance to the selective agent, drug resistance also develops against other drugs. The development of cross-resistance in trypanosomes to many of the drugs used for treatment can be expected because of the close chemical relationship between most of the trypanocides (Leach and Roberts, 1981). Common receptor sites or molecules involved in the transport of trypanocides into trypanosomes, and modifications to those sites during the development of resistance, may be important factors in the development of cross-resistance (Williamson, 1970; Fairlamb *et al.*, 1992).

Most of the present information on the problems of cross-resistance with trypanocides has been interpreted from field data of Whiteside (1961) (Table 6). However, recent investigations conducted by Ndoutamia *et al.* (1993) have confirmed, under controlled laboratory conditions, the ease with which resistance to quinapyramine in *T. congolense* can be generated, and that such trypanosomes display the cross-resistance profile observed in the field (Whiteside, 1960; Mwambu and Mayende, 1971a, b).

Investigations on cross-resistance in the field led Whiteside (1960, 1962) to advise the use of "sanative pairs", an example of which is homidium and diminazene, since neither of these appear to produce cross-resistance to each other.

1.2.3c Natural resistance

Natural resistance of a trypanosome strain or species to a drug is that variation in drug sensitivity shown by a trypanosome that is not dependent on previous exposure to the drug concerned. In this case, the feature responsible for resistance is an integral property of the species that has arisen through the processes of evolution, in which all organisms develop mechanisms for survival and self-propogation. For instance, it is believed that in West Africa, the initial appearance of resistance to both homidium and diminazene in *T. vivax* (Jones-Davies and Folker, 1966a, b; Jones-Davies 1967a, b) probably reflected the level of natural resistance of these populations

Table 6: The cross-resistance patterns of drugs used in cattle trypanosomiasis (after Whiteside, 1961)

Trypanosomes	Response of trypanosomes to:			
resistant to	Quinapyramine	Homidium	Metamidium	Diaminazene
Quinapyramine	R	++	+	++
Homidium	+	R	+	0
Metamidium*	+	++	R	0
Diminazene	0	0	0	R

R: direct resistance
+: Cross-resistance to curative dose
++: Cross-resistance to higher dose
0: No cross-resistance
*: A mixture of phenanthridines that contained 45% isometamidium (Kinabo and Bogan, 1988).

1.2.4 Factors that may contribute to the apparent resistance phenotype

1.2.4a Depressed immune status of the host

It is known that an antibody response by the host to the surface antigens of trypanosomes facilitates the rapid elimination of parasites following drug treatment (De Gee *et al.*, 1983). The trypanocidal effect of DL-alpha-difluormethyl-ornithine (DFMO) (Eflornithine$^{(R)}$, Merrrel Dow Company), an ornithine decarboxylase inhibitor, was greatly reduced in immunosuppressed mice (De Gee *et al.*, 1983). Since depressed immune responses resulting from other, unrelated parasitic infections or malnutrition are frequent occurrences in tropical livestock (Holmes *et al.*, 1974; Griffin *et al.*, 1981), the response to treatment should be considered in relation to the immune status of the host.

1.2.4b Extravascular trypanosomes resisting treatment

Members of the subgenus *Trypanozoon* such as *T. brucei* and *T. evansi* (Jennings *et al.*, 1979) and also *T. vivax* (Whitelaw *et al.*, 1988) can invade body compartments other than the vascular system. Depending on the drug used, in some of these extravascular sites the typanocidal level may be low or nonexistent. They may therefore represent sites of escape from chemotherapy (Whitelaw *et al.*, 1985, 1988) and account for some cases of apparent drug resistance.

1.2.5 Stability of drug resistance

Field studies carried out by Knowles (1927) demonstrated that suramin resistance remained stable for one year or longer in *T. evansi* infected horses. Laboratory investigations also demonstrated that there was little if any change in the drug sensitivity of a multidrug-resistant *T. b. brucei* after *in vitro* propagation as bloodstream forms for up to 272 days (Kaminisky and Zweygarth 1989a). In addition, *in vitro* transformation of the *T. b. brucei* multidrug-resistant bloodstream forms into procyclic, epimastigote and metacyclic forms did not alter their drug-resistant profile. Ndoutamia *et al.* (1993) demonstrated that quinapyramine-resistant clones of *T. congolense* remained resistant to

quinapyramine during six months passage in mice in the absence of drug pressure. These findings do not comply with earlier findings which demonstrated that the passaging of uncloned populations in the absence of quinapyramine resulted in significant reductions in resistance to quinapyramine in *T. equiperdum* (Hawking, 1958) and *T. congolense* (Hawking, 1963). However, since Peregrine *et al.* (1991) have demonstrated heterogeneity in resistance to isometamidium amongst sub-clones of a cloned population of *T. congolense*, it is unclear whether the reduction in resistance observed by Hawking was due to a real reduction in sensitivity of individual trypanosomes or whether it occurred as a result of selection of a more sensitive subpopulation. Therefore, stability or instability depends on the mechanisms of resistance and the method by which resistance has been selected.

1.2.6 Detection of drug resistance

Drug resistance in African trypanosomes is the most common reason for the failure of treatment with trypanocides. Such failures in the field have led to the requirement for assays to quantify the resistance. Many assays for determining drug resistance *in vitro* have been described (Borowy *et al.*, 1985a, b; Kaminsky and Zweygarth, 1989b; Ross and Taylor, 1990). However, these systems are not yet field applicable; limited numbers of field isolates can be characterised, and all of the systems take months to produce definitive data. There is therefore a requirement for assays that will rapidly quantify the drug resistance phenotype of large numbers of trypanosome isolates. The development of direct assays for drug resistance, that can rapidly characterise the drug-resistance phenotype of large numbers of trypanosome isolates, will most likely depend upon a detailed understanding of the molecular bases of drug resistance.

1.2.7 The future

In addition to improving the present methods of detecting drug resistance, it is clearly evident from the preceding sections that there is an urgent need to develop new

compounds, chemically unrelated to those now in use, due to increasing problems of acquired and cross resistance. Drug resistance will continue to remain a major problem in the control of trypanosomiasis unless the mechanisms of resistance are understood. Such information will potentially offer a means of overcoming this problem in a sustainable and economically viable manner with new therapeutics if sufficient funds are available. In the meantime, trypanocides must be used in a rational manner so as to limit the spread of resistance.

In the future, the identification of new anti-trypanosmal compounds will be dependent upon research on biochemical pathways that are unique to trypanosomes. In *T. b. brucei*, for instance, the trypanocidal action of two chemically unrelated compounds has recently been shown to be mediated by interference with biochemical pathways involved in purine metabolism (Byers *et al.*, 1992; Carter and Fairlamb, 1993). Since an inability to synthesize purines *de novo* is common to trypanosomes (Hassan and Coombs, 1988), research on development of purine analogues may be a promising area in the future for developing trypanocides. The unique carbohydrate metabolism and energy production pathways in African trypanosomes (Opperdoes, 1987) also provide several novel targets for chemotherapeutic intervention (Sogin *et al.*, 1986; Misset and Opperdoes, 1987).

Efforts to ellucidate the metabolic pathways of polyamine synthesis have been most productive in the last decade, resulting in the identification of targets for new anti-trypanosomal compounds such as DL-α-diflouromethyl ornithine (DFMO), which is now in extensive clinical trials to treat human sleeping sickness (Pepin *et al.*, 1991). The United States Food and Drug Administration has, in November 1990, registered the drug for gambiense sleeping sickness, and thus for the first time in 40 years a new and effective drug is available for the treatment of African trypanosomiasis. Despite the success of DFMO, its use is limited in humans and impractical for animals because of its complicated mode of application involving high doses given several times daily intravenously, or orally for at least two weeks (Kuzoe, 1991).

1.3 The genome of *Trypanosoma brucei*

1.3.1 Chromosome number

Trypanosomes lack histone H1 and so their chromosomes do not condense into cytologically recognisable structures at metaphase during the cell division cycle (Vickerman and Preston, 1970). Therefore, cytological staining techniques cannot be used in determining the chromosome number. However, the application of pulsed field gel electrophoresis (PFGE) (Schwartz and Cantor, 1984) initially to *T. brucei,* and subsequently to other trypanosomes, has contributed greatly to the present information on trypanosome karyotypes. Early investigations revealed that there are large differences between species in their molecular karyotypes (van der Ploeg *et al.*, 1984a). Furthermore, trypanosome clones from the same serodeme can differ in their karyotypes (Bernards *et al.*, 1986). By PFGE, the trypanosome DNA can be classified into three general classes of chromosomes (van der Ploeg *et al.*, 1984b; Majiwa *et al.*, 1986b); the genome of *T. b. brucei*, for example, is organised into 100 minichromosomes ranging in size from 50 to 100 kbp, intermediate size chromosomes between 150 to 800 kbp, and the large chromosomes that are greater than 900 kbp in size. Recently, van der Ploeg *et al.* (1990) showed a total of 18 individual large chromosomes up to 5.7 mbp in size by separating the chromosomes using a range of PFGE conditions. Furthermore, by determining the chromosomal location of different genetic markers, it was demonstrated that 14 of these chromosomes could be organised into seven pairs of homologous chromosomes (van der Ploeg *et al.*, 1990). In most instances the putative chromosome homologues differed in size by about 20%. In some cases, however, non-homologous chromosomes co-migrated as a single band, and some DNA remained at, or close, to the well of the pulse field gels. As yet there is no evidence that the non-migrating DNA represents very large chromosomes; van der Ploeg *et al.* (1990) suggest that this DNA represents different DNA molecules, among which are the kinetoplast DNA network (van der Ploeg *et al.*, 1984a), as well as chromosome-sized DNA molecules which are non specifically trapped.

1.3.2 Ploidy

The size of the genome of one stock of *T. brucei* has been estimated to be 7.4 X 10^7 base pairs per diploid genome (Borst *et al.*, 1982). Evidence based on isoenzyme studies (Tait, 1980, 1983), analysis of restriction fragment length polymorphisms (RFLPs) (Gibson *et al.*, 1985), DNA content with kinetic complexity (Borst *et al.*, 1982), and measurements of DNA content by cytophotometry (Entilis *et al.*, 1987), indicates that bloodstream forms of trypanosomes are diploid. Extension of flow cytometry to other life-cycle stages of trypanosomes showed that each stage has an identical DNA content and, by analogy to blood -stream forms of *T. b. brucei*, are thus also diploid (Shapiro, 1984; Sternberg *et al.*, 1988a). The direct evidence for the existence of diploidy, and a system of gene exchange, has been convincingly obtained following the deliberate transmission of two different *T. brucei* subspecies through the tsetse fly vector (Jenni *et al.*, 1986). The hybrid progeny of this cross fell into two categories: (1) subtetraploid heterokaryons, which may have arisen from a simple fusion event and which have an unstable DNA content (Jenni *et al.*, 1986; Pandavoine *et al.*, 1986; Wells *et al.*, 1987), and (2) diploid hybrids showing Mendelian inheritance of some characters, implying the involvement of meiosis at a stage as yet undetermined during cyclical development in the tsetse fly (Le Page *et al.*, 1988; Sternberg *et al.*, 1988b; Tait *et al.*, 1988).

Studies on the inheritance of karyotypes following genetic exchange has revealed that minichromosomes of hybrids are principally composed of a mixture of the parental ones. In addition, there are minichromosomes of non parental sizes and some parental chromosomes that are absent (Wells *et al.*, 1987). These observations were made in hybrids with elevated DNA contents compared to the parental strains, and are now believed to be the product of an irregular mating event (Wells *et al.*, 1987). Analysis of intermediate chromosomes has also revealed their non-Mendelian manner of inheritance (Le Page *et al.*, 1988; Gibson, 1989), where some hybrids inherit all the intermediate chromosomes from one parent, while others inherit variable numbers of intermediate

chromosomes from both parents. These results suggest that the intermediate chromosomes are not segregated as diploid homologues. They are therefore considered as haploid chromosomes, inherited in a random manner (Tait *et al.*, 1993). Investigations on the inheritance of the larger chromosomes (Gibson, 1989; Gibson and Garside, 1991; Tait *et al.*, 1993) has convincingly shown their segregation and recombination to be as in a normal diploid Mendelian genetic system. The results of such experiments have, however, shown that genetic exchange is not obligatory, as only a limited number of flies harbouring mature infections produce hybrid trypanosomes (Tait and Turner, 1990), with between 10 and 100% of clones being identical to one or other of the parents (Gibson *et al.*, 1989; Sternberg *et al.*, 1989). To date, the available data also suggest that the genetic exchange systems in *T. brucei* can involve both cross and self-fertilization (Tait *et al.*, 1993). There remain however a number of unanswered questions regarding genetic exchange in *T. brucei*. Firstly, since there is no evidence for the occurrence of haploid gametes at any stage of the trypanosome life-cycle (Sternberg *et al.*, 1988b; Tait *et al.*, 1988), the stage at which mating occurs has yet to be determined. Secondly, it is not known whether meiosis results in haploid gametes which then fuse, or whether meiosis occurs after fusion of the diploid parental cells.

1.3.3 DNA rearrangements in African trypanosomes

1.3.3a Antigenic variation

Antigenic variation, in the manner and extent that it occurs in African trypanosomes, is unique to these organisms and appears to be the primary mechanism for evasion of the host's immune response (Cross, 1990). Antigenic variation is manifested through frequent gene rearrangements within the trypanosome genome, enabling the trypanosomes to evade the immune system of their hosts by the sequential expression of variant surface glycoproteins (VSGs) (Vickerman, 1969, 1978). The VSG forms a homogenous dense surface coat, accounting for about 10% of the total protein in bloodstream forms of *T. brucei* (Cross, 1975); usually only one VSG is expressed at any one time, covering the entire outer trypanosome membrane.

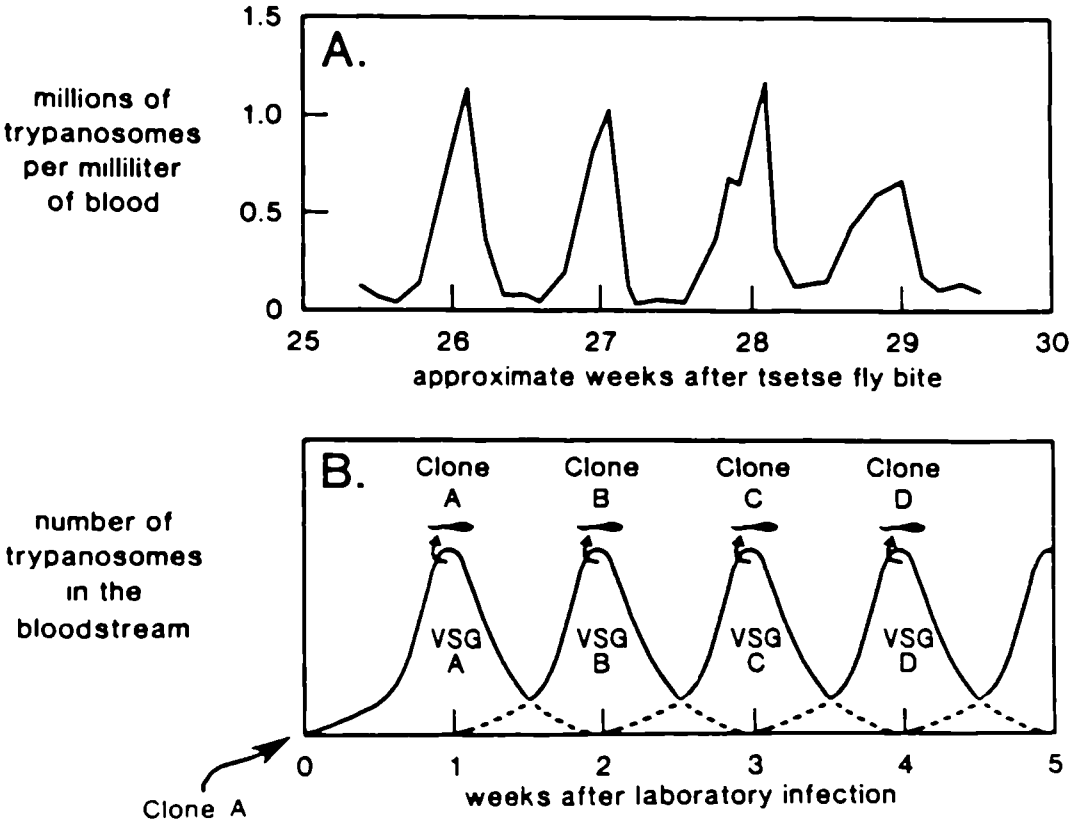
The classically described course of a trypanosome infection is that of a relapsing parasitaemia in which successive waves of parasites contain trypanosomes with different VSGs. Most of the parasites in each wave are destroyed by host antibodies against these VSGs. A small number of trypanosomes survive, however, because individual parasites from the population switch spontaneously from the expression of one VSG to another (Miller and Turner, 1981). Since different VSG variants generally differ dramatically in amino acid sequence (Bridgen, Cross and Bridgen, 1976; Vickerman, 1978; Johnson and Cross, 1979), new antibodies must be raised against the VSG of the switched population and its descendants, enabling the trypanosome population as a whole to stay one step ahead of the host's immune response. Figure 2 presents a schematic summary of antigenic variation. It has been demonstrated that a single trypanosome infection in an experimental rabbit can result in the successive expression of over 100 different antigenic types (Capbern *et al.*, 1972). Furthermore, DNA hybridisations have revealed that each trypanosome has a large repertoire, estimated at about 1,000, of silent, or basic-copy VSG genes (van der Ploeg *et al.*, 1982a). Recent data show that this finite repertoire can be further amplified by mosaic gene formation (Barbet and Kamper, 1993) and point mutation (Baltz *et al.*, 1991), producing an almost limitless capacity to vary.

When a tsetse fly bites an infected animal, trypanosomes can be ingested with the blood meal. In the midgut of the insect, *T. congolense* and *T. brucei* differentiate into procyclic or procyclic-like forms and lose the VSG, but express another membrane protein termed the procyclic acidic repetitive protein (PARP) (Clayton *et al.*, 1990) or procyclin (Overath *et al.*, 1983; Roditi *et al.*, 1987, 1989). In the midgut, the trypanosomes undergo several transformations before a final differentiation in the salivary glands or the proboscis (depending on the life-cycle of the species) to metacyclic forms. The metacyclic forms, which are infective for mammalian hosts, re-acquire a VSG coat but exhibit a limited repertoire of metacyclic VSGs. On inoculation into the mammalian host, the metacyclics switch to the expression of bloodstream VSGs, the first of which is often the VSG originally ingested by the fly (Hajduk and Vickerman, 1981).

Figure 2

(A) A schematic demonstration of the successive waves of parasitemia during a *Trypanosoma brucei rhodesiense* infection of a human patient (redrawn from Donelson, 1989).

(B) A schematic demonstration of a controlled *T. b. rhodesiense* infection in an immunocompetent laboratory animal. A single trypanosome expressing VSG_A (clone A) is injected into the blood and gives rise to the first peak of parasitaemia, in which all of the parasites are expressing VSG_A. The host immune system kills most of the parasites during the next few days, but one or more trypanosomes switches to the expression of VSG_B, which is not recognised by the anti-VSG_A antibodies. These trypanosomes begin the next wave of the process again. From each peak of parasitaemia individual trypanosomes can be cloned to investigate the VSG structure and the molecular mechanism of antigenic variation. Since the switch to a new VSG occurs spontaneously and individual trypanosomes can have different growth rates. the synchrony of the parasitaemia peaks is lost later in the infection.


A.
millions of trypanosomes per milliliter of blood
1.5
1.0
0.5
0
25
26
27
28
29
30
approximate weeks after tsetse fly bite
B.
number of trypanosomes in the bloodstream
Clone A
Clone B
Clone C
Clone D
VSG A
VSG B
VSG C
VSG D
0
1
2
3
4
5
weeks after laboratory infection
Clone A

1.3.3b Rearrangement of VSG genes

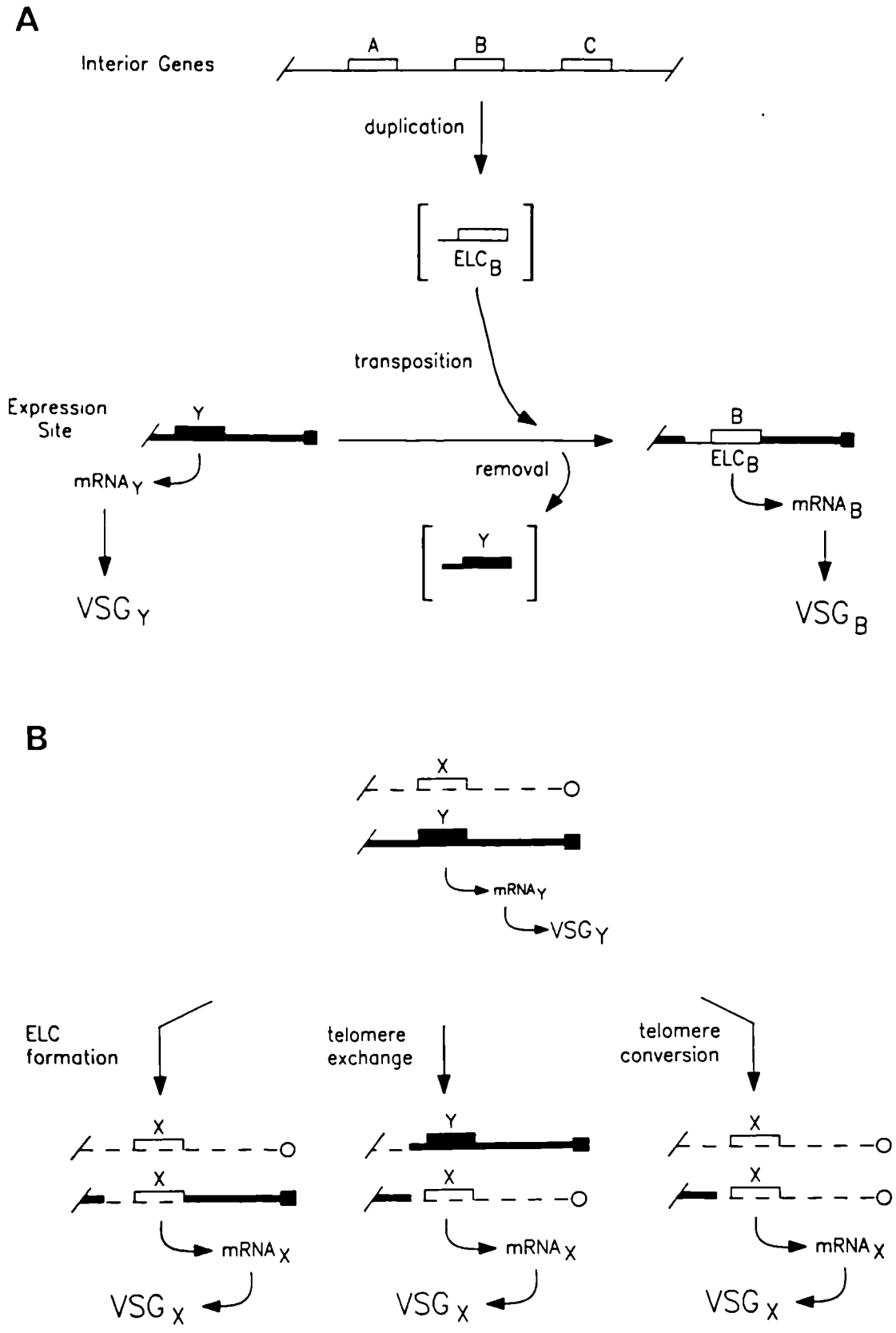
VSG genes are situated at one or two general chromosomal locations; either at an internal DNA site or near a double-strand DNA break which has been identified as a chromosomal end or telomere (Williams *et al.*, 1979, 1981; De Lange and Borst, 1982; Young *et al.*, 1982). An expressed VSG gene is invariably localised in a telomeric expression site (ES) (De Lange and Borst, 1982). Hybridisation studies suggest that there may be as many as 20 different ESs (Cully *et al.*, 1985); expression sites occur on chromosomes of both intermediate and large sizes. Only one antigen is generally expressed at any time, although several telomeres can be used alternately for antigen gene expression. Antigenic switching can occur by replacement of the VSG gene in the active ES by another previously silent or basic copy VSG gene. The basic copy must be duplicated and translocated to an ES before their transcription can occur. The duplicated VSG gene was named an "expression linked copy" (ELC) by Hoeijmakers *et al.* (1980). This duplicative translocation is a gene conversion event (Bernards *et al.*, 1981; van der Ploeg *et al.*, 1982b; Michels *et al.*, 1983; Pays *et al.*, 1983), a term used to describe the non-reciprocal transfer of nucleotide sequences from one DNA region to another. The ELC was found to be sensitive to DNase I, suggesting that it is the gene undergoing transcription (Pays and Steinert, 1981). The telomere-linked basic copy VSG genes, on the other hand, need not undergo gene conversion to be transcribed. They have the potential to be expressed *in situ*, even though they sometimes do undergo duplication prior to their transcription (Laurent *et al.*, 1983; Pays *et al.*, *1983*; Buck *et al.*, 1984; Young and Williams, 1984; Pays and Steinert, 1988). Attempts to understand the differences involved between the two basic observations of VSG-gene location and VSG-gene transcription have been the source of much of the interest in trypanosome antigenic variation by molecular biologists. The possible rearrangements associated with the expression of VSG genes are summarised in Figure 3a and 3b (Donelson, 1989). The one factor that has stood the test of continued experimentation is that it is necessary, but not sufficient, for a VSG to be linked to a telomere if it is to be transcribed. Furthermore, several lines of evidence indicate that there are several different telomeres at which a

Figure 3

(A) Summary of the activation of VSG genes that are located at interior chromosomal sites (redrawn from Donelson, 1989).

The VSG genes, represented by A, B and C can only be transcribed after undergoing a duplicative transposition to form an expression-linked copy (ELC) gene in an expression site linked to a telomere (indicated by the black square). The transposed segment usually includes the VSG coding region and 1 to 2 kb in front of the gene. Often the VSG previously in that expression site, gene Y, is deleted from the genome during the formation of the new ELC. Sometimes, however, the ELC goes to another telomere-linked expression site and gene Y lingers as an unexpressed telomere-linked gene in the old expression site.

(B) Summary of the DNA rearrangements associated with the expression of VSG genes that are already located near telomeres. At the top of the diagram, gene X is an unexpressed VSG near the telomere (open circle) of a chromosome (dashed line). Gene Y is undergoing transcription from an expression site near the telomere (black square) of another chromosome (solid line). The left pathway shows the formation of an ELC of gene X at the expression site on the solid chromosome with the concomitant deletion of gene Y. The centre pathway illustrates the activation of gene X, and inactivation of gene Y, by a reciprocal exchange of the telomeric regions, including genes X and Y, between the dashed and solid chromosomes. The right pathway shows the displacement of the telomere, plus gene Y, on the solid chromosome with a duplicated copy of the telomere and flanking region of the dashed chromosome. This telomere conversion event results in the activation of the duplicated gene X on the solid chromosome.


A
Interior Genes
A
B
C
duplication
ELC B
transposition
Expression Site
Y
mRNA Y
VSG Y
removal
Y
B
ELC B
mRNA B
VSG B
B
X
Y
mRNA Y
VSG Y
ELC formation
telomere exchange
telomere conversion
X
X
mRNA X
VSG X
Y
X
mRNA X
VSG X
X
X
mRNA X
VSG X

VSG gene can be transcribed (Longacre *et al.*, 1983; Pays *et al.*, 1983), although, the factor(s) that activates one, and only one, of these telomere-linked VSG genes to the exclusion of others remains unknown. The recent finding of relatively frequent DNA rearrangement and recombinational events at ES inactivation, in a region encoding VSG gene ES promoter(s) (Gottesdiener *et al.*, 1991, 1992), suggests their possible role in ES control.

1.3.4 Transcription of VSG genes

In trypanosomes, several genes exist in tandem arrays whose primary transcripts span several gene copies. This has been shown for the *T. brucei* tubulin (Imboden *et al.*, 1987; Mulich and Boothroyd, 1988), actin (Ben Amar *et al.*, 1988) and calmodulin (Tschudi and Ullu, 1988) genes. Genes with different specificities may belong to the same polycistronic transcription unit (Ben Amar *et al.*, 1988).

VSG expression sites are also long multigene transcription units (Cully, Ip and Cross, 1985; Kooter *et al.*, 1987; Murphy *et al.*, 1987; Alexandre *et al.*, 1988; Pays *et al.*, 1989). The VSG gene is the last coding sequence of a large transcription unit, which also includes other genes. The best studied of these is a family of genes called the expression site associated genes (ESAGs) (Cully, Ip and Cross, 1985; Cully *et al.*, 1986; Kooter *et al.*, 1987). Transcription of these genes is simultaneously regulated with the downstream VSG gene in the expression site. If the VSG gene is transcribed, then the corresponding ESAG is also transcribed. If the VSG is not transcribed, then the neighbouring ESAG is not transcribed. Salmon *et al.* (1994) have recently demonstrated that ESAGs 6 and 7 most likely represent the transferrin receptor in *T. brucei*.

It is believed that expression of VSG genes is controlled at the level of transcription since VSG-specific cDNA hybridises under high stringency only to RNA from trypanosomes expressing that VSG and not to RNA from trypanosomes producing other VSGs (Hoeijmakers *et al.*, 1980; Pays *et al.*, 1980). More recently, however, post-transcription control of expression has been reported; Pays *et al.* (1990) have demonstrated that the beginning of the AnTat 1.1 VSG gene expression site, about 45 kilobases upstream from the antigen gene, remains transcribed in procyclic forms. The

permanent activity of the promoter can readily account for the systematic reappearance of the VSG antigen type present in the ingested bloodstream form following cyclical transmission through the tsetse fly. Investigations by Pays *et al.* (1989) indicate that the abortive transcription of the VSG gene expression site appears to be linked to RNA processing abnormalities.

1.3.5 Discontinuous transcription

Other than elucidating the molecular mechanism of antigenic variation, studies on expression of VSG genes shed light on other molecular aspects of gene regulation in trypanosomes. Discontinuous transcription, which is a process by which a mature mRNA is derived from two independent transcription units and events, is one such process. Historically, the study of discontinuous transcription started with the analysis of the 5' end of a VSG mRNA, and comparing that with the corresponding genomic ELC. This produced the surprising result that the 5'-most 35 nucleotides of the mRNA were not contiguous with the genomic copy of the VSG gene (Boothroyd and Cross, 1982). In addition, it was found that VSG mRNAs from different telomeric expression sites also possessed the identical 35 nucleotides at their 5' ends (Boothroyd and Cross, 1982). Further investigations led to the finding that all trypanosome mRNAs have the same sequence at their 5' ends, now known to be 39 nucleotides (Perry *et al.*, 1987). This conserved 39 nucleotide sequence has been called the spliced leader or miniexon sequence (Nelson *et al.*, 1982). About 200 copies of the miniexon sequence are present in the *T. b. brucei* genome, of which the majority are clustered in 1.35 kbp tandem repeats (Michels *et al.*, 1983; Nelson *et al.*, 1982; Dorfman and Donelson, 1984). Demonstration that the primary transcript of the miniexon is 140 nucleotides with a miniexon at its 5' end (Campbell *et al.*, 1984a; Kooter and Borst, 1984; Milhausen *et al.*, 1984), and that VSG genes may be transcribed from chromosomes that do not carry a copy of the miniexon repeat (van der Ploeg *et al.*, 1984c; Guyaux *et al.*, 1985), proved that the two exons are transcribed discontinuously. Kooter and Borst (1984) have shown that different RNA polymerases are responsible for transcription of the miniexon and the

VSG gene, since they differ in sensitivity to inhibition by α-amanitin. It appears that during the maturation of trypanosome mRNAs the miniexon is transferred from the miniexon-derived RNA precursor to pre-mRNAs transcribed elsewhere in the genome by a trans-splicing mechanism (Guyaux *et al.*, 1985; Borst, 1986; Murphy *et al.*, 1986; Sutton and Boothroyd, 1986; Ralph *et al.*, 1988).

1.4.1 Understanding drug resistance at the molecular level

The only currently available means for control of African trypanosomiasis are vector control, drug treatment, and in the case of bovidae, the use of trypanotolerant livestock. These three strategies have many shortcomings. For instance, there is yet no obvious way of achieving sustainable control of trypanosomiasis by effectively controlling the 31 species and sub-species of tsetse flies in their different habitats across the tsetse-infested zone of Africa.

As far as drug treatment is concerned, the problem of drug resistance in certain forms of animal trypanosomiasis is now so acute that it poses a grave threat to future control of trypanosomiasis using the existing repertoire of drugs (Leach and Roberts, 1981). The level of drug resistance which becomes particularly important in the field is resistance to the standard curative doses (Hawking, 1963).

The optimum solution for control of trypanosomiasis would be the development of a cheap, simple vaccine with a wide range of protection against different species of trypanosomes. However, as Stephen (1986) states, "The tantalizing prospect of producing a vaccine which would protect animals against the establishment of parasitaemic infections has cost a good sized fortune; expended thousands of person-hours; killed untold numbers.of experimental animals; filled hundreds of pages of scientific journals with articles that are seldom quoted; led dozens of scientists to jump onto the vaccine band-wagon and just as smartly off again in a state of frustration". There is truth in this statement, but when one considers the annual economic and human loss due to trypanosomiasis his point of view appears extreme. Although the information

presently available on the mechanisms of antigenic variation make it highly unlikely that a vaccine against trypanosomes can be developed based on these molecules, the potential for a vaccine based on invariant surface molecules, or pathogenic molecules secreted by these parasites, still remains. Molecular studies on trypanosomes are also contributing to our understanding of unique DNA structures and novel mechanisms of gene regulation, but are yet to have an impact on the disease caused by these parasites.

Lastly, there is an urgent requirement for detailed information on the genetic basis of resistance to trypanocidal drugs and on the mode of action of the drugs. Such information would be invaluable as it would allow the development of rapid diagnostics for drug resistance, thereby enabling the potential development of new, urgently required, therapeutic strategies to complement and improve the presently used regimens. This is exemplified in cancer research: advances have recently been made in understanding the molecular mechanisms of drug resistance in cancer cells. These investigations have contributed to improvements in the chemotherapeutic strategies for the treatment of cancer (Endicott and Ling, 1989).

1.4.2 Mechanisms of resistance

Comparisons of drug resistance mechanisms in bacteria, insects, plants and man reveal several common features, in that certain classes of proteins can be responsible for conferring resistance to various drugs in a diverse number of organisms.

Drug resistance can arise as a consequence of various biochemical mechanisms:
(a) Reduced drug delivery, which is a consequence of factors independent of the target cell. In mammals, blood circulation is a factor of crucial importance in the delivery of the drug to the target cells. Also, the brain, for example is difficult to target in chemotherapy due to the blood brain barrier (Jennings *et al.*, 1979).
(b) Decreased or defective transport of drugs into cells is a general mechanism of resistance, the importance of which varies considerably, depending on both the lipophilicity of the drug and the structure of the cell membrane (Goldenberg and Begleiter, 1984).

(c) Increased drug efflux by proteins which act as membrane-located, energy-dependent, efflux pumps (Chopra, 1984, 1986).

(d) Drug metabolism due to enzymes which can act by either potentiating or reducing the toxicity of the drugs (Arca *et al.*, 1988).

(e) Reduction in the active drug concentration achieved by drug sequestration. This occurs by increased intracellular drug binding, thus preventing drug interaction with a target site (Kelly *et al.*, 1988).

(f) Increased intracellular concentration of the target site(s) (Alt *et al.*, 1978).

(g) Structural alterations of the cellular target(s) in a manner which reduces the affinity of the drug for the target site(s). This type of drug resistance is usually the result of point mutations in the structural gene(s) encoding the target, and is usually associated with drugs whose target is well defined (Simonsen and Levinson, 1983).

(h) By-passing of a metabolic block through the production of a novel protein. In this instance the new protein, which has the same function as the target protein, no longer interacts with the drug (Smith and Amyes, 1984).

(i) Increased rates of repair of damaged target site(s). This represents an important mechanism of resistance to alkylating agents and particularly radiation (Walker, 1985). DNA repair has been implicated in resistance to chemical carcinogens and a wide range of anti-cancer drugs (Kessel, 1986).

It is now realised that resistance to a particular drug can be achieved by a multitude of mechanisms. Recent work has shown that where drug resistance is due to changes in either drug transportation or detoxification, protection against more than one chemical is invariably observed. This can be manifested as cross-resistance to either structurally related or structurally unrelated chemicals. The latter phenomenon has been referred to as multidrug, or pleotropic, drug resistance (Hayes and Wolf, 1990).

The molecular changes which account for drug resistance include gene amplification, gene deletion, point mutations, the loss of cis-acting regulatory elements, the loss or dysfunction of trans-acting factors, transcriptional activation, hypo' or hyper' methylation or production of heat shock proteins which have been implicated in

protection against both physiological and chemical stress (Li *et al.*, 1982). All these effects could occur within genes directly involved in combating the cytotoxic compounds, or in genes involved in regulation or processing of genes combating the cytotoxic compounds. In theory, the number of ways in which cells could become drug resistant are almost limitless.

1.4.2a Multidrug-resistance

Since the early 1980s, considerable attention in cancer research has focused on multidrug-resistance (MDR), *where tumour cells selected for resistance to a single agent*, such as vinblastine or actinomycin D, display high levels of resistance to a broad range of structurally diverse drugs (Gerlach *et al.*, 1986). The basis of this multidrug resistance appeared to be due to reduced cellular accumulation of drugs resulting from an energy-dependent increase in drug efflux (Dano, 1973; Inaba *et al.*, 1979). The MDR phenotype could be reversed by a variety of pharmacological agents which promote drug accumulation. These include calcium channel blockers such as verapamil and calcium-calmodulin antagonists (Kessel, 1986).

In 1976, Juliano and Ling found that several MDR Chinese hamster ovary (CHO) cell lines expressed an increased amount of a 170 kilodalton membrane glycoprotein, termed the P-glycoprotein. They further showed that the level of resistance to colchicine correlated to the level of expression of the P-glycoprotein and that there was an inverse relationship between the accumulation of certain vinka alkaloids and expression of P-glycoprotein, suggesting that this membrane protein may be involved in drug transport. The mammalian P-glycoprotein was found to be encoded by a multigene family (Gottesman and Pastan, 1988; Endicott and Ling, 1989). On the basis of similarities between sequences, the P-glycoprotein genes in mammals have been divided into two major classes: *mdr*1 and *mdr*2. The *mdr*1 class of genes have been further subdivided into *mdr*1a and *mdr*1b (Kane and Gottesman, 1989).

Structure and function of the *mdr*1 gene

The human *mdr*1 gene, encodes a 1280 amino acid sequence consisting of two homologous halves (Chen *et al.*, 1986). Each half of the protein includes a hydrophobic region with six predicted transmembrane segments and a relatively hydrophillic region which contains consensus sequences for a nucleotide-binding site. Each half of the protein has a high degree of primary amino acid sequence and structural similarity with bacterial transport proteins, particularly hemolysin B (Higgins *et al.*, 1986). The hypothesised energy-dependent efflux pump function of P-glycoprotein received indirect confirmation from biochemical studies in which P-glycoproteins purified from a human leukemia cell line were shown to bind drugs and to hydrolyse ATP *in vitro* (Cornwell *et al.*, 1986, 1987; Hamada and Tsuruo, 1988).

Homology with bacterial transport proteins and the presence of potential channel-forming membrane-spanning segments represents a structural motif which is a characteristic feature of pore-forming proteins (Henderson and Maiden, 1987). The presence of two highly conserved ATP-binding regions is consistent with a role for an active efflux pump, responsible for the removal of drugs from multidrug-resistant cells. However, more direct evidence has been obtained from investigations demonstrating that transfection of *mdr*1a and *mdr*1b cDNAs into drug-sensitive cells produces the MDR phenotype (Gros *et al.*, 1986; Croop *et al.*, 1987; Veda *et al.*, 1987; Pastan *et al.*, 1988). Surprisingly, not all *mdr* genes have the ability to produce the MDR phenotype. Finally, whilst the P-glycoprotein encoded by the *mdr*1 class of genes can confer drug resistance, the *mdr*2 class of genes encode a protein whose physiological function is presently unknown. It is now evident that increases in either the expression of *mdr*1 genes, or changes that affect their function as efficient efflux pumps, are of central importance in producing the MDR phenotype .

Amplification of specific genes has been implicated in a variety of adaptive responses of cells to environmental stresses (Schimke, 1984). An early link was established between the MDR phenotype and gene amplification as highly multidrug-resistant cell lines revealed cytogenetic changes that are hall-marks of gene amplification

(Stark, 1986). The term gene amplification is usually applied to changes in relative copy number of a limited portion of the genome; specific amplification of genes encoding the drug target is one means by which cells acquire resistance to the action of selective agents (Schimke, 1984; Stark and Wahl, 1984). Following the cloning of *mdr* genes, examination of the DNA of highly resistant cells by Southern blotting revealed a marked increase in the copy number of the *mdr* genes in many of these cell lines.

Other than gene amplification, Choi *et al.* (1988) have shown that point mutations in the human *mdr*1 gene can also produce a P-glycoprotein that is more effective in its efflux property.

Gene amplification and point mutations are not the only mechanisms which can result in an MDR phenotype. Increased expression of P-glycoprotein mRNA has also been observed in cell lines displaying an MDR phenotype, in the absence of amplification of *mdr* genes (Shen *et al.*, 1986).

The expression of P-glycoproteins in normal human tissues has been recently determined using monoclonal antibodies and DNA probes (Endicott and Ling, 1989). These studies have shown that there is expression of P-glycoproteins in normal human liver, kidney, pancreas, stomach, intestine, colon and spleen. Many of these organs play a role in chemical detoxification, suggesting a possible normal role for P-glycoproteins in transport and secretion. It is suggested that P-glycoproteins may transport different compounds in different tissues, or, by analogy to its role in drug transport in MDR cells, it may be a common route for the export of toxic compounds (Endicott and Ling, 1989).

Lastly, P-glycoproteins are widely distributed in nature. For instance, in addition to mammals, other eukaryotes such as the malaria parasite *Plasmodium falciparum* (Foote *et al.*, 1989; Wilson *et al.*, 1989), the yeast *Saccharomyces cerevisiae* (McGarth and Varshavsky, 1989) and the kinetoplastid protozoan flagellate *Leishmania tarentolae* (Ouellette *et al.*, 1990) also possess *mdr*1-like genes.

1.4.2b Drug resistance in Plasmodium and Leishmania

The appearance of chloroquine-resistant (CQR) *Plasmodium falciparum* has thwarted the goal of global control of malaria. Chloroquine is a diprotic base and appears to raise the pH of the parasite food vacuole and interfere with its function (Yayon, 1984). It is suggested that the plasmodium parasite has a chloroquine-concentrating mechanism that is absent from the mammalian acid vesicles. This mechanism accounts for the relative inability of the parasite to buffer a base load in the presence of chloroquine, and is thus responsible for the selective action of chloroquine against it (Krogstad, 1987).

Experiments have demonstrated that the CQR phenotype is associated with a lower accumulation of chloroquine than that observed in sensitive parasites (Yayon *et al.*, 1984). This has been attributed to the resistant parasite having an efflux mechanism by which chloroquine is released at a rate 50-times that of susceptible parasites (Krogstad, 1987). The phenomenon of chloroquine efflux, and resistance, in *P. falciparum* is energy dependent and can be partially blocked by calcium channel antagonists (Martin *et al.*, 1987), similar to the MDR phenotype characterised in mammalian cells (Fojo *et al.*, 1985). These observations initiated the use of sequence conservation in the *mdr* gene family to identify *P. falciparum mdr* homologues (Wilson *et al.*, 1989). Two genes were identified and designated Pf*mdr*1 and Pf*mdr*2. Foote *et al.* (1989) demonstrated that the malaria *mdr* copy number and transcripts are amplified in some chloroquine-resistant parasites but not in any of the sensitive isolates examined. Furthermore, as for the mammalian MDR phenotype, specific point mutations in the *mdr* gene were also implicated in CQR (Foote *et al.*, 1990). However, genetic crosses between CQR and chloroquine-susceptible strains indicated that there was no direct linkage between CQR and the plasmodium P-glycoprotein gene homologues (Wellems *et al.*, 1990). This suggests that if mutations in Pf*mdr* genes play a role in CQR, this is likely to be just a component of a multigenic process (Foote *et al.*, 1990; Newbold, 1990).

In *Leishmania* spp, the H-circle amplicon was first observed in *L. major* lines resistant to one folate analog methotrexate (MTX) (Coderre *et al.*, 1983). It was

subsequently observed in lines selected with terbinafine and primaquine (Ellenberger and Beverley, 1989), and in two natural isolates of *L. tarentolae* (Petrillo-Peixoto and Beverley, 1988). The occurrence of H-circles in these diverse settings suggests that amplification may confer simultaneous resistance to many drugs, as with the MDR phenotype in mammalian cell lines (Schimke, 1984). Ouellette *et al.* (1990) have shown that the amplified H circle of MTX-resistant *L. tarentolae* contains a P-glycoprotein gene (*ltpgpA*). Callahan and Beverley (1991) demonstrated, by transfection, that amplification and over-expression of the *ltpgpA* gene confers resistance to *L. major* against arsenite and particular antimonial agents; drugs that are not typically transported by human P-glycoproteins (Endicott and Ling, 1989). In addition, *L. tarentolae* lines with the H amplification were not resistant to a variety of compounds considered substrates of the mammalian P-glycoprotein (Ouellette *et al.*, 1990). These experiments were unable to demonstrate that *ltpgpA* conferred an MDR phenotype as in mammals. Callahan and Beverley (1991) showed that *ltpgpA* proteins represent a functionally distinct group of P-glycoproteins that exhibit a substrate specificity similar to that of prokaryotic heavy metal pumps. More recently, Henderson *et al.* (1992) demonstrated that an MDR phenotype as in humans, was conferred to *L. donovani* by amplification of a gene homologous to the mammalian *mdr*1 gene (*ldmdr1*). Henderson *et al.* (1992) also demonstrated that the *ldmdr1* locus was unrelated to H-region amplification, thus showing that *Leishmania* species can acquire two dissimilar MDR phenotypes associated with the amplification of genes highly homologous to the mammalian *mdr* gene.

Although the H-region was first described in MTX-resistant *L. major*, transfecting with *lpgpA* did not confer resistance to antifolates, and the MTX resistance is not reversed by calcium channel blockers verapamil (Callahan and Beverley, 1991). These discrepancies have been explained with the recent discovery of a new gene called *ltdth* in *L. tarentolae* (Papadopoulou *et al.*, 1992), that by transfection has been shown to confer high levels of resistance to different antifolates tested. Such investigations suggest that a large number of loci mediating a wide spectrum of reactions can be amplified under

appropriate conditions, suggesting that no particular class of enzymes or loci are especially prone to amplification (Beverly *et al.*, 1988).

Over-expression of genes as a consequence of gene amplification has been reported in several MTX-resistant lines of *Leishmania* species, beginning with *L. major* (Coderre *et al.*, 1983). Further studies showed a correlation between resistance to MTX and elevated DHFR activity, which led to the isolation of the gene encoding the target of MTX, the dihydrofolate reductase thymidylate synthase (DHFR-TS) gene product, a novel bi-functional protein (Beverley *et al.*, 1987).

It is now known that there are at least three mechanisms by which resistance to MTX occurs in *Leishmania* (Beverley *et al.*, 1988). The mechanisms identified are: (1) amplification of the DHFR-TS gene, encoded on a segment of DNA termed the R region, (2) decreased cellular MTX accumulation, and (3) amplification of a region of DNA termed the H-region, which is completely distinct from the R-region. Ellenberger and Beverley (1987, 1989) have shown that these three mechanisms can occur independently or in combination in the same cell, indicating that the parasite has diverse mechanisms it can marshal to survive chemotherapeutic attack. Amplified DNA such as the R region occurs generally as extrachromosomal circular DNA, and can be readily detected due to a high copy number and unusual mobility properties in conventional or pulsed-field electrophoresis gels (Beverley, 1988). Multiple *Leishmania* spp. bearing amplified R-regions have been derived, and the location of the amplified segments of the chromosomal DNA have been determined (Beverley *et al.*, 1984; Beverley, 1991). The region amplified always includes the coding region for DHFR-TS, as well as substantial flanking DNA ranging in size from 30-90 kbp.

Mutations in the DHFR-TS gene have been implicated in pyrimethamine resistance in *Plasmodium falciparum*. Pyrimethamine, a substrate analogue, binds to, and inhibits, the dihydrofolate reductase activity of the essential bifunctional enzyme DHFR-TS (Ferone *et al.*, 1969; Garret *et al.*, 1984). It has been shown that a number of *P. falciparum* DHFR mutants exhibit the pyrimethamine resistance (Pyr^R) phenotype

(Banyal *et al.*, 1986; Zolg *et al.*, 1989). Inselburg *et al.* (1989) have also suggested that chromosome-size changes that contribute to either gene duplication or gene regulatory processes probably play an important role in the development of higher levels of Pyr^R. The same workers have also noted that maintaining a Pyr^R culture of *P. falciparum* in the absence of drug led to the selection or alternation of a parasite population with a diminished level of resistance.

The outcome of studies on Pyr^R has had a great impact on control of *P. faciparum*. For instance, it is now known that with control of pyrimethamine usage, the selective drug pressure that maintains some highly resistant organisms can be eliminated, thereby allowing the sensitivity to pyremethamine to be restored (Inselburg *et al.*, 1989).

1.4.2c Mechanisms of drug resistance in African trypanosomes.

To date, very little is known about the molecular mechanisms involved in drug resistance of trypanosomes. Recently, however, Carter and Fairlamb (1993) have reported that the *in vitro* trypanolytic effect of melarsen oxide, an arsenical used to treat human African sleeping sickness (Apted, 1980), can be specifically abrogated by adenine, adenosine and dipyridamole, all of which compete for uptake by an adenosine transporter. Their investigations have also shown that melarsen-sensitive trypanosomes have two high-affinity adenosine transport systems; a P1 type, which also transports inosine, and a P2 type, which transports adenine and the melaminophenyl arsenicals. These studies have shown that melarsen-resistant trypanosomes lack the P2 type adenosine transporter, suggesting that resistance to these arsenicals is due to loss of uptake.

By fluorescence analysis of the interaction of isometamidium with clones of *T. congolense*, Sutherland *et al.* (1991) have observed an inverse relationship between the intensity of trypanosome-associated fluorescence and the level of resistance to isometamidium expressed by the clones. More recently, Zilberstein *et al.* (1993) have demonstrated that the alterations in fluorescence that occur when isometamidium interacts with isometamidium-sensitive *T. congolense* are due to interaction of the drug

with an intracellular component(s). This work has also demonstrated that isometamidium is transported across the plasma membrane via a protein carrier. The role of these processes in drug resistance, however, are yet to be determined.

The work presented in this thesis was the result of an attempt to identify genes that may be involved in drug resistance in a multidrug-resistant *T. b. brucei*, CP (Chemotryp Project) 547 (CP 547). This isolate has been characterised with regard to its drug-resistance profiles both *in vitro* and *in vivo* (Zweygarth and Röttcher, 1988; Kaminsky and Zwegarth, 1989b; Kaminsky *et al.*, 1989). It was isolated in 1985 from a naturally infected cow in Jilib Somalia. It is a pleomorphic *T. b. brucei* (Zweygarth and Röttcher, 1988) and has been shown to be resistant to diminazene, homidium, isometamidium, quinapyramine, mel B and pentamidine, but fully sensitive to suramin. The drug-sensitivity profile of the stock has been shown to remain relatively stable *in vitro* conditions for at least 275 days (Kaminsky and Zweygarth, 1989 b). Table 7 (Zweygarth and Röttcher, 1988) shows the *in vivo* patterns of drug sensitivity of *T. b. brucei* stocks CP 462, a sensitive isolate, and CP 547, to currently used trypanocides. In the present work the sensitive control population used was *T. b. brucei* ILTat 1.4. In additional studies, the multidrug-resistant trait of CP 547 could not be reversed by verapimil (Kaminsky *et al.*, 1991), a drug that reverses the mammalian MDR trait. This observation suggests that, as for *Leishmania* (Henderson *et al*,. 1993) and possibly *P. falciparum*, trypanosomes may acquire MDR phenotypes that are dissimilar to the mammalian MDR phenotype.

In view of the problem of drug resistance and the difficulties and expenses involved in the development and introduction of new trypanocides, it is important to improve on the presently available drugs, both in terms of modifying them and/or using them in improved combinations. One of the strategies to combat resistance relies on reversal of resistance, which has recently been experimentally demonstrated for chroloquine resistance in human and rodent malaria (Krogstad, 1987). Such approaches could also be extended to control of trypanosomiasis, but require an intense knowledge both of the mode of action of the present drugs and the mechanisms by which

Table 7: **Patterns of drug sensitivity in mice of *T.b. brucei* stocks CP 462 and CP 547 to commercial trypanocides**

Drug[a]	Dose (mg/kg)	Results (No. cured/No. treated mice)[b]	
		CP 462	CP 547
Diminazene aceturate	6.3	7/8	-
	16	8/8	-
	50	-	0/8
	80	-	0/8(5)
Homidium chloride	6.25	8/8	-
	50	-	1/8
Isometamidium chloride	1	8/8	-
	3	8/8	0/8
	10	-	5/8
Quinapyramine sulphate	8	4/8	0/8
Suramin	5	8/8	8/8
Mel B	2.5	8/8	1/8
	10	-	8/8
Pentamidine isethionate	10	7/8	1/8
	20	8/8	4/8
	50	-	8/8

[a] All drugs were injected ip

[b] Number in parenthesis indicates deaths due to toxicity

trypanosomes can overcome the toxic effects of trypanocides. The initial objective of this study was to identify and characterise molecular differences between a multidrug-resistant isolate (CP 547), and a reference drug-sensitive population, ILTat 1.4. The aforementioned R- and H- region amplifications result in extrachromosomal circular DNA elements which are readily detected. In light of the involvement of such elements in drug resistance in *leishmania* spp., we were interested to determine whether similar amplified sequences could be identified in a multiple-drug resistant *T. b. brucei* field isolate, CP 547, and whether such elements could be used as DNA-based diagnostics for drug resistance. Finally, we were interested to determine whether gene amplification is a mechanism employed by drug-resistant trypanosomes to overcome the toxic effects of drug pressure as has been observed in *Leishmania* spp. (Segovia, 1994).

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials used:

Suppliers:

The following abbreviated forms are used for some suppliers:

Amersham:	Amersham International, Amersham, U.K.
BDH:	British Drug Houses, Poole, Dorset, U.K.
Bio-Rad:	Bio-Rad Laboratories, Richmond, California, U.S.A.
Boehringer:	Boehringer Mannheim, West Germany
BRL:	Bethesda Research Laboratories Inc, Gaithersburg, MD, U.S.A.
Sigma:	Sigma Chemical Company, Poole, Dorset, U.K.
NEB:	New England Biolabs, Beverley, MA, U.S.A.
Promega:	Promega Corporation, 2800 Woods Hollow Road, Madison, U.S.A.
S and S:	Schleicher and Schuell, Dassel, Germany

a)	Ordinary chemicals: All chemicals used were BDH or equivalent analytical grade
b)	Enzymes: NEB, BRL, Amersham, Promega.
c)	Animals: Balb C mice and Sprague Dawley Rats were both obtained from the animal breeding unit at ILRAD
d)	Miscellaneous X-ray film: Fuji Photo Film Co. Ltd., Japan

Polaroid film: Fabrique au Royaume-Uni par Polaroid (U.K.) Ltd.

Nytran membrane: from Schleicher and Schuell

Radiochemicals: Amersham

2.1.1 Buffers:

Abbreviations for some buffers referred to in the text:

CGA: Citrate glucose anticoagulant

100 mM trisodium citrate

40 mM glucose, (pH 7.7)

PSG: Phosphate saline glucose

57 mM Na_2HPO_4

2.6 mM $NaH_2PO_4(H_2O)_2$

42.8 mM NaCl

55.5 mM glucose

SSC: Saline sodium citrate

150 mM NaCl

15 mM trisodium citrate

prepared as a 20 X stock.

TBE: Tris borate EDTA

90 mM TRIS

90 mM boric acid

2.5 mM EDTA, (pH 8.0)

Prepared as a 10 X stock

T.E: Tris EDTA

10 mM Tris-HCl (pH 7.4)

0.1 mM EDTA, (pH 8.0)

TE: Tris EDTA

10 mM Tris-HCl (pH 7.4)

10 mM EDTA, (pH 8.0)

TNE: Tris sodium EDTA

100 mM NaCl

5 mM EDTA

25 mM Tris-HCl (pH 8.0)

Prepared as 20 X stock.

TAE: Tris acetate EDTA

40 mM Tris-HCl

20 mM HAc

2 mM EDTA (pH 8.0)

Prepared as a 50 X stock.

2.1.2 Markers:

DNA markers lambda DNA cut with *Hind* III (NEB)

1	23,130 bp	5	2,322 bp
2	9,416 bp	6	2,027 bp
3	6,557 bp	7	564 bp
4	4,361 bp	8	125 bp

DNA markers ϕ X 174 cut with *Hae* III (NEB)

1	1,353 bp	6a	281 bp
2	1,078 bp	6b	271 bp
3	872 bp	7	234 bp
4	603 bp	8	194 bp
5	310 bp	9	118 bp
		10	72 bp

RNA markers (Promega)

1	9,488 bp	5	1,898 bp
2	6,225 bp	6	872 bp
3	3,911 bp	7	562 bp
4	2,800 bp	8	363 bp

ProMega-markers for PFGE

1) *S. pombe* chromosomes: 3.5, 4.7 and 5.7 Mb

2) *S. cerevisiae* chromosomes: 1900, 1640, 1120, 1100, 945, 915, 815, 785, 745, 680, 610, 555, 450, 375, 295, 225 kbp

3) Lambda ladders: ranging from 50 kbp to 1000 kbp increasing by 48.5 kbp in a 20 step ladder.

2.1.3 Media:

2 x YT (per litre)
16 g bactotyptone
10 g yeast extract
5g NaCl

NZCYM medium (per litre)
10 g NZ amine
5 g NaCl
1 g casamino acids
5 g Bacto-yeast extract
5 g $MgSO_4$ $7H_2O$
0.5 mg ampicillin

L.B. Medium (per litre)
10 g Bacto-typtone
5 g Bacto-yeast extract
10 g NaCl

SOB Medium (pH 7.0) (per litre)
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 (per litre)
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 (per litre)
5.8 NaCl
2 g $MgSO_4$
50 ml 1M Tris-HCl pH 7.5
5 ml 2% gelatin

2.1.4 Drugs

Diminazene aceturate (Berenil, Hoechst AG, FRG) and isometamidium chloride (Samorin, May and Baker, UK) were purchased commercially. Concentration was calculated on the basis of the active principle of 44% diminazene aceturate in Berenil. The drugs were dissolved in triple distilled water on the day of use.

Ampicillin (Penbritin, Dawa Pharmaceuticals Ltd., Nairobi, Kenya) was stored dissolved at 100 mg/ml and stored at -20°C. It was added to media and agar to a concentration of 100 µg/ml when required.

2.2 Bacterial strains and vectors

Escherichia coli JM109 (*recA*1 *supE*44 *endA*1 *hsdR*17 *gyrA*96 *relA*1 thi F') (Yanisch-Perron *et al.*, 1985). *E. coli* Y1090 (*supF hsdR araD*139 *lacU*169 *rspL trpC*22::Tn10(*tet*)pMC9) (Young and Davis, 1983).

Vectors

pUC19 (Promega, USA) is a 2.69 kbp vector which contains a polylinker with 20 unique restriction sites and a *lacZ* promoter for blue/white colour selection (Yanisch-Perron *et al.*, 1985).

pBluescript (KS) (Strategene, USA). This is a 2,964 bp phagemid derivative of pUC19 and possesses T3 and T7 promoters flanking a polylinker, which contains 21 unique restriction sites and a *lacZ* promoter for blue/white colour selection (Yanisch-Perron *et al.*, 1985).

lambda gt11 (*lac*5 *nin*5 cI1857 S100; Young and Davis, 1983); (Promega, USA). This bacteriophage vector possesses a unique *Eco*R I cloning site located in the *lacZ* promoter allowing for blue/white colour selection (Young and Davis 1983).

All strains of bacteria with or without plasmids were stored at -70°C in 50 % glycerol, 50% culture medium.

2.3 Trypanosome management

2.3.1 Trypanosomes used

CP 547 is a pleomorphic *T. b. brucei* was isolated in 1985 from a naturally infected cow in Jilib, Somalia. This stock causes relapsing parasitemia in mice and is resistant to diminazene, isometamidium, quinapyramine, and melarsoprol (Kaminsky and Zweygarth, 1989b).

ILTat 1.4 is a *T. b. brucei* monomorphic clone of a derivative of EATRO (East African Trypanosomiasis Research Organisation) 795, which was isolated from a cow in

Uhembo, Kenya (Miller and Turner, 1981). The clone is highly virulent in mice and is susceptible to diminazene, isometamidium, suramin and pentamidine (Kaminsky *et al.*, 1989).

ILTat 1.1 is a pleomorphic clone that is also a derivative of EATRO (East African Trypanosomiasis Research Organisation) 795 (Miller and Turner, 1981).

IL 3388 (Hirumi, 1980) is a *T. b. brucei* clone a derived from EATRO 217, isolated from Uganda in 1960.

ILB24 is a *T. b. brucei* clone (Cunningham *et al.*, 1962; Hirumi, 1980). Reference records available from ILRAD.

ILB10 is a *T. b. brucei* clone isolated by Murray. K. form a cow in The Gambia in 1976. Reference records available from ILRAD.

ILTat 221 was derived from a stock 427 isolated in Uganda from a sheep in 1960. (Cunningham, *et al.*, 1962).

ILTar 2 was derived from stock 247 isolated in the Serengeti in Tanzania in 1973 (Geigy, 1973)

GUTat 3.1 is a sensitive *T. b. brucei* clone (Black *et al.*, 1982) isolated from Uganda in 1966.

ANTat 1.8 is a *T. b. brucei* clone (Magnus *et al.*, 1981).

2.3.2 Routine propagation of trypanosomes

Stabilates were thawed on ice and expelled from capillary tubes with 0.5 ml of PSG. The number of viable trypanosomes was estimated by counting motile organisms in a haemocytometer. Irradiated mice (650 rad.) were infected with an intraperitoneal injection of 10^6 trypanosomes from the capillary. The course of the infection was followed by examination of tail blood using the wet blood film method. High parasitaemia (greater than 5×10^8 trypanosomes per ml) was usually obtained after 4 days. Infected blood was collected from anaesthetised mice by cardiac puncture in a solution of 3% sodium citrate glucose anticoagulant (CGA).

When larger numbers of trypanosomes were required, infected mouse blood was used to infect rats by intraperitoneal injection of blood diluted in 0.2-0.5 ml PBS, containing 2×10^7 trypanosomes. The ensuing parasitaemia was monitored by microscopic examination of tail blood using the wet blood film method. Rats were anaesthetised and bled by cardiac puncture as for mice.

2.3.3 Enumeration of trypanosomes

Trypanosomes were counted in a Neubauer haemocytometer, as described by Lumsden *et al.* (1973). Only motile trypanosomes were counted and dividing stages counted as one trypanosome.

2.3.4 Preparation of trypanosome stabilates

The method used for cryopreservation of trypanosomes is based on the work of Cunningham *et al.* (1963). Briefly, infected mouse or rat blood was collected into a 2 ml syringe containing about 0.2 ml of CGA. The blood was then mixed with an equal volume of 20% glycerol in PSG, and transferred into capillary tubes in aliquots of approximately 50 µl. The capillaries were sealed at one end only with plasticine, and then cooled, first on ice, and then in the vapour phase of a liquid nitrogen tank using a slow cooling plug (Union Carbide) or an insulated bottle, both of which allowed cooling at about one degree per minute. The frozen stabilates were stored in liquid nitrogen.

2.3.5 Trypanosome cloning

Trypanosomes were cloned by a modification of the dilution method of Walker (1968). Infected blood was diluted in RPMI 1640 medium containing 10% foetal calf serum. A dilution containing about 10^3 trypanosomes per ml was prepared. Small droplets (about 1 µl) of this dilution were placed on coverslips which were then inverted over the depression of a cavity microscope slide containing a drop of the dilution buffer. These drops were examined with a phase contrast microscope. To the droplets containing single living trypanosomes, additional medium containing 10% FCS was added and the

diluted drop was drawn into a 1 ml syringe. The recovered mixture was then used to infect irradiated mice (900 rads) by intravenous injection. The presence of only a single trypanosome in each droplet was confirmed by independent observers. Blood was collected from the infected mice after 3 days and transferred into additional irradiated mice in which the parasitaemia was followed by examination of tail blood. Blood from positive mice was then cyropreserved (section 2.3.4).

2.3.6 Characterisation of drug sensitivity

In order to characterise the drug sensitivities of the various populations, stabilates of each clone were expanded in irradiated mice. Prior to the first peak of parasitaemia, blood was collected into ethylene diaminetetra-acetate (EDTA) (final concentration 7.5% w/v) and the parasitaemia estimated using a Neubauer haemocytometer. Groups of non-irradiated mice (6/group) were innoculated i.p with 1.0×6^6 bloodstream forms and treated 6 h later with varying doses of isometamidium chloride (0.5-15 mg/kg body weight) or diminazene aceturate (20-60 mg/kg body weight). Trypanocides were administered i.p. in 0.2 ml of sterile water. Following treatment, mice were monitored twice weekly for 60 days, for the presence of parasites, by examination of tail blood using the wet blood film method. Using standard logic analyses, the sensitivities of each clone to isometamidium chloride and diminazene aceturate were expressed as 50% curative dose (CD_{50}) values, i.e., the dose of drug required to cure 50% of infected mice. Kendall's rank correlation test (Sprent, 1989) was used to determine whether there was any correlation between the isometamidium chloride and diminazene aceturate CD_{50} values of each clone.

2.3.7 Isolation of trypanosomes (Lanham and Godfrey, 1970; and Grab and Mbwayo, 1982)

At peak parasitaemia, infected mice or rats were anaesthetised and the parasitaemic blood collected. To the parasitaemic blood, an equal volume of Percoll solution (100 ml of Percoll Pharmacia containing 8.55 g sucrose, 2.0 g glucose and 0.34 g

HEPES was added). The mixture was then centrifuged at 16,000 rpm, 4°C for 20 min in a Beckman J-20 rotor. The buffy coat of trypanosomes was collected and diluted 3X with cold phoshate/glucose/saline buffer (PSG) and applied to a column of DEAE cellulose (Whatman DE-52), previously equilibrated with PSG buffer pH 8.0 and poured in a suitable plastic syringe barrel plugged with glass wool. The column was washed with PSG to obtain the trypanosomes separated from blood cells absorbed to the DEAE cellulose. The trypanosomes were used for isolation of DNA and RNA.

2.4 Nucleic acid preparation:

2.4.1 Isolation of trypanosome DNA (Blin and Stafford, 1976).

Reagents:

	T.E
	TNE
Proteinase K:	Stock prepared at 20 mg/ml in sterile water
RNase:	2 mg/ml RNase A (Serva) in water, incubated in boiling water (93°C in Nairobi) for 15 minutes and stored at -20°C.
10% SDS:	10% w/v sodium dodecyl sulphate in sterile water stored at room temperature.

Procedure:

Once trypanosomes were purified by DEAE cellulose chromatography (section 2.3.7), they were counted, washed once in PSG and resuspended at 0.3-10 x 10^9 trypanosome per ml in TNE in 30 ml Corex tubes. This suspension was brought to a final concentration of 0.1% SDS by mixing quickly, but gently, by inversion of the tube. The lysate was incubated at 37°C until completely clear (2-5 minutes) followed by addition of 0.05 volumes of RNase A (2 mg/ml) which was added and mixed by inverting and rolling the tube gently. Proteinase K was added to a final concentration of 100 µg/ml and the digestion allowed to continue for 4 h at 37°C. The lysate was extracted with an equal volume of phenol equilibrated with TNE. The aqueous and phenolic phases were mixed

gently by continuous inversion of the tube for about 3 minutes. The mixture was centrifuged at 3,000 x g for 5 minutes, and the aqueous phase was removed with a plastic pipette cut off to give a 2 mm aperture. The DNA solution was then precipitated in the form of a loose fibrous clot with the addition of 2½ volumes of absolute ethanol (at room temperature). The precipitate was recovered by pulling it out with sterile flat tipped forceps and transferred twice into fresh 70% ethanol with agitation. It was then dried under vacuum and resuspended in T.E at a final concentration of 200-500 µg/ml. DNA concentrations were estimated by measuring the absorbance at 260 nm, assuming that A_{260} of 1 represents 50 µg per ml of DNA. The typical yield was 1 mg of DNA per 10^{10} trypanosomes. The purified trypanosome DNA was stored at 4°C.

2.4.2 Isolation of trypanosome RNA (Chomczynski and Sacchi, 1987)

Reagents:

Solution D:

a) 4 M guanidinium thiocyanate
25 M sodium citrate pH 7.0
0.5% sarcosyl
100 mM 2-mercaptoethanol

b) 2 M sodium acetate pH 4.0
Phenol - water saturated
Choloroform-isoamyl alcohol (49:1)
Isopropanol, 75% ethanol and 0.5% SDS

Procedure

A trypanosome pellet of 2 x 10^9 cells was dissolved in 1 ml of solution D in a 30 ml corex tube. Subsequently, 0.1 ml of 2 M sodium acetate, 1 ml of phenol saturated with water and 200 µl of chloroform-isoamyl alcohol were added to this lysate. The contents were mixed by vigorous vortexing for 10 seconds and cooled on ice for 15 minutes. Subsequently, the suspension was aliquoted into sterile Eppendorf tubes and centrifuged in a microfuge at 10,000 x g for 20 minutes at 4°C. The aqueous phase,

containing the RNA, was transferred into fresh sterile Eppendorf tubes and mixed with one volume of isopropanol and placed at -20°C for 20 minutes to precipitate the RNA. The RNA was pelleted by spinning in an Eppendorf centrifuge for 10 minutes at 4°C. The resultant pellet was re-suspended in solution D and then re-precipitated with 1 volume of isopropanol at -20°C for 15 min. The RNA was spun down as before and the pellet was washed in 500 µl of 70% ethanol, vacuum dried and dissolved in 100 µl of sterile water. The total RNA was quantified by determination of absorbance at 260 nm (A_{260} X 42 X dilution gave the concentration of RNA in µg/ml). RNA aliquots were stored at -80°C.

2.4.3 Affinity purification of trypanosome poly(A)$^+$RNA

Reagents:

1 X loading buffer:	20 mM Tris-HCl pH 7.6
(Prepared as 1X and 2X stocks)	0.5 M NaCl
	1 mM EDTA
	0.1% SDS
Oligo(dT):	Collaborative Research Inc. U.K.
Sterile RNase free water	

Procedure:

Poly(A)$^+$ RNA selection by oligo(dT)-cellulose chromatography was done as described by Edmonds *et al.* (1971). The oligo(dT) cellulose (Collaborative Research Inc., U.K.) with an average of 14-16 T residues and a binding capacity of 2.5 mg poly(A)$^+$ RNA/gm of cellulose, was weighed to obtain 100 mg of resin per 1 mg total RNA. The oligo(dT) cellulose was equilibrated with 1 X sterile loading buffer thrice by mixing and discarding the fine particles that do not settle after 30 seconds. A final resuspension was diluted in 2 ml of 1 X loading buffer and poured into a 1 ml syringe in which the nozzle was plugged with sterile siliconised glass-wool. The column was washed with 3 bed-volumes of 1 X loading buffer.

The total RNA dissolved in water was heated to 70°C for 5 min. To this an equal amount of 2 X loading buffer was added and the mixture loaded on to the column. The flow-through was collected and re-heated and re-applied to the column thrice. The flow through which contains the non-polyadenylated RNA was kept on ice if required. The column was then washed with 3 bed volumes of 1 X loading buffer. The poly(A)$^+$ RNA was eluted with 100 µl aliquots of sterile warmed water (50°C) until the final volume of the eluent was 1 ml. To enrich the poly(A)$^+$ further, the whole process could be repeated on the eluted poly(A)$^+$ enriched RNA. The final eluent of poly(A)$^+$ RNA was then precipitated on ice for 20 min with an equal volume of isopropanol and 1/10th volume of 3 M sodium acetate. This was centrifuged at 14,000 rpm or 30 min at 4°C in an Eppendorf minifuge. The supernatant was discarded and the mRNA pellet was vacuum dried and dissolved in 50 µl of sterile distilled water. The poly(A)$^+$ RNA was then aliquoted at a concentration of 1 µg/µl and analysed by RNA agarose electrophoresis. The aliquots of poly(A)$^+$ RNA were stored at -70°C until used.

2.4.4 Isolation of plasmid DNA (Maniatis *et al.*, 1982)

Small scale (miniprep) plasmid purification by alkaline lysis:

E. coli JM109, transformed with plasmids, were grown overnight in 3 ml of 2xYT medium containing 100 µg/ml of ampicillin in sterile tubes. 1.5 ml of this culture was transferred into a sterile Eppendorf tube which was spun at 14,000 rpm for 10 seconds in a microfuge. The medium was aspirated and the bacterial pellet was then resuspended in 100 µl of ice-cold solution I (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0). To this suspension 200 µl of solution II (0.2 M NaOH, 1% SDS) was added followed by mixing of the contents by inverting the tubes and then placing on ice for 5 min. Finally, solution III (60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid and 28.5 ml of water, giving a resulting solution of 3 M with respect to potassium and 5 M with respect to acetate) was mixed with the bacterial lysate, vortexed and the mixture centrifuged at 14,000 rpm for 5 min at 4°C in a microfuge. The supernatant was transferred to a fresh tube and extracted subsequently with an equal volume of buffered

phenol/chloroform. In this procedure, the SDS lyses the cells and the high pH denatures the DNA by disrupting the hydrogen bonding between nucleotide base pairs. The buffer of solution III rapidly adjusts the pH to the range where the DNA re-anneals. Bacterial chromosomal DNA, on the other hand, is circular and much bulkier. With its strands completely separated, it does not re-anneal at an appreciable rate. Thus, while the plasmid DNA re-anneals, the chromosomal DNA precipitates out of solution.

The plasmid DNA in the aqueous layer was then precipitated by adding 2.5 volumes of absolute ethanol at room temperature, mixed by vortexing and allowed to stand at room temperature for 2 min. The double stranded DNA was then pelleted by centrifugation of the tube at 12,000 x g for 5 min at 4°C. The supernatant was removed and the tube placed in an inverted position on a paper towel to allow all the fluid to drain off. The pellet was rinsed with 1 ml of 70% ethanol at 4°C and the pellet of nucleic acid allowed to dry in the air for 10 min. The DNA was dissolved in 50 µl of T.E (pH 8.0) containing DNase-free pancreatic RNase (20 µg/ml; Sigma), vortexed briefly and stored at -20°C until use.

2.4.5 Large scale preparation and purification of plasmid DNA

Apart from the difference in the volume of cell culture, the maxi-preparation of plasmid DNA was similar to the method of alkali lysis employed in making DNA minipreparations as described in the above section. The bacterial cells were harvested from a 500 ml culture by centrifugation ar 4000 rpm for 15 min at 4°C in a Sorvall G53 rotor. The pellets were first suspended in 100 ml of ice-cold STE buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8.0 and 1mM EDTA, pH 8.0), after a thorough drain of the centrifuge bottle. The bacterial cells were collected by recentrifugation and the washed pellet resuspended in 10 ml of solution I. A 1 ml volume of freshly prepared solution of lysozyme (10 mg/ml in 10 mM Tris-HCl pH 8.0), was followed by the addition of freshly made solution II. The content of the centrifuge bottle was thoroughly mixed by gently inverting the bottle several times. The bottle was stored at room temperature (23°C) for 10-15 min before 15 ml ice-cold solution III was added. This was stored for 10 min on

ice after mixing. A flocculent white precipitate, consisting of chromosomal DNA, high molecular weight RNA, and potassium/SDS/protein/membrane complexes, forms during this storage. The bacterial lysate was centrifuged at 4000 rpm for 15 min at 4°C in a Sorvall G53 rotor. The supernatant was filtered through 4 layers of cheese-cloth into a 250 ml centrifuge bottle and transferred to a Corex tube. A 0.6 volume of isopropanol was then added, mixed well and the tube stored for 10 min at room temperature. Because salt may precipitate out if centrifugation is carried out at 4°C, the nucleic acids were recovered by centrifugation at 5000 rpm for 15 min at room temperature in a Sorvall G53 rotor. The pellet was rinsed with 70% ethanol at room temperature, the ethanol drained off and the DNA allowed to dry by placing the tube in an inverted position on a pad of paper towels at room temperature. The DNA was dissolved in 1 ml of TE, pH 8.0.

The plasmid DNA was purified by equilibrium centrifugation in CsCl-ethidium bromide continuous gradients as described in Sambrook *et al.* (1989). The DNA solution was made up to 9 ml with TE, pH 8.0, and for every millilitre of DNA solution, 1g of solid CsCl was added. The solution was mixed gently until the salt dissolved. An aliquot of 0.8 ml of a solution of ethidium bromide (10 mg/ml in water) was added for every 10 ml of the DNA/CsCl solution. The ethidium bromide solution, which floats on the surface, was mixed with the denser DNA/CsCl solution. This solution gives a final density of 1.55 g/ml with a refractive index of 1.3860 and an ethidium bromide concentration of approximately 740 ug/ml. Using a disposable syringe fitted with a large-gauge needle, the clear red solution was transferred to a Beckman Quick-seal tube. The tube was sealed with a Beckman sealer and inserted into a Beckman vertical VTi 65 or VTi 80 rotor. The density gradient was centrifuged at 45,000 - 55,000 rpm for 16-22 hr at 20°C. Two bands of DNA, located in the centre of the gradient, were visible in ordinary light and most clearly under UV illumination. The upper band, which usually contains less material, consisted of linear bacterial (chromosomal) DNA and nicked circular plasmid DNA. The lower band consisted of closed circular plasmid DNA. The deep-red pellet on the bottom of the tube predominantly consisted of ethidium bromide/RNA complexes.

The closed circular plasmid DNA was collected by the following procedure: the tube was clamped on a stand close to a UV illuminator, which was used to visualise the bands of DNA. For reasons of safety and in order to minimise damage to the DNA from UV irradiation, the UV lamp remained off for most of the operation. A 21-gauge hypodermic needle was inserted into the top of the tube to allow in air. Then an 18-gauge hypodermic needle was attached to a 1 ml plastic syringe and, as the UV lamp was turned on, the needle was inserted (bevelled side up) into the tube horizontally, so that the bevelled side of the needle was positioned just below the band of closed circular plasmid DNA and parallel to it. The plunger of the syringe was then immediately, but, carefully, withdrawn in order to collect the banded DNA into the syringe, taking care not to include the upper band. The collected DNA was then transferred into a Sterilin tube and the ethidium bromide was removed from the purified DNA by extraction with either one of two organic solvents, as described below.

To the solution of DNA in a glass or plastic tube, an equal volume of either 1-butanol, saturated with water, or isoamyl alcohol was added. The two phases were mixed by gentle vortexing and the mixture centrifuged at 800 x g for 3 min at room temperature. Using a Pasteur pipette, the lower aqueous phase was transferred to a clean tube and the extraction repeated four to six times, until all the pink colour disappeared from both the aqueous and the organic phase. The CsCl was then removed from the DNA solution by dialysis for 24-48 hr against several changes of TE (pH 8.0). The dialysis tubing (Spectropor) had been treated by boiling for 10 min in a large volume of 2% (w/v) sodium bicarbonate and 1 mM EDTA (pH 8.0). The tubing was then rinsed thoroughly in distilled water, then boiled for 10 min in 1 mM EDTA (pH 8.0). The tubing was allowed to cool down and then stored at 4°C submerged in the 1 mM EDTA solution. The dialysed DNA solution was carefully removed from the dialysis bag and precipitated with 2.5 volumes of ethanol, followed by centrifugation at 10,000xg for 15 min at 4°C. The precipitated DNA was then dissolved in 500 µl to 1 ml of TE (pH 8.0) and the OD_{260nm} of the final solution measured in a Beckman DU-50 series spectrophotometer. The concentration was calculated using the formula: OD_{260nm} x dilution factor x 50 = µg/ml

(Maniatis *et al.*, 1982). The purified, recombinant plasmid DNA was then stored at -20°C until used for physical mapping, Southern analysis and sequencing.

2.4.6 Alkaline lysis of CP 547 trypanosome DNA

In an attempt to enrich for circular extrachromosomal DNA from *T. b. brucei* total CP 547 DNA, the alkaline lysis purification procedure for bacterial plasmids was used. Briefly, 5 x 10^8 trypanosomes were treated exactly as the 1.5 ml bacterial culture (section 2.4.4) to enrich for circular *T. b. brucei* DNA.

2.5 Nucleic acid manipulations

2.5.1 Restriction enzyme digestions

For all the restriction enzymes used, information for buffer conditions and temperatures of incubation were obtained from the NEB catalogue (1989-1992). The buffers used can now be obtained as 10 X stocks from NEB.

An excess of enzyme was always used. For digestion of trypanosome DNA, the product of the number of units of enzyme and the number of hours of incubation was always sufficient for at least ten-fold over-digestion. The percentage of glycerol in the total reaction volume was always kept below 5%.

2.5.2 Agarose gel electrophoresis and blotting

2.5.2a DNA electrophoresis

Gels containing between 0.7% and 1.5% (w/v) agarose (Ultra-Pure, BRL) in 0.5 X TAE buffer were used for Southern blots and for restriction enzyme analysis of genomic, plasmid and phage DNA as described in Maniatis *et al.* (1982). Electrophoresis was also used for the preparative separation of extrachromosomal DNA and restriction enzyme fragments. The gels were prepared by boiling agarose in TAE until completely dissolved, cooling to 55°C, and pouring into the gel former with an appropriate comb.

The gel former with the set gel was then placed in the electrophoresis tank containing sufficient TAE running buffer to cover the gel.

Sample buffer (10 X TAE, 50% glycerol, containing 1% bromophenol blue and 1% xylene cyanol FF) was added to samples before electrophoresis to give a 1 x TAE final concentration. For preparative electrophoresis, several sample wells were connected together by placing adhesive tape over the sample comb teeth. The voltage was generally set at 5-10 V/cm of gel. After electrophoresis was completed, the DNA was stained with ethidium-bromide so that the DNA fragments in the gel could be visualised on a UV transilluminator. The gels were then photographed with a Polaroid camera.

2.5.2b Pulsed Field Gel Electrophoresis (PFGE)

The technique of pulsed field gel electrophoresis was developed by Schwartz and Cantor (1984) for the resolution of chromosome-sized DNA molecules from yeast. The method exploits the dependence of the relaxation time of extended DNA molecules on their length. Briefly, the DNA molecules are extended within an agarose gel matrix by application of an inhomogeneous electric field, and then subjected to an approximately orthogonol field. The extended molecules will be unable to move through the gel matrix under the influence of the second field until their extended conformation has relaxed. Smaller molecules, relaxing more rapidly, will move sooner. Continuous alternation of the two fields will result in a greater net mobility of the smaller molecules. The apparatus used in this work was the Pulsaphor system (Pharmacia LKB Biotechnology). The HEX electrode array was used. With this electrode, two different fields, North/South and East/West fields can be applied. The HEX electrode gives a homogeneous field resulting in straight migration of the DNA in lanes when the pulsed field technique is used.

2.5.2c Preparation of DNA for PFGE

To avoid shearing of large DNA molecules during extraction, cells were lysed *in situ* in agarose plugs (Schwartz and Cantor, 1984). Briefly, the pelleted trypanosomes were suspended in phosphate saline glucose buffer, pH 8, to a concentration of 4×10^9 trypanosomes per ml. The parasite suspension was then mixed with an equal volume of 1.5% low melting point agarose (Ultra Pure LMP, electrophoresis grade; BRL), previously maintained at 50°C, to give a final concentration of 2×10^9 trypanosomes/ml. The mixture was then poured between glass plates separated by 1 mm plastic spacers. These plates were previously blocked on 3 sides by clamping in the spacers. On pouring in the mixture, the plates were placed on ice to allow rapid setting of the agarose. The agarose blocks were then immersed into a lysing mixture of 10 mM Tris (pH 7.5), 0.2 M EDTA (pH 8.0), 1% SDS, 1 mg/ml proteinase K and incubated in this for 36 h at 50°C. The proteinase K-treated agarose blocks were washed in several changes of 10 mM EDTA and stored in this buffer at 4°C.

2.5.2d gamma-irradiation of DNA in agarose plugs

PFGE of DNA in agarose plugs exposed to different levels of gamma-irradiation as described by van der Bliek *et al* (1988) was used to show the presence of large circular DNAs within the CP 547 isolate. Briefly, the agarose embedded DNA was cut in 2cm X 1mm sizes and immersed in 200 µl of buffer (10 mM EDTA, 10 mM Tris-HCl, pH 7.5) in a standard 1.5 ml propylene microcentrifuge tube. Six such samples were all placed at ambient temperature at a distance of 16 cm from a single point Caesium source (Conservatone Lisa IA) emitting 3 Gray/min in the irradiation chamber (The source was calibrated with Alanine dosemeters, NPL U.K). Individual sample tubes were withdrawn at 3.3, 10, 20, 33, 50 and 60 min, exposing the samples to a radiation dose of 10, 30, 60, 100, 150 and 180 Gray respectively. The samples remained at room temperature for at least 30 min prior to electrophoresis.

Electrophoresis

The conditions of voltages, switch frequencies and run-times used for each pulsed field gel (PFG) are specified in the figure legends. All the DNA samples were resolved in 1.5% agarose gels run in 1 X TBE circulating buffer. At the completion of the run, the gels were placed for 2-3 h in 1 µg/ml ethidium-bromide and destained overnight in 0.1 µg/ml ethidium-bromide. Southern blotting was carried out essentially as for regular agarose gels (section 2.5.2e).

2.5.2e Southern blots (Southern, 1975)

After photography, DNA in gels was denatured with gentle agitation in 0.5 M NaOH, 1.5 M NaCl for 45 minutes, and neutralised in 1 M Tris-HCl (pH 7.4), 3 M NaCl for one hour at room temperature. Volumes of the soaking solutions were enough to allow complete submersion of the gel.

The gels were then placed upside down on a sheet of Whatman 3 MM paper resting on a 1.5 cm thick sponge in a shallow tray filled with 20 X SSC. A sheet of nytran (Schleicher and Schuell), cut to the size of the gel, was first soaked in water and then placed on top of the gel. This was followed by 20 similarly cut sheets of Whatman 3 MM paper and an approximately 4 cm stack of paper towels. The stack was lightly weighed and blotting was allowed to proceed for 15-24 h. The nytran was then removed and baked under vacuum at 80°C for 2 h, or, if nitrocellulose filters were used, the nucleic acids on the filter were cross-linked and fixed on the membrane by a 2 min exposure to ultraviolet light. These Southern blots were stored at 4°C until required.

2.5.2f RNA electrophoresis

Electrophoresis of RNA was carried out in a manner similar to that described for DNA (Section 2.5.2a), except that 10 mM phosphate (Na_2HPO_4/NaH_2PO_4), pH 6.8 was used as running buffer (Pellé and Murphy, 1993). Briefly, the RNA samples (2-20 µg) were glyoxylated (Thomas, 1983), mixed with 6 X RNA sterile gel loading buffer (60 mM sodium phosphate, pH 6.8, 30% (w/v) glycerol, 0.25% (w/v) bromophenol blue,

0.25% (w/v) xylene cyanol FF, 1.2% (w/v) SDS) and volumes adjusted to a final concentration of 1 X of the RNA loading buffer). These samples were then denatured by heating to 65°C for 5 minutes in a water bath. The RNA was loaded and the electrophoresis performed in 0.01 M sodium phosphate buffer (pH 7.0), which is constantly recirculated by the use of a peristaltic pump.

2.5.2g Northern blotting

The agarose gels with RNA were transferred immediately after electrophoresis to nytran filters; no further treatment of the gel was necessary. The gel was placed in contact with the nytran filter and blotted essentially as described for Southern blotting. The blots were dried at room temperature and the RNA was fixed onto the filter by cross-linking with ultraviolet irradiation (254 nm).

2.5.3 Recovery of DNA from agarose gels

2.5.3a Geneclean®(BIO 101, Inc., La Jolla, CA)

This procedure is adapted from the glass-milk extraction of DNA method of Vogelstein and Gillespie (1979). This procedure involves isolation of DNA from agarose gels run in TAE running buffer. The slice of agarose gel containing the required DNA is removed and weighed. At least 2.5 volumes of saturated NaI solution was then added to the slice and the mixture was then placed in a 50°C water-bath until the gel was completely dissolved. For every µg of DNA, 5 µl of 'glass-milk' suspension was added and the suspension mixed well and kept on ice to allow the DNA to bind on to the 'glass-milk' particles. Subsequently the 'glass-milk' was spun down for 5 sec in a microfuge and the NaI aspirated off. The 'glass-milk' with bound DNA was washed four times with 'New-Wash' solution by re-dissolving gently and spinning. The DNA was then eluted off the 'glass-milk' with a small volume of sterile water at 55°C for 5 min.

2.5.3b DNA extraction by centrifugation

The section of the agarose gel containing the DNA fragment of interest was cut out and cut into very small pieces. These pieces were put into a 500 µl Eppendorf tube which had a hole at its base made with an 18 gauge hypodermic needle. The hole was plugged with siliconised glass wool which acted as a sieve. The tube was placed into a 1.5 ml Eppendorf tube. These were then spun at 8,000 rpm for 10 minutes in an Eppendorf centrifuge. The glass wool blocks the agarose, but the buffer containing DNA is collected in the lower Eppendorf tube. This DNA solution is then extracted once with phenol followed by a phenol/chloroform extraction. Finally the DNA is purified by ethanol precipitation.

2.5.4 Gel purification of the 6.6 kbp extrachromosomal element

i) Geneclean®(section 2.5.3a)

ii) Electrophoresis onto DEAE - cellulose membranes (Sambrook *et al.*, 1989)

Geneclean®(section 2.5.3a) outlined above, and electrophoresis onto DEAE-cellulose membrane (section 2.5.4a) gave the highest yields for the 6.6 kbp extrachromosomal element DNA and was therefore routinely used for its purification.

2.5.4a Electrophoresis onto DEAE-cellulose membrane (Dretzen *et al.*, 1981)

Reagents:

DEAE-cellulose membrane (Schleicher and Schuell NA-45)
Low-salt wash buffer
50 mM Tris-HCl (pH 8.0)
0.15 M NaCl
10 mM EDTA (pH 8.0)

High-salt elution buffer
50 mM Tris-HCl
1 M NaCl
10 mM EDTA (pH 8.0)

The membrane was soaked for 5 min in 10 mM EDTA, pH 8.0. The EDTA was replaced with 0.5N NaOH for a further 5 min. The membrane was then washed six times in sterile water and stored in sterile water at 4°C until used.

Electrophoresis of the uncut total DNA was performed with size markers. The gel strip that corresponded to the migration of a 5 kb to 7 kb linear marker was excised. This gel strip was placed perpendicular to its initial position in the gel former. This strip was held in place by surrounding it with melted agarose which forms a supportive mould on solidification. The DEAE-cellulose membrane was positioned in an incision made at the end of the gel slice away from the 6.6 kb size marker (lambda DNA digested with *Hind* III). The DNA was then transferred electrophoretically from the gel slice onto the DEAE-cellulose membrane. The distance of the migrated 6.6 kbp element DNA was monitored by the parallel mobility of the 6.6 kbp size marker. When all of the DNA had left the gel slice and was trapped on the DEAE membrane, the membrane was rinsed in the low salt wash to rinse off the agarose. The DNA was eluted from the membrane by submerging the membrane in the elution buffer for 30 minutes at 65°C. The eluate was extracted once with buffered phenol alone, followed by one extraction with phenol/chloroform before it was precipitated with two volumes of ethanol and 0.2 volumes of 10 M ammonium acetate at 4°C. The DNA was recovered by centrifugation at 14,000 rpm in an Eppendorf microfuge for 20 min at room temperature.

2.5.5 Labelling of DNA

2.5.5a Radiolabelling

1) Radiolabelling with [α-^{32}P]dCTP of small quantities (less than 100 ng) of DNA was carried out using a Random Priming kit (Amersham, U.K.) according to the suppliers instructions. Likewise for labelling of DNA available in larger quantities, (more than 100 ng), a Nick Translation kit (Boehringer Mannheim) was used and the suppliers instructions were followed. Since very small quantities of the 6.6 kbp extrachromosomal DNA could be recovered from agarose gels, it was always labelled by random priming.

Radioactive [α-^{32}P]dCTP was obtained from Amersham Inc. (10 mCi/ml, 3000 Ci/mmol).

Labelled DNA was separated from unincorporated nucleoside triphosphates by centrifugation through columns of Sephadex G-50 in 1 ml syringes (Maniatis *et al.*, 1982). To measure the incorporated radioactivity, a sample of the eluted DNA was counted in Aquasol in a liquid scintillation counter (Beckman).

2) Kinasing 5'-ends of oligonucleotides (Sambrook *et al.*, 1989)

The 5' hydroxyl termini of oligonucleotides were labelled using the catalytic activity of T4 polynucleotide kinase (Promega, USA). The enzyme catalyses the transfer of the g-phosphate of ATP to the 5'-hydroxyl termini of oligonucleotides. The kinasing reaction was carried out exactly as outlined in Sambrook *et al.* (1989). Radioactive [g-^{32}P]dATP was supplied by Amersham (> 5000 Ci/mmol).
Labelled oligonucleotides were used directly from the labelling reaction with no further manipulation.

2.5.5b Hybridisation conditions

For both Southern and Northern blots, identical conditions were used which are a modification of the procedure described by Wahl *et al.* (1979). The same conditions were used for both the prehybridisation and hybridisation steps.

Stock solutions	Final concentration
20 X SSC	4X
10% SDS	0.1%
10% NaPPi-HCl (pH 7.4)	0.1%
50 X Denhardts solution	5X

50 X Denhardts solution
1% w/v of polyvinylpyrrolidone (molecular weight - 350,000)
1% w/v of Ficoll 400
1% w/v of BSA fraction V

These components were dissolved in water and stored at -20°C.

2.5.5c Hybridisation procedure

The filters were placed in a heat-sealable plastic bag into which the pre-hybridisation solution was added, and incubated in a shaking water bath for 2 h or more at 65°C. The Denhardt's solution was used to minimise non-specific binding of the probe to the membrane because single stranded DNA, unlike RNA, has high affinity for nylon membranes.

Radiolabelled probe DNA was denatured by incubation in a boiling water bath for 3-5 minutes and added to the hybridisation solution. The probe concentration was between 5 X 10^5 and 10^6 cpm per ml of hybridisation solution.

After hybridisation, the filters were removed from the bags, and washed in 0.1 X SSC, 0.1% SDS, unless otherwise indicated. After rinsing for 2-3 minutes at room temperature, the filters were washed twice for 45 minutes at 65°C with one change of washing solution. Filters were then blotted with 3 MM paper, air dried and exposed to X-ray film at -80°C for 12-72 h with intensifying screens (Du Pont lightening plus). Radioactive particles entering autoradiographic film cause ejection of electrons from silver halide crystals. These electrons attract positively charged silver ions, generating precipitates of silver atoms, hence formation of the autoradiographic image. There is a five-fold enhancement in the intensity of the image when an intensifying screen is placed on the X-ray film, as radioactive particles that pass through the film hit the screen, which is coated with oxides of heavy metals and cause it to emit protons that are captured by the silver halide crystals in the emulsion.

2.5.6 Preparation of plasmid vector for cloning

Plasmid DNA (0.5-1.0 µg) was digested with the required enzyme in a reaction volume of 50 µl. A tenth of this reaction was electrophoresed in a 1% agarose gel to ascertain that digestion was complete. The linearised plasmid was then extracted with phenol/chloroform and precipitated with 2 volumes of ethanol. The digested plasmid was then de-phosphorylated by removal of the 5' phosphate group from both ends thus

preventing inter- and intra- molecular ligation of the plasmid. The dephosphorylation reaction was carried out as described by Maniatis *et al.* (1982). Briefly, the digested DNA was dissolved in water and the reaction was carried out in a total reaction volume of 50 µl. The phosphatase reaction was set up as follows:

10 X calf intestinal phosphate buffer (CIP)	5 µl
(0.5 M Tris.Cl (pH 9.0), 10 mM $MgCl_2$, 1mM $ZnCl_2$, 10mM spermidine)	
Linearised plasmid in water	43 µl
Calf intestinal alkaline phosphatase (CIAP)	2 µl
(1 unit/µl Boehringer Mannheim, Germany)	

The reaction was incubated at 37°C for 30 min and subsequently stopped by heat inactivation at 70°C for 15 minutes followed by phenol/chloroform extraction. The aqueous phase containing the dephosphorylated DNA was precipitated with ethanol and the DNA dissolved in sterile water at a concentration of approximately 100 ng/µl.

2.5.7 Ligations

Ligations were generally carried out in 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$ and 10 mM DTT, and 25 µg/ml BSA. This buffer was prepared and stored as a 10 X stock. ATP (Sigma) was stored as a 10 mM stock and added to a concentration of 1 mM in ligation reactions. 2 µl of T4 DNA ligase (Amersham, 2.5 U/µl) was used for each ligation. All sticky end ligations were incubated at 14°C overnight. For blunt end ligations, the final concentration of ATP was decreased to 0.05 mM, and the ligations were incubated at room temperature at 4°C overnight.

2.5.8 Preparation of competent bacterial cells, and transformation.

This was performed essentially as described by Hanahan (1988)

Reagents:

FSB: 10 mM potassium acetate (pH 7.4), 45 mM $MnCl_2.4H_20$, 100 mM KCl, 3mM hexamine cobalt chloride and 10% (w/v) glycerol.

DMSO: Aliquots from a newly opened bottle of DMSO (Merck, Analytical grade) were stored in full, tightly capped, 400 µl tubes at -20°C. Aliquots were used only once.

Procedure:

A 500 ml conical flask containing 50 ml of SOB medium was innoculated with 0.5 ml of an overnight culture of JM109. The culture was incubated with agitation at 37°C until the $O.D._{600}$ reached 0.4 to 0.5 (approximately 4 hr). Forty five ml of this culture was cooled on ice for 15 min and then centrifuged at 2,500g in a Heraeus Christ minifuge II for 10 min at 4°C. The pelleted cells were resuspended in 15 ml of ice cold FSB and left on ice for 15 min. The centrifugation was repeated and the cells were resuspended in 4 ml of FSB. After 10 min on ice, 140 µl of DMSO was added and the cell suspension was gently swirled and replaced on ice for another 5 min. A second aliquot of 140 µl of DMSO was added and the tube placed on ice for another 5 min. The cell suspension was then transferred to pre-cooled Eppendorf tubes in aliquots of 220 µl each and instantly frozen in liquid nitrogen. The aliquots were stored at -80°C until used.

During transformations, the competent cells were left to thaw on ice. 100 µl of the competent cells were transferred into a 5 ml pre-chilled polystyrene tube. Between 0.1 and 50 ng of DNA from the ligation was added to the cells and the mixture gently swirled and left on ice for 30 min. The mixture was then heat pulsed at 42°C for 90 seconds after which it was replaced on ice. After addition of 800 µl of SOC medium, the transformed cells were incubated at 37°C for one hour. Meanwhile, NZYM plates containing 50 µg/ml ampicillin were prepared for colour selection by spreading 120 µl of 100 mM isopropyl ß-thiogalactopyronoside (IPTG; Gold Biotechnology, Manchester, MO, USA) and 60 µl of 2% chromogenic ß-galactosidase substrate, X-gal (5-bromo-4 chloro-3 inolodyl-ß-D-galactopyranoside; Boehringer Mannheim Biochemicals), the latter being dissolved in dimethylformamide. Following the incubation of the heat-pulsed cells, about 200 µl of the transformation sample was spread on the plates which were then allowed to dry prior to incubation at 37°C overnight.

2.5.9 Hybridisation screening for putative recombinants

Transformant colonies and recombinant plaques were grown to 1-2 mm in diameter and processed according to the method described by Grunstein and Hogness (1975). Dry nitrocellulose filters (Schleicher and Schuell, Germany) were placed onto the agar surface and marked for alignment by stabbing with a 23 gauge hyperdermic needle dipped in Indian ink. The filters were peeled away as soon as they were completely wetted and placed, colonies or plaques upwards, on 5 ml puddles of denaturing solution (0.5 M NaOH and 1.5 M NaCl) for 5 minutes. The filters were then gently blotted on a dry Whatmann 3 MM paper and transferred on to a 5 ml puddle of neutralizing solution (1 M Tris-HCl pH 7.4 and 3 M NaCl) for 10 minutes. The filters were then given a quick rinse in 2 X SSC and allowed to air dry. The dry filters were stacked between Whatmann 3 MM paper and baked under vacuum at 80°C to enable the DNA to fix on the filters. Plates with colonies were replaced at 37°C to enable the re-growth of the lifted colonies. Subsequently, the plates were sealed with parafilm and stored at 4°C. Triplicate sets of each filter could be generated from each plate with recombinant colonies or plaques, allowing the simultaneous hybridisation of the filters with different probes. The filters were hybridised (Section 2.5.5) using the appropriate radiolabelled probe. The positive colonies were selected after autoradiography by aligning the X-ray films with the plates. The positive clones were grown in liquid culture and processed to isolate the plasmid DNA (Section 2.4.4)

2.6 Library construction

2.6.1 Construction of a genomic library

a) Sonication

A genomic library was generated from total genomic DNA of *T. b. brucei* CP 547 by a modification of the procedure of Huynh *et al.* (1988). Briefly, 40 µg. of the trypanosome DNA was brought up to 2 ml with 0.2 M NaCl in a 10 ml sterilin tube. The material was sonicated for 2 seconds with a setting 5 on a with a Branson Sonifer® cell

disruptor (model B 30) equipped with wattmeter (model A 410) (Branson sonic power co. Danburg, CT). A special microtip was used delivering 300 watts at 1 sec pulses (2 X) at 4°C. The sonicated DNA was ethanol precipitated and the precipitate was washed in 70% ethanol, spun down and the DNA pellet vacuum dried. The sonicated DNA was resuspended in 180 µl of water, of which 8 µl was tested on an agarose gel to examine the level of shearing.

b) Fill-in reaction

Since the DNA had been sonicated, its 3' or 5' overhanging ends were repaired with T4 DNA polymerase in a reaction generating blunt ends. To the 172 µl of sonicated DNA, 20 µl of 10 X T4 DNA polymerase buffer (500 mM Tris-HCl (pH 8.0), 50 mM $MgCl_2$, 50 mM DTT, 500 µg/ml BSA) and 4 µl dNTP (5 mM) was added. The reaction was initiated with 4 µl T4 DNA polymerase (NEB, 3 U/µl) for 60 minutes at 14°C. The reaction was stopped by phenol/chloroform extraction. The DNA was ethanol precipitated followed by a wash in 70% ethanol, spun, and vacuum dried.

c) Methylation of *Eco*R I sites and linker ligation

In order to make the blunt ended DNA suitable for ligation to a cloning vector, it was methylated by *Eco*R I Methylase, ligated to *Eco*R I linkers and digested with *Eco*R I to create cohesive ends. The methylation step was necessary to protect *Eco*R I sites in the genomic DNA. The sonicated DNA was resuspended in 158 µl water and to this 20 µl of 10 X methylase buffer (100 mM NaCl, 100 mM Tris-HCl pH 7.5, 1 mM EDTA, 100 µl of 10 mg BSA/ml) was added. The methyl group was obtained from the addition of 20 µl of 10 X S-adenosylmethionine SAM (NEB). To the mixture, 2 µl of *Eco*R I methylase 400 U/ml (NEB) was added and the ingredients were mixed gently by pipetting up and down. The reaction was carried out by 2 hr incubation at 37°C. The reaction was stopped by phenol/chloroform extraction. The methylated DNA was then ethanol precipitated and subsequently washed in 70% ethanol. The DNA was pelleted by spinning and then vacuum dried. To ligate the *Eco*R I linkers, the DNA was resuspended

in 86 µl of distilled water, to which 10 µl of 10 X ligase buffer and 2 µl of *Eco*R I linkers 2 µg/µl (NEB) were added. The reaction was initiated by 2 µl of T4 DNA ligase (Amersham 2.5 U/µl) and incubated at 12°C overnight.

d) Removal of excess linkers

After the overnight incubation, the ligation reaction mixture was centrifuged briefly and placed in a 65°C waterbath for 10 min to inactivate the ligase. To digest the ligated and excess linkers, the tube was then placed on ice for 5 min before addition of 85 µl of sterile distilled water and 10 µl 10 X *Eco*R I buffer. The reaction was initiated with the addition of 5 µl (20 U/µl) of *Eco*R I and incubated at 37°C overnight.

e) Potassium acetate gradient

The DNA was separated from the excess linkers by size fractionation. This was done in a (5-20% w/v) potassium acetate (KAc) density gradient spun in an ultracentrifuge using the Beckman SW 27 rotor. Twenty percent KAc, 2 mM EDTA, 1 µg/ml ethidium-bromide was prepared and filtered through a 0.45 µm (or 0.22 µm) filter. A volume of 2.6 ml of 20 % KAc solution was added to the back chamber of a small gradient maker. Air bubbles were removed from the tubing connecting the two chambers by allowing the solution to flow into the front chamber followed by tilting to allow bubbles to float upwards. The passage between the chambers was closed and 2.5 ml of the 5% solution added to the front chamber. The apparatus was then placed on a stirring plate, with the magnetic bar stirring as fast as possible. The stopcock connecting the two chambers was first opened followed by the front stopcock, allowing the flow of the solutions to form a gradient. A 5 ml polyallomer SW55 tube (Beckman) was filled from the bottom with the KAc gradient. The gradient was overlaid with the entire DNA sample. A balancing tube was prepared, and the gradient spun for 3 hr at 70,000 X g at 20°C. The fractions were collected from the gradient by piercing the tube with a vacutainer needle. All but the top 2 cm of the gradient were collected as 300 µl fractions and the size of each fraction tested on an agarose gel. The fractions with fragments

between 2 and 6 kb were pooled and ethanol precipitated with two volumes of absolute ethanol at -20°C. The DNA was spun at 12,000 X g for 30 min, the ethanol decanted, and the DNA pellet washed with 70% ethanol and vacuum dried. The DNA was then resuspended in 80 µl of sterile distilled water.

f) Cloning of genomic DNA into bacteriophage lambda gt11

The target DNA was cloned into bacteriophage lambda gt11. The *Eco*R I cohesive ends of the target DNA were inserted into the *Eco*R I site of the lambda gt11 located within the *lac*Z gene, downstream from the ß-galactosidase initiation codon. On the day before the experiment, an overnight culture of *E. coli* Y1090 was started by inoculating a single colony into 50 ml of LB medium supplemented with 0.5 ml of 20% maltose and 0.5 ml of 1 M $MgSO_4$. This was shaken overnight at 37°C and the cells were pelleted by centrifugation and then resuspended in 25 ml of 10 mM $MgSO_4$ and stored at 4°C.

Four standard ligations were set up using 3 µl, 5 µl, 7 µl and 10 µl of the target DNA in a total volume of 20 µl. In all the ligations a constant concentration of 0.5 µg of commercial Protoclone lambda gt11 and 0.5 µl of T4 DNA ligase (Amersham, 2.5 U/µl) were used. The ligations were incubated for 3 hr at 14°C. Subsequently, the ligated DNA was packaged using the Packagene *in vitro* packaging system (Promega). This was mixed gently by tapping the bottom of the tubes several times and the mixture incubated for 2 hr at room temperature. To this, 480 µl of SM phage storage buffer was added, followed by 15 µl of chloroform. This was mixed gently by inversion and the chloroform allowed to settle to the bottom of the tube.

g) Titration of recombinant lambda gt11 library

Dilutions of 1/1,000 and 1/10,000 of the packaging extracts were made in SM phage buffer. The diluted phage (100 µl) was used to infect 100 µl of freshly prepared *E. coli* Y1090 cells and the phage was allowed to absorb for 30 min at 37°C. Y1090 contains (a) a plasmid, over-expressing a *lac* I repressor which prevents *lac*Z-directed gene

expression. The repressor is inactivated by the addition of IPTG to the medium; (b) a deficiency in the *lon* protease which increases the stability of recombinant fusion proteins; (c) *sup*F to suppress the (S100) phage mutation causing defective lysis.

Molten (45°C) LB top agar (3 ml), into which 40 µl of 1 M IPTG and 40 µl of 40 µg/ml of the chromogenic indicator 5-bromo-4-chloro-3-indolyl ß-D-galactosidase (X-gal) had been added, was placed into the tubes containing the bacteria. The tubes were mixed gently and poured immediately onto LB plates. The top agar was allowed to harden and the plates were incubated inverted at 37°C overnight. The size of the library was determined by counting the number of plaque forming units per millilitre of the original packaging extract.

2.6.2 Construction of a cDNA library

Messenger RNA (Section 2.4.3) was copied into single stranded cDNA using a kit purchased from BRL (Bethesda, Maryland, USA) following the manufacturers instructions. The reaction conditions were designed to convert 2-20 µg poly(A^+) RNA into double stranded (ds) cDNA. To a sterile microcentrifuge tube on ice, the following components from the kit and the mRNA were added for the first strand synthesis: 5 X first strand buffer (10 µl), 10 mM dNTP mix (2.5 µl), oligo (dT) primer (3 µl, final concentration 50 µg/ml) and 20 µl of diethylpyrocarbonate (DEPC)-treated triple distilled water and poly(A^+) RNA (10 µg). The final composition of the first strand reaction was 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 500 µM each dATP, dGTP, dCTP and TTP, 50 µg/ml oligo(dT) and 100 µg/ml poly(A^+) RNA. To the mixture was added 1 µCi of [α-^{32}P] dCTP tracer, before initiating the reaction by addition of 2.5 µl of cloned Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (final concentration 10,000 U of enzyme/ml). The reaction was incubated at 37°C for one hour and then transferred to ice. This was then processed for the second strand cDNA synthesis reaction by adding the following reagents: DEPC-treated water (289.5 µl), 10 mM stock dNTP mix (6 µl), 10 X second strand synthesis buffer (40 µl), [α-^{32}P] dCTP (1.25 µl), *E. coli* DNA polymerase I (10 µl) and *E. coli*

RNase-H (1.75 µl). The composition of this mixture was 25 mM Tris-HCl (pH 8.3), 100 mM KCl, 5 mM $MgCl_2$, 250 µM each dATP, dCTP, dGTP and TTP, 5mM dithiothreitol, 250 U/ml DNA polymerase I, and 8.5 U/ml RNase-H. The tube was vortexed gently and incubated at 16°C for 2 h, after which the reaction mixture was moved to ice and the reaction stopped by addition of 25 µl of 250 mM EDTA (pH 7.5).

The cDNA in the reaction mixture was extracted with an equal volume of phenol/chloroform and ethanol precipitated. The pellet was redissolved in 200 µl of sterile T.E buffer, adjusted to 700 mM ammonium acetate and reprecipitated with ethanol. The pellet was washed with 70% ethanol, dissolved in 20 µl of sterile T.E buffer, before using 2 µl for gel analysis. The volume of the double stranded (ds) cDNA was adjusted to carry out the methylation reaction, the linker ligation, the removal of excess linkers and the cloning of the cDNA in bacteriophage lambda gt11, essentially as for the genomic library (Section 2.6.1). A cDNA Library of 6×10^5 recombinant was obtained with over 95% recombinants per plate. The screening of the recombinants was done essentially as previously described (section 2.5.9).

2.7.1 Polymerase Chain Reaction (PCR)

No single protocol of PCR is appropriate to all situations. Consequently, each new PCR application generally required optimisation. The standard conditions used to amplify most target sequences in a total volume of 100 µl is outlined by Innis *et al.* (1990):

Template DNA (1-5 ng)

20 pmoles of each primer

20 mM Tris-HCl (pH 8.3) 20°C

1.5 mM $MgCl_2$

50 mM KCl

0.05% Tween 20

0.05% Nonidet

50 μM each dNTP

2 units of *Taq* (*Thermus aquaticus*) DNA polymerase (Promega). The mix was overlaid with 75 μl of mineral oil. The tubes were placed in a Perkin Elmer programmable thermal cycler which was set using the following temperature profile for 35 to 40 cycles:

Denaturation at 94°C, for 1 min

Primer annealing at 55°C for 30 s

Primer extension at 72°C for 1.5 min

Cycling concluded with a final extension at 72°C for 5 minutes. Reactions were stopped by chilling to 4°C. The primers used in this work are listed:

ILO: 2236

Miniexon (universal): TAG GCG GCG CTA GAA CAG TTT CTG TAC TAT ATT

ILO: 2235

Oligo d(T)

TAG GCG CGC TTT TTT TTT TTT TTT TTT TTT

ILO· 1148

Nla III repeat (F)

GAA ACC CAA TCA CCC C

ILO: 1149

Nla III repeat (R)

CCC CAC TAA CCC AAA G

In this work PCR amplification technology was also exploited to amplify unknown sequences using random hexanucleotides.

2.7.2 Amplification of membrane-bound DNA using random hexanucleotides in PCR

Total undigested DNA (2 μg) from CP 547 was electrophoresed in a 1% agarose gel and transferred to a nylon filter (Hybond-N, Amersham Int., U.K.). The 6.6 kbp

element could not be visualised by ethidium-bromide staining, but could be localised on the membrane by hybridisation with ^{32}P-labelled total trypanosome DNA. It was estimated that there was a maximum of 1 ng of the 6 kbp element bound to the filter. The area on the filter encompassing the area to which the 6.6 kbp element was bound (approximately 0.2 cm^2) was excised and washed twice in 20 ml of distilled water for 15 min. The washed filter was then boiled in 50 µl of sterile water containing 10 µg of random hexanucleotide primers (Sigma, U.K.) for 5 min then placed on ice (10 µg of primer was found to give optimum results in the reaction). The PCR was then carried out in a 100 µl reaction volume containing 100 µM dNTP, 3 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 0.05% NP 40, 0.5% Tween 20, 50 mM KCl and 2.5 units of *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). The PCR profile was 95°C for 45 seconds, 30°C for 1.5 min and 72°C for 3 min, for a total of 40 cycles. DNA from the gel-purified element in solution was also amplified by PCR using the same conditions. The amplified DNA from either source was subsequently purified from the primers and from unincorporated nucleotides on a Sephadex G-50 spin column. The PCR products were then ^{32}P-labelled by random priming or nick translation.

2.7.3 Polymerase chain reaction amplification of unknown sequences using arbitrary primers

Welsh and McClelland (1990) and Williams *et al.* (1990) have described a method for the analysis of DNA polymorphisms by using arbitrary primers in the PCR. More recently, Waitumbi and Murphy (1993) have used arbitrarily primed PCR (AP-PCR) to show intra- and inter-species differences in trypanosomes.

AP-PCR conditions were similar to standard PCR conditions except that the reaction contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 0.05% NP40, 0.05% Tween 20, 200 µM of dNTPs, 0.6 mM primer and 2.5 units of *Taq* DNA polymerase (Promega) and were performed on a Perkin Elmer thermal cycler. The cycling conditions were 94°C for 1 minute, 40°C for 1.5 minutes and 72°C for 2 minutes, for 40 cycles.

2.7.4 ILRAD oligonucleotide numbers

The arbitrary primers used in this work are listed:

508	CGGCCCCTGT	1058	GCACGAGGCG
509	TGGTCAGTGA	1059	GGCCCGTCCG
522	TAAGCATCAACACCACGTGG	1060	CTGAGCCCCC
523	CGAGCCGCGG	1061	CGGGGCGGCT
524	CGCGCCCGCT	1062	CTCCGTCGCG
525	CGGACGTCGC	1069	GCCACGAGGC
526	GCCGTCCGAG	1070	GGAGGCTGCG
527	GCGCGCAGCG	1070	GGAGGCTGGG
539	CGGCGGAGCT	1071	GCAGGACCGG
540	GAGGGGGCGT	1080	GCGCACGGCG
541	GCGGCTGCCA	1100	GCTTGTTCTGGATGGCCTC
542	GGGTGCGCGG	1110	CCTCGGTGAG
543	GTGTCCGGCG	1112	TCCGGCCGCT
548	CACCGAGTCCGTGTCACC	1113	GTGACCCGGG
868	CAGCCTCGGC	1114	GGACCGCACG
869	CAGGACGGAG	1114	GGACCGCACG
872	CCCGCCATCT	1115	GGCTGACGCG
873	CATGTGCAGG	1122	GCTGGCTCGC
874	GAGGTGGCGC	1123	AGACACCGCC
875	GTCCGTGAGC	1124	TGCGCCAGGC
876	GGGACGTCTC	1125	CGGGAGAGCG
878	GTCGCGGAGC	1126	GTTGGCTCGC
906	CCTTCATTCATCCTAC	1126	GTTGGCTCGC
914	CAGGGGGCCA	1127	CCGCGCCGGT
922	GGCCCTATCA	1128	CGGAGGCTTC
944	GGCCGCCACATTGG	1129	TGTGGTGGGC

967	TCTACCACCGATACAGATGG	1131	AACGAGGGCC
1047	CCCACGGGGG	1187	GTGGCGGGCG
1048	GGGGCCTTGG	1188	CGCAGCCGTG
1050	CTGCTGGCAC	1189	CCGGTTCTCC
1051	CCCCGCCTGT	1190	GGGGGGCTGC
		1191	CGCAGCCTGC
		1242	CGCATGGGTTGTCAGTGG

2.8 DNA sequencing

2.8.1 Deletions of target DNA for double-stranded DNA sequencing

To generate unidirectional deletions of predictable sizes in the insert, a commercial Exo III/Mung Bean deletion kit (Stratagene) was used. Following the manufacturers instructions, this system allowed deletions in the insert, but not in the pBluescript plasmid vector DNA. The principle involves double digestion to completion of the recombinant plasmid with a unique (not in the insert) 3' overhang restriction site, and a unique 5' site between the insert and the 3' site chosen. The polylinker has the unique 3' restriction site on the outside edge of the polylinker, and 5' sites internally. Thus, with the progression of the Exonuclease III, nested deletions of the insert can be constructed. Subsequent treatment with Mung Bean nuclease digests the single stranded DNA to create blunt ends that can be ligated to circularise the plasmid for use in double stranded sequencing. For each time point, 5 µg of double digested DNA was treated in 12.5 µl of 2 X Exo buffer (100 mM Tris-HCl, pH 8; 10 mM $MgCl_2$, 20 µg/ml tRNA), 2.5 µl 100 mM 2-mercaptoethanol and 1 µl Exonuclease III (100 U/µl of DNA). A total volume of 25 µl was made up with sterile water. At 30°C, approximately, 230 bp were deleted per min. The reaction of each time point was stopped by heat inactivation at 68°C for 15 min and then placed on ice. Subsequently, each time point tube was incubated with 15 U of Mung Bean nuclease for 30 min at 30°C. This reaction was stopped with 4 µl of 20% SDS, 10 µl 1 M Tris-HCl (pH 9.5) and 20 µl 8 M lithium chloride. The DNA was extracted with 250 µl phenol/chloroform, ethanol precipitated

followed by a wash in 70% ethanol, spun, and vacuum dried. The blunt end Exo/Mung DNA was then ligated and the plasmid amplified for use in double stranded sequencing.

2.8.2 Sequencing reaction protocols

A modification of the dideoxy or chain termination sequencing method developed by Sanger and Coulson (1977) was used. The method utilises DNA polymerase to synthesize a complementary copy of the single stranded DNA template. The enzyme catalyses chain elongation at the 3' end of the primer DNA which is annealed to the template DNA. The deoxynucleotide added to the growing primer chain is directed by base-pair complementation to the template DNA. Chain growth involves the formation of a phosphodiester bridge between the 3'-hydroxyl group at the growing end of the primer and the 5' phosphate group of the incoming deoxynucleotide, resulting in an overall chain growth in the 5' to 3' direction.

This method capitalises on the enzymes ability to use 2', 3'-dideoxynucleotide triphosphates (ddNTPs) as substrates. When a ddNTP is incorporated at the 3' end of the growing primer chain, the chain is terminated as it lacks the required 3'-hydroxyl group for chain elongation. By using four different ddNTPs in four separate enzymatic reactions, populations of oligonucleotides are generated that terminate at positions occupied by every A, C, G or T in the template strand. These populations of oligonucleotides are then resolved by electrophoresis and visualised on X-ray film due to incorporation of radionucleotides during chain elongation.

In this work, sequencing of double stranded DNA strands was carried out. A commercial kit (Sequenase, United States Biochemical, USA) was used.

For the sequencing of double stranded plasmid DNA, between 3 and 5 μg of plasmid DNA in a volume of 16 μl of water was denatured by 2 μl of 2 M NaOH and 2 μl of 0.1 M EDTA (pH 8.0). The mixture was incubated for 5 minutes at 65°C. The denatured plasmid was neutralised with 2 μl of 3 M sodium acetate (pH 5.2), and the DNA precipitated with 60 μl of ice-cold ethanol. After washing the pelleted DNA in 70% ethanol, it was re-dissolved in 7 μl of distilled water before the addition of the

sequencing buffer and primer Annealing of the primer to the denatured target DNA was carried out in a total volume of 10 µl by adding 1 µl Universal primer (100-200 ng) and 2 µl 5 X reaction buffer (200 mM Tris-HCl, pH 7.5), 100 mM $MgCl_2$, 250 mM NaCl). The mixture was mixed well and warmed to 70°C for 2 minutes and left to cool to room temperature over a period of 20-30 minutes and then placed on ice.

The labelling reaction was then carried out by adding to the annealed template-primer 1 µl of 100 mM DTT, 2 µl of diluted (1:5) labelling mix, 0.5 µl of [α-^{35}S] dATP and 2 µl of diluted (1:7) Sequenase^{R}. This mixture was incubated for 2-5 min at room temperature.

In the termination reaction, 2.5 µl of ddNTP termination mix (80 µM of each dNTP A, C, G, T) was placed in the respective tubes labelled A, C, G and T. To each of these tubes 3.5 µl of the labelling incubation mix was added and the tubes were incubated at 37°C for 5 min. The reaction was stopped with 4 µl of a stop solution. Prior to loading on the gels, the samples were heated to 75°C for 2 minutes.

Sequencing gels were prepared as a 6% polyacrylamide mix made from a 40% stock solution (2 g bisacrylamide, 38 g of acrylamide in 100 ml of distilled water). To 10 ml of 10 X TBE (108 g Tris, 55 g boric acid, 9.3 g EDTA) and 15 ml of 40% acrylamide, 42 g of urea was dissolved and made up to a total volume of 100 ml. This was warmed to dissolve the urea and then cooled to 4°C. Polymerisation was initiated by addition of 600 µl of 10% ammonium persulphate and 100 µl of TEMED (Ultra-pure, BDH, U.K.). The gels were cast in a BRL model 50 sequencing gel apparatus using 0.2 mM spacers and shark tooth sample combs. Both plates were siliconised by wiping with 5% dimethyl dichlorosilane in carbon tetrachloride followed by ethanol. The sequencing reactions were heated at 75°C for 2 min before loading 3 µl of the samples into the sample wells.

The gels were run with a Pharmacia model ECPS 3000/150 power supply. Electrophoresis was carried out at a constant power of 70 watts in 1 x TBE running buffer.

The short runs generally took 1½-2 hr and the long runs 5 hr. On completion, the gel plates were carefully separated and the plate holding the gel was gently immersed into

fix solution (19% acetic acid, 10% methanol) for 15 minutes to wash out the urea. The gels were transferred onto Whatman 3 MM filter paper by pressing in onto the gel and gently rolling the gel off the plate. It was then wrapped in saran wrap and dried at 80°C under vacuum. Exposure for autoradiography was done with direct contact between the dried gel and the emulsion-coated side of an X-ray film, which was left overnight in a cassette at room temperature. The sequencing gels were read using a sonic digitiser (Science Accessories Corp., Stratford, Conn., USA) interfaced to a microcomputer. The sequencing data were analysed using a Dnasis/Prosis program (Hitachi Software Engineering, Brisbane, CA, USA) on an IBM PS/2 Model 80 computer with a 386 microprocessor.

CHAPTER 3

RESULTS

3.1.1 Search for amplified genomic sequences in a multidrug-resistant *T. b. brucei* isolate (CP 547).

Current data indicate that gene amplification is an important mechanism employed by parasites to survive drug selection (Beverley, 1991). However, the possibility of gene amplification being employed for the evasion of drug pressure by drug-resistant African trypanosomes has not been carefully examined. In drug-resistant *Leishmania* spp. amplified DNA often constitutes as much as 5%-10% of total parasite DNA (Coderre *et al.*, 1983) and the amplified DNA is readily detected as abundant DNA fragments in undigested and restriction enzyme digested total genomic DNA (Coderre *et al.*, 1983; Beverley *et al.*, 1988). Attempts were therefore made to look for the presence of amplified sequences that may correlate with drug resistance within a multidrug-resistant *T. b. brucei* isolate (CP 547).

In an experiment designed to detect amplified DNA sequences, total genomic DNA was purified from bloodstream forms of the drug-resistant CP 547 isolate and a drug-sensitive ILTat 1.4 clone. 2 μg of undigested and overnight restriction enzyme digested genomic DNA from both the drug-resistant and the drug-sensitive populations was electrophoresed in two identical 1.2% agarose gels, and stained with ethidium-bromide (Figure 4a). From these ethidium-bromide stained gels, no differences were apparent between the resolved DNA from the drug-resistant and the drug-sensitive populations. Southern blots of the two identical gels were generated and one blot was hybridised with ^{32}P-labelled total DNA from the drug-resistant CP 547, while the second blot was hybridised with ^{32}P-labelled total DNA from the drug-sensitive ILTat 1.4 clone. The two blots had almost identical hybridisation profiles for the restriction enzyme

digested DNAs, despite the use of the two different DNA probes (Figure 4b). The hybridisations disclosed some of the well characterised repetitive DNA sequences within *T. b. brucei*, namely TRS-1 (ingi) (Kimmel *et al.*, 1987; Murphy *et al.*, 1987), the 177 bp Alu repeat (Sloof *et al.*, 1983), the linearised minicircles (Borst and Hoeijmakers, 1979) and the well characterised miniexon repeat unit (Cornelissen *et al.*, 1986; Walder *et al.*, 1986) (Figure 4b). In addition to these well characterised repetitive sequences, hybridisation was also evident to other, as yet uncharacterised, repetitive sequences. However, hybridisation to a band migrating as a 6.6 kbp DNA fragment was observed in the lane of undigested DNA from the drug-resistant CP 547 isolate, but not the drug-sensitive clone (Figure 4b, lane 1). In addition, there was a weaker hybridisation signal with a band migrating as a 13 kbp fragment in the undigested DNA samples of CP 547 (Figure 4b), possibly a dimer or a higher hybridising form of the 6.6 kbp fragment. Taking into consideration that the 6.6 kbp element was not visible on the ethidium-bromide stained gels, and it is possible to visualise 10 ng of DNA, it was estimated that there are less than 70 copies of the 6.6 kbp element per genome of the CP 547 isolate.

With agarose gel electrophoresis, the kinetoplast DNA networks are trapped within the gel slot and do not migrate into the gel. The undigested chromosomal DNA enters the gel but does not migrate past the 20 to 40 kbp region. The migration of the 6.6 kbp fragment in the undigested DNA samples thus indicates that this element is neither of chromosomal nor kinetoplast origin, but is of extrachromosomal origin. That the revelation of this extrachromosomal element was not just an artefact was verified by re-expanding the population of CP 547 from the original stabilate and then comparing DNA purified from it with DNA purified from two characterised drug sensitive *T. b. brucei* clones, ILTat 1.1 and 221 (Kaminsky and Zweygarth 1989a) in addition to two *T. b. brucei* populations ILTar 2, and IL B10 whose drug-sensitivities are unknown.

3.1.2 Initial characterisation of the 6.6 kbp DNA

Since the extrachromosomal 6.6 kbp element represents the first identification of an extrachromosomal element in African trypanosomes, further characterisation of it was

warranted. In addition, some extrachromosomal nucleic acid elements are known to be important carriers of genetic information (Esser *et al.*, 1986; Wickner *et al.*, 1986). They can originate from several different sources, some common examples being organellar genomes, amplified genomic DNA sequences, viral genomes and plasmids. Hybridisation of total genomic DNA from the drug-sensitive trypanosomes to the 6.6 kbp element in CP 547 (Figure 4b) indicates that sequences similar to those on the element are also present within the genomes of the drug-sensitive clone, although not as extrachromosomal DNA. Thus, the presence of the element in only the drug-resistant isolate suggested a form of amplification which may be responsible for the drug resistance phenotype, analogous to gene amplification in drug-resistant *Leishmania* spp. (Beverley *et al.*, 1988). In drug resistant *Leishmania* spp., extrachromosomal elements such as H and R circles are known to be directly associated with drug resistance (Section 1.4.2). The characterisation of such elements and amplified sequences in parasitic organisms is of particular importance, as knowledge about them may shed light on particular biological nuances of the parasite which may be exploited in devising new control measures. For instance, the outcome of similar studies on the amplified sequences of the DHFR-TS gene in pyrimethamine-resistant *P. falciparum*, has had an important impact on the control of malaria. It is now known that with the control of pyrimethamine usage the selective drug pressure that maintains some highly resistant organisms can be removed and the sensitivity to pyrimethamine restored (Inselburg *et al.*, 1989).

The characterisation of the 6.6 kbp element was further warranted because of its small size and potential exploitation as a vector for experimental transformation of *T. brucei* or other African trypanosome species. Such a vector could be of significant value in molecular biology studies of trypanosomes.

3.1.2a Purification of the 6.6 kbp element

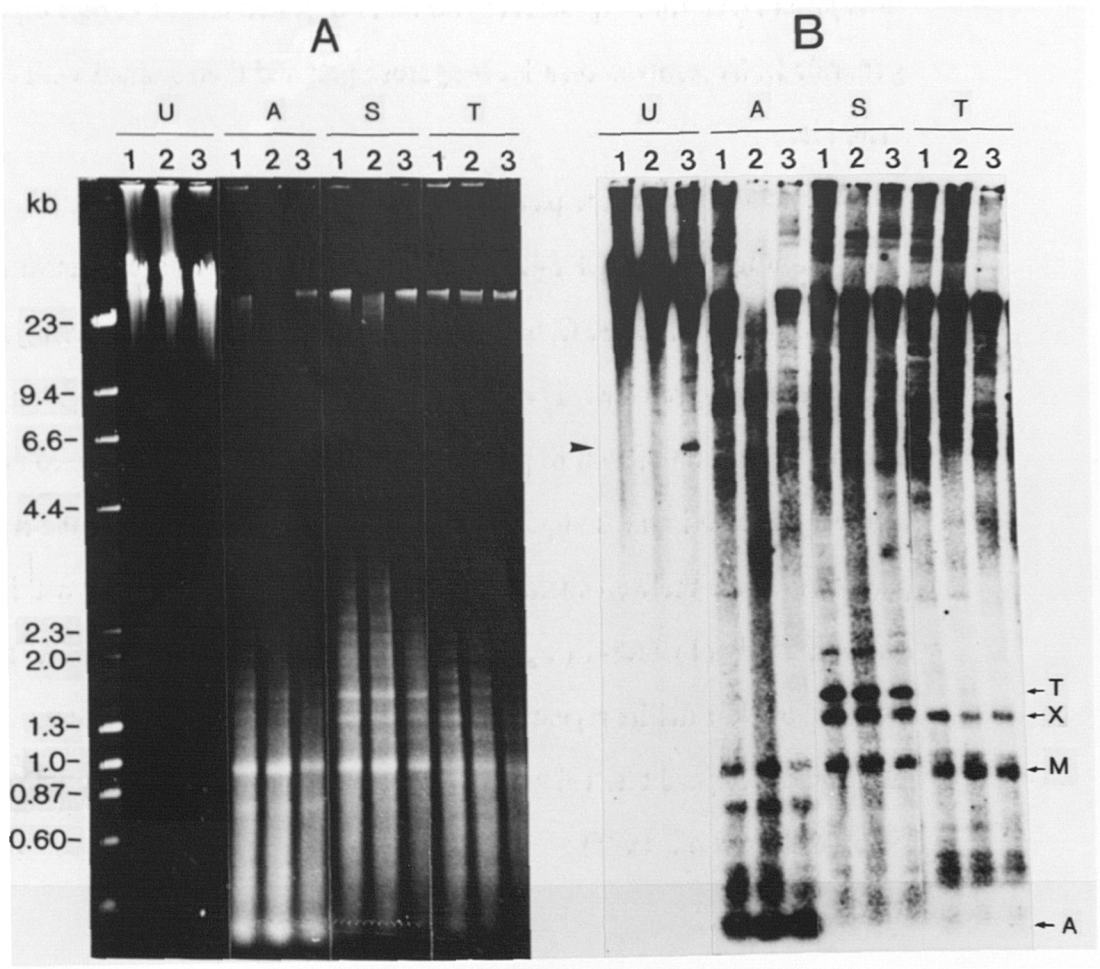
Since the 6.6 kbp element is not visible in ethidium-bromide stained gels, its copy number was expected to be low. Therefore, initial purifications involved running

Figure 4

Comparison of the drug-resistant *T. b. brucei* CP 547 isolate and drug-sensitive clones ILTat 1.4 and GUTat 3.1. In the figure are tracks of drug-sensitive ILTat 1.4 (lane 1) and GUTat 3.1 (lane 2) and the multidrug-resistant isolate CP 547 (lane 3).

(A) 2 μg of total DNA from each population was grouped together as (U) undigested, (A) *Alu* I digested, (S) *Sau*3A I digested and (T) *Taq* I digested total genomic DNA resolved on a 1.2% agarose gel; and then stained with ethidium-bromide.

(B) A Southern blot of the panel A was hybridised with ^{32}P-labelled total DNA from the drug-resistant CP 547 parental population. The filter was washed at high stringency (0.1 X SSC, 65°C) and exposed to X-ray film overnight at -80°C with intensifying screens. The size of the DNA size markers are given in kbp. The arrow head on the left of panel B indicates the 6.6 kbp extrachromosomal element detected in the undigested CP 547 DNA. The arrows on the right of panel B indicate the well characterised repetitive DNA sequences within *T. b. brucei*; namely (T) TRS-1 (ingi) (Kimmel *et al.*, 1987; Murphy *et al.*, 1987), (A) the 177 bp Alu-satellite repeat (Sloof *et al.*, 1983), (M) linearised minicircles (Borst and Hoeijmakers, 1979) and (X) miniexon repeat unit (Cornelissen *et al.*, 1986; Walder *et al.*, 1986)


A
B
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1 2 3 1 2 3 1 2 3 1 2 3
kb
23-
9.4-
6.6-
4.4-
2.3-
2.0-
1.3-
1.0-
0.87-
0.60-
T
X
M
A

approximately 300 µg of undigested total DNA from CP 547 on large sized, 20.5 cm x 24 cm, preparative 1% agarose gels. The agarose gel slice corresponding to the 6.6 kbp region was then excised and the DNA extracted either by the geneclean® method or electroelution. Major losses were incurred during the purification of the relatively small quantity of DNA contained in the large agarose slices. During geneclean® the agarose gel slice of an approximate weight of 15 gm was dissolved in about 45 ml of saturated sodium iodide solution. Although a ten-fold excess of glass milk (50 µl) was added to the 50 ml sodium iodide and DNA mixture, the chances for the glass milk beads to interact and then bind to the DNA were greatly reduced in the very dilute DNA solution. Similarly, with eletroelution, the 6.6 kbp DNA was greatly diluted which made its precipitation not only inefficient but also resulted in co-precipitation of large amounts of contaminating components from the agarose. Generally, 500 µg of total genomic DNA was required to isolate a sufficient quantity of the 6.6 kbp element to perform one ^{32}P-labelling experiment by random priming.

The ability to label the 6.6 kbp element with random primers and the Klenow fragment of *E. coli* DNA polymerase I verified that the 6.6 kbp element was DNA. Attempts to radiolabel the 6.6 kbp element by the nick translation method resulted in poor ^{32}P incorporation, most likely because there was insufficient DNA and/or because gel components inhibited the reaction.

From the specific activity of the radiolabelled probe generated from the gel isolated 6.6 kbp element, it was estimated that from the 500 µg of genomic DNA approximately 20 ng of DNA from the 6.6 kbp region could be isolated. Using this value and assuming a 50% loss during the purification, it was estimated that the copy number of the 6.6 kbp element was approximately one per genome.

3.1.2b Initial efforts at cloning sequences from the 6.6 kbp element

The gel-purified 6.6 kbp DNA was used as a radiolabelled probe on the same Southern blot shown in Figure 4b after removal of the original probe. In such blots, curiously, the 6.6 kbp probe hybridised back to the 6.6 kbp element very faintly or not at

all, whereas it hybridised strongly to restriction fragments from both the drug-resistant and the drug-sensitive populations (Figure 5a). Hybridisations of additional Southern blots like those of Figure 5a, generated from different preparations of the element, indicated that the hybridisation profiles of the restriction fragments could be organised into classes representing the relative frequency of hybridisation to a particular restriction fragment. The first class represents restriction fragments that hybridised most frequently with the different preparations of the 6.6 kbp probes (given in Table 8). However, among this class some fragments were not always apparent in the different Southern blots. The second class represented restriction fragments that hybridised infrequently with the different 6.6 kbp probes. The third class represented fragments that were unique to the different probes. The different Southern blots contained hybridising restriction fragments from the three classes in varying combinations. Figure 5c shows a representative example of an identical set of Southern blots of total CP 547 DNA digested with some commonly used restriction enzymes, each probed with a different isolation of the gel-purified 6.6 kbp DNA.

At this stage of the analysis, the variability with respect to infrequent hybridisations to some restriction fragments was attributed to incomplete transfer of DNA during blotting and the presence of varying amounts of contaminating sheared genomic DNA which co-purified with the different preparations of the 6.6 kbp region. The latter explanation was, however, incomplete considering that the different 6.6 kbp probes did not hybridise strongly to any of the well characterised *T. b. brucei* repetitive sequences which are the most likely sequences expected among contaminating DNAs. This indicated that the 6.6 kbp region is relatively pure and free of contaminating genomic DNA. Therefore, it was possible that the inconsistencies in the hybridisations were due to variations within the 6.6 kbp region itself or due to variations within sequences hybridising to the 6.6 kbp region. Evidence to support this contention is presented later in section 3.5.1c.

In the Southern blots of different restriction enzyme digests it was not clearly evident which (if any) enzymes cut the 6.6 kbp element, and what its resulting profile

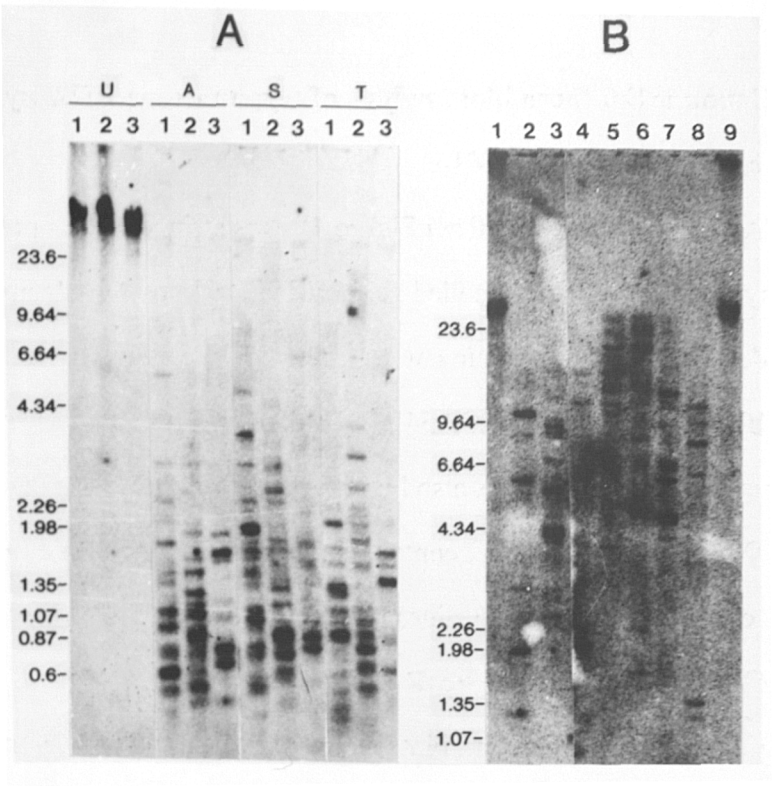
Figure 5

Genomic Southern blot analysis of trypanosome DNA by hybridisation with the purified 6.6 kbp DNA.

(A) The Southern blot from Figure 4B was stripped of its previous probe and re-hybridised with ^{32}P-labelled gel purified 6.6 kbp DNA from CP 547 in order to identify putative genomic copies of the 6.6 kbp element.

(B) A Southern blot of CP 547 DNA digested with six base pair cutting restriction enzymes was also hybridised with ^{32}P-labelled gel purified 6.6 kbp DNA. Lanes (1) and (9) contain undigested total genomic DNA while lanes 2 to 8 contain genomic DNA digested with (2) *Sph* I, (3) *Pst* I, (4) *Sal* I, (5) *Sma* I, (6) *Kpn* I, (7) *Sac* I, (8) *Eco*R I.

(C) Two sets of Southern blots were hybridised with different preparations of the 6.6 kbp element (numbered 1 to 4). Probes 1 and 2 were hybridised consecutively to one Southern blot which was stripped between probing. Similarly probes 3 and 4 were hybridised to a second Southern blot. The position of the size marker fragments is indicated by the lines on the left of each blot. Differences in hybridisation signals between probes are shown by the brackets and asterisk on the right of each pair of lanes.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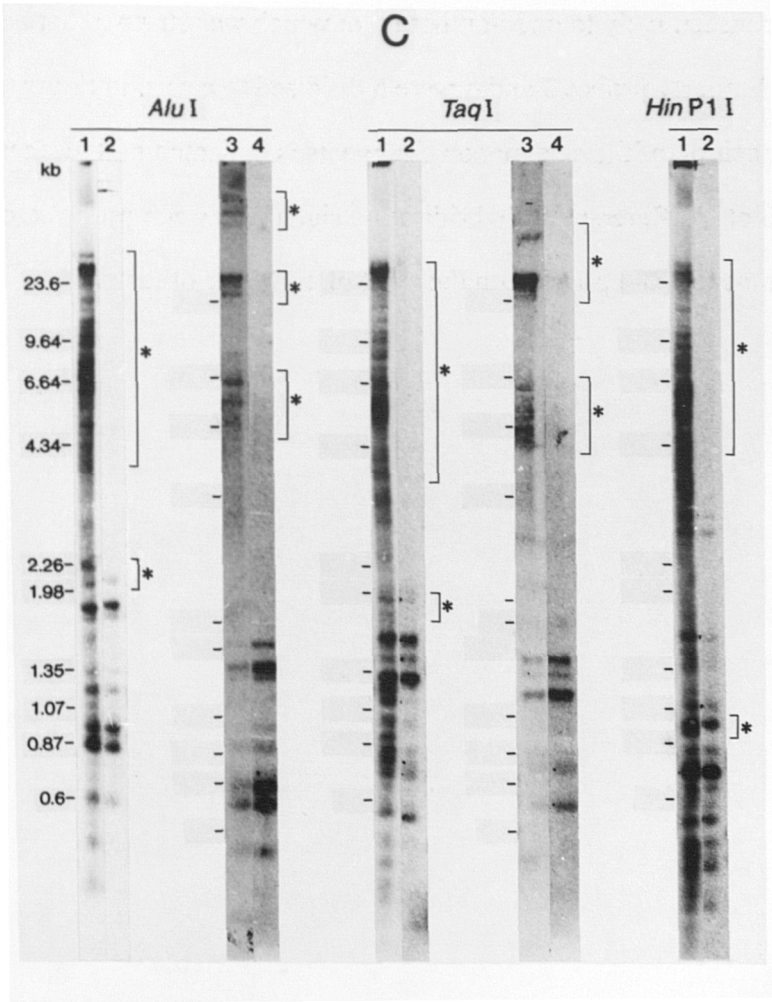
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was. This was partly because hybridisation of the 6.6 kbp probe to the various genomic copies masked the digestion profile for the 6.6 kbp element. Furthermore, because of the differences between the hybridisation profiles of the drug-resistant isolate in comparison to the drug-sensitive clone, it was difficult to distinguish the restriction fragments originating from possible digestion of the 6.6 kbp element (Figure 5a and 5b). This problem was compounded by the fact that hybridisation to the element itself was weak or nonexistent. Nevertheless, the hybridisation profiles with the gel-purified 6.6 kbp probe were obviously selective since there was lack of hybridisation to known repetitive sequences. At this point it was unclear why there was weak or lack of hybridisation to the 6.6 kbp element with probes generated from the region encompassing the 6.6 kbp element. The possible reasons for some of these problems became apparent later, but at this stage attempts to clone fragments from the element were initiated.

As there was only sufficient purified 6.6 kbp DNA for either cloning or restriction analysis, it was decided that it would be preferable to attempt to directly clone the element or sequences from it. Since it had been difficult to determine the restriction enzymes that might cut the 6.6 kbp element, the approach involved taking aliquots of the gel-purified 6.6 kbp DNA and digesting each aliquot with restriction enzymes that cut within the polylinker sequence of the *E. coli* plasmid vector pBluescript. Efforts to clone the digested 6.6 kbp DNA into pBluescript digested with the same restriction enzymes yielded only very few recombinants. DNA was purified from these recombinants and subsequently tested as ^{32}P-labelled probes on Southern blots containing undigested CP 547. None of the clones hybridised to the 6.6 kbp element, and the restriction enzyme digestion profiles were too ambiguous to establish whether the cloned sequences were derived from the 6.6 kbp element or were due to contaminating genomic sequences.

The apparent failure to clone sequences from the purified 6.6 kbp element was attributed to several possible reasons: (1) The presence of contaminating components from the agarose which may have been sufficient to inhibit the restriction enzymes used; (2) The 6.6 kbp region may not be double stranded DNA and therefore cannot be cut by restriction enzymes; (3) The 6.6 kbp region may lack restriction enzyme sites either

through containing simple sequence repeats or modified bases; (4) Since very small quantities of 6 8 kbp DNA could be isolated from the gel, accurate quantitation was not possible. Therefore, the optimum quantities required for efficient cloning experiments could not be achieved.

Since these problems were difficult to overcome, an alternative approach was adopted which involved attempting to clone genomic copies of sequences contained on the 6.6 kbp element. The rationale behind this approach was that if a genomic copy could be cloned, then it would be easier to determine which enzymes cut the 6.6 kbp element and thus allow the cloning of a full length copy. This approach involved cloning of DNA purified from the regions corresponding to restriction enzyme fragments which hybridised to the gel-purified 6.6 kbp (Figure 5a and 5b). Additional restriction fragments to which the gel-purified 6.6 kbp DNA hybridised were identified by using some commonly used six base cutting restriction enzymes (Figure 5b). Table 8 lists the restriction fragments that were selected as putative genomic copies of the 6.6 kbp element.

The 15 restriction fragments marked with an asterisk were selected using the following criteria:

(1) Some of the restriction fragments of choice were those that were identified in the drug-resistant isolate only. The rationale behind this was that since these fragments were absent in the drug-sensitive population, they most likely represent digested fragments from the 6.6 kbp element or genomic copies unique to the drug-resistant isolate.

(2) Restriction fragments were chosen if they consistently hybridised to the probes generated from the 6.6 kbp DNA purified from different CP 547 stock expansions. The rationale behind this selection approach was to try and eliminate fortuitous hybridisation signals due to different levels of contaminating genomic sequences in the various probes generated. It was likely that the degree of contamination with genomic DNA would differ in the separate 6.6 kbp preparations, whereas consistent quantities of the element were expected. Therefore, the probes generated from seperate preparations were

Table 8: Putative genomic copies of the 6.6 kbp element

Restriction enzyme	Fragment sizes (kbp)
Alu I	1.80
	1.65
	1.40
	1.20*
	0.84
	0.60
*Sau*3A I	6.60*
	5.90
	1.90
	1.75*
	0.90
	0.54
Taq I	5.90
	3.50
	1.40*
	0.60*
*Bst*U I	5.80
	3.05*
	2.55
	1.10*
	0.72
*Eco*R I	13.50*
	10.80
	9.20*
	1.58*
*Bam*H I	16.50
	10.50
	9.00
Sph I	1.90*
	7.80*
	1.20*
	0.70

Key: * DNA fragments most likely representing genomic copies of the 6.6 kbp element which were selected for further analysis.

expected to give consistent hybridisations to sequences specific to the element. It was hoped that such a selection would reduce the possibility of studying artefacts.

(3) Preference was given to restriction fragments hybridising with a greater intensity in the lane with DNA from the drug-resistant isolate in comparison to the corresponding fragment from the drug-sensitive population. The rationale behind this was that the greater intensity may be due to additional copies arising from sequences derived from the 6.6 kbp element, in addition to the genomic copies.

(4) Hybridising restriction fragments that corresponded to faint bands on the ethidium-bromide stained gels were given preference. Strong bands on the ethidium-bromide stained gels were avoided as these most likely contained repetitive sequences.

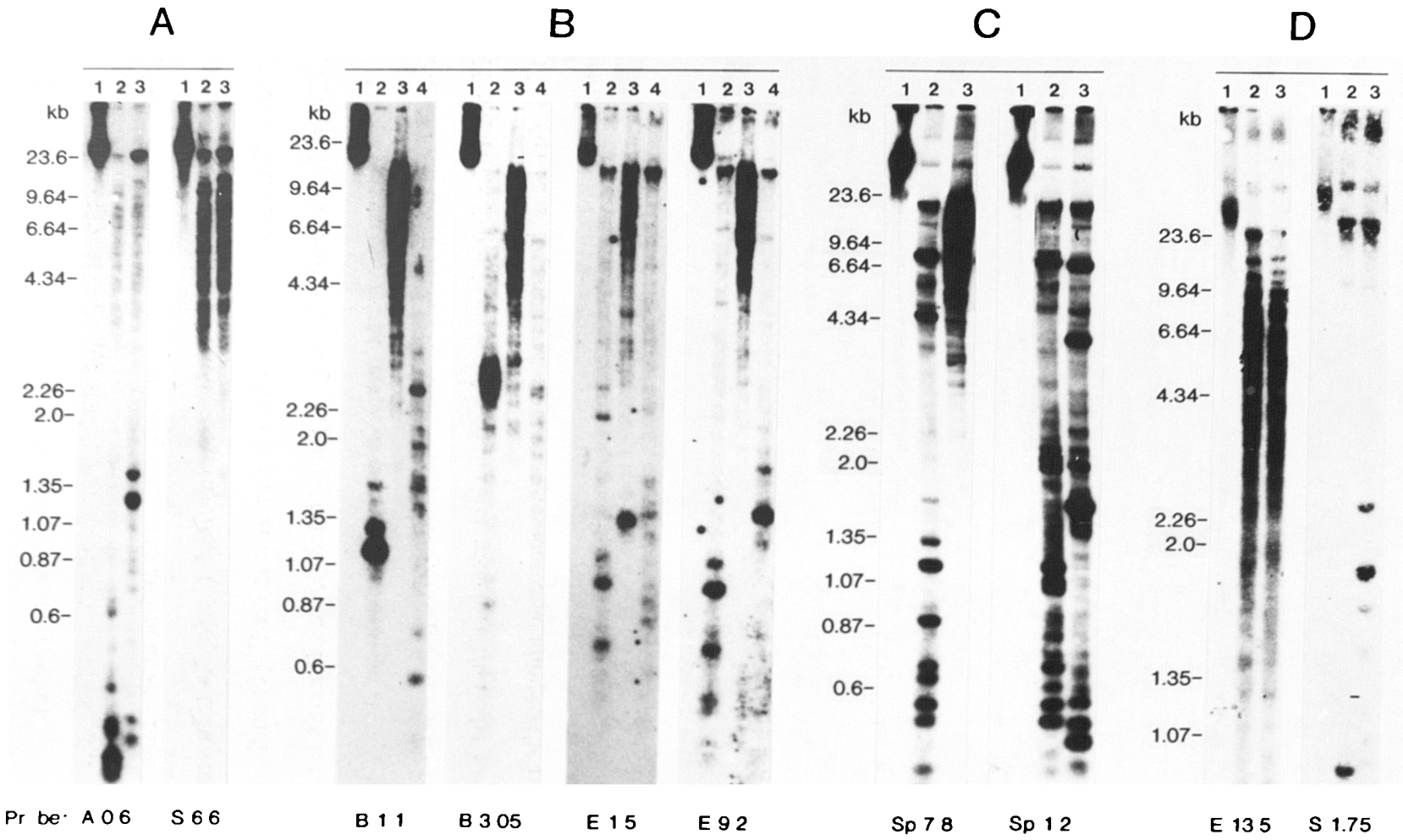
(5) Restriction fragments localised at a close proximity to known repetitive sequences were avoided so as to eliminate the possibility of cross-contamination by these repeats during the gel purification of the selected region.

To purify DNA corresponding to these 15 restriction fractions, aliquots of 8 μg of CP 547 genomic DNA were each digested with the respective restriction enzyme of choice and then resolved by agarose gel electrophoresis. The selected fractions were excised and the DNA extracted by the geneclean® method. The purified DNA from each of these 15 regions was then radiolabelled and used as a hybridisation probe on test Southern blots generated from CP 547 DNA. Each test blot had at least a lane of undigested genomic DNA, a lane of DNA digested with the restriction enzymes used to purify the fragment and representatives of genomic DNA digested with some of the restriction enzymes used to purify the different fragments. Figure 6 shows a representative example of results of some of these hybridisations. Of the 15 restriction fractions used as probes, none hybridised back to the 6.6 kbp element. Some of these probes hybridised to repetitive sequences within the genome as depicted by the *Alu* I 0.6 kbp fraction (Figure 6, blot A, probe A 0.6) which hybridised to the well characterised 177 bp Alu-satellite repeat, and *Sph* I 7.8 (Figure 6, blot C, probe *Sph* I 7.8) which hybridised to uncharacterised repetitive sequences. The hybridisation profiles revealed

Figure 6

Southern blot analyses of DNA from the CP 547 parental stock using size selected total genomic DNA fragments as hybridisation probes.

DNA from CP 547 parental stock (2 μg) digested with different restriction enzymes was resolved in 1% agarose gels and Southern blotted. In set A the lanes represent, (1) undigested DNA, (2) *Alu* I, (3) *Sau*3A I; in set B, (1) undigested DNA, (2) *Alu* I, (3) *Eco*R I, (4) *Sau*3A I; in set C, (1) undigested DNA, (2) *Sph* I, (3) *Eco*R I; in set D, (1) undigested DNA, (2) *Eco*R I, (3) *Sau*3A I. The different ^{32}P-labelled DNA probes and their relative sizes in kbp are indicated at the bottom of each blot. The probes were generated by resolving total CP 547 DNA that was digested with the enzymes, (A) *Alu* I, (S) *Sau*3A I, (B) *Bst*U I, (E) *Eco*R I, (Sp) *Sph* I, and the region corresponding to the required fragment size, gel-purified.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Probe: A06
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B305
E15
E92
Sp78
Sp12
E135
S1.75

that despite the critical selection of the restriction fragments, the putative genomic copies did not hybridise to the 6.6 kbp element.

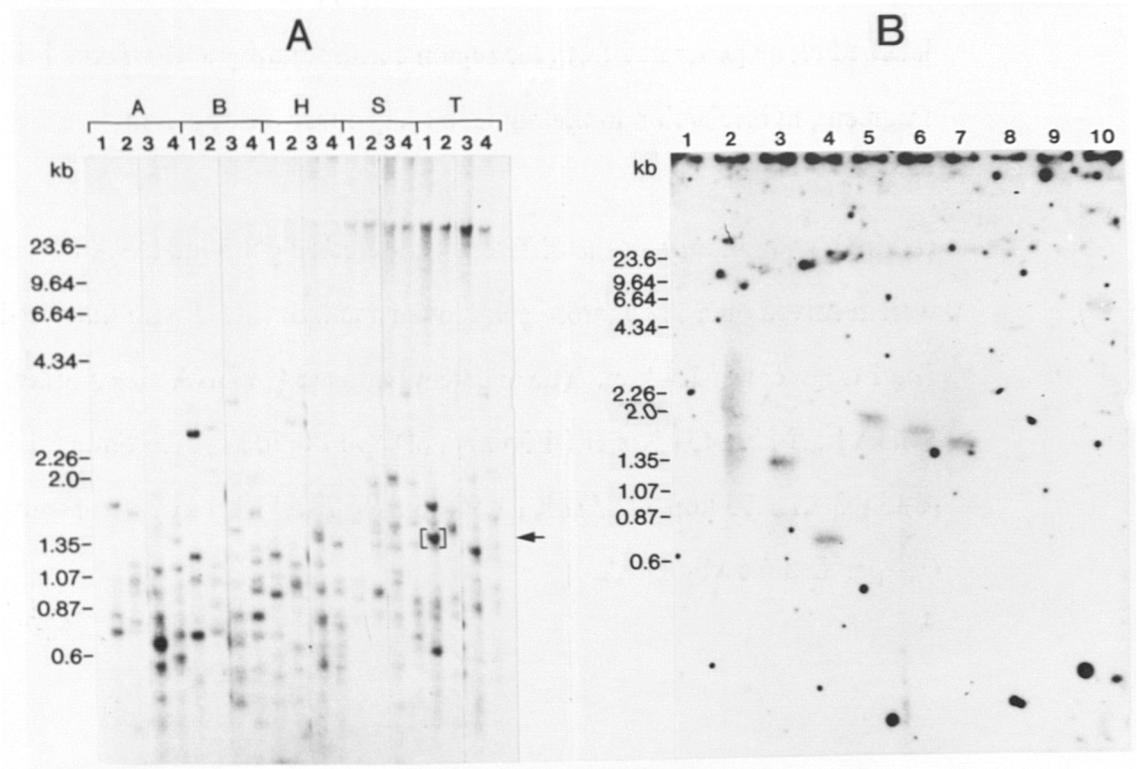
In order to increase the chances of obtaining sequences specific to the 6.6 kbp element, an alternative approach was then tried. In this approach, the purified DNA from the region corresponding to the restriction fragment of choice was first checked for cross-hybridisation to any of the other purified restriction fragments. Figure 7 shows one representative example whereby a ^{32}P-labelled probe generated from the DNA region corresponding to the *Taq* I 1.4 kbp region (Figure 7a) was shown to cross-hybridise to some of the other selected fractions, one of which was the *Eco*R I 1.58 kbp region (Figure 7b, lane 7). On verifying this cross-hybridisation, clones were then generated from the DNA purified from the *Taq* I 1.4 kbp region and subsequently screened with ^{32}P-labelled DNA isolated from the region corresponding to the *Eco*R I 1.58 kbp region. Cloning from one restriction enzyme digested size-class and screening with the cross-hybridising one was expected to reduce the chances of selecting false positives because, presence of the same genomic contaminants in different restriction fragment size-classes was unexpected. Subsequently, to cross-check specificity to the 6.6 kbp element, DNA was prepared from the isolated clones, and used as ^{32}P-labelled hybridisation probes. Even by this procedure, none of the clones hybridised to the 6.6 kbp element; one clone from the *Taq* I 1.4 kbp region hybridised to the miniexon sequence. Seven regions shown to cross-hybridise to other putative genomic copies were used in generating clones, but none of the clones hybridised to the 6.6 kbp element. This approach required large quantities of genomic DNA for the preparation of restriction fragments and for generating the many test Southern blots of undigested and restricted CP 547 DNA. Therefore, to ease the requirement for large quantities of genomic DNA, simple Southern blots containing lanes with only undigested DNA were generated for the initial testing of specificity of hybridisation to the 6.6 kbp element. Although such strips economised on the quantities of genomic DNA, no further information regarding the restriction enzyme profiles was obtained for the tested clones. The lack of this information at the time did not seem essential, but in retrospect such information could have been very useful. Although there

Figure 7

Cross-hybridisation analysis of the different size-selected genomic DNA fragments.

(A) This is a representative Southern blot probed with ^{32}P-labelled 6.6 kbp DNA whereby the lanes contain genomic DNA from (1) CP 547 population, (2) ILTat 1.4, (3) ILTat 1.1, (4) GUTat 3.1 digested with (A) *Alu* I, (B) *Bst*U I, (H) *Hinp* I, (S) *Sau*3A I, and (T) *Taq* I. The arrow on the right indicates the *Taq* I 1.4 kbp fragment hybridising sequence selected as a putative genomic copy of the 6.6 kbp element. However when this blot was stripped and reprobed with one ^{32}P-labelled clone generated from the region corresponding to the *Taq* I 1.4 kbp fragment, hybridisation to the miniexon sequence was apparent.

(B) In this Southern blot the different size-selected genomic DNA fragments were resolved on a 1% agarose gel, blotted and hybridised with the ^{32}P-labelled *Taq* I fragment of 1.4 kbp. The different lanes are (1) DNA size marker, (2) *Sau*3A I 5.9 kbp, (3) *Bam*H I 1.35 kbp, (4) *Sph* I 0.70 kbp, (5) *Sau*3A I 1.90 kbp, (6) *Sau*3A I 1.75 kbp, (7) *Eco*R I 1.58 kbp, (8) *Bst*U I 1.1 kbp, (9) Blank, (10) Gel-purified 6.6 kbp DNA.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were other putative genomic copies which were not tested, this approach was not pursued because, in addition to being expensive, cloning of DNA from the different regions followed by screening and eventual testing of the clones proved to be very time consuming and did not appear to yield the desired results.

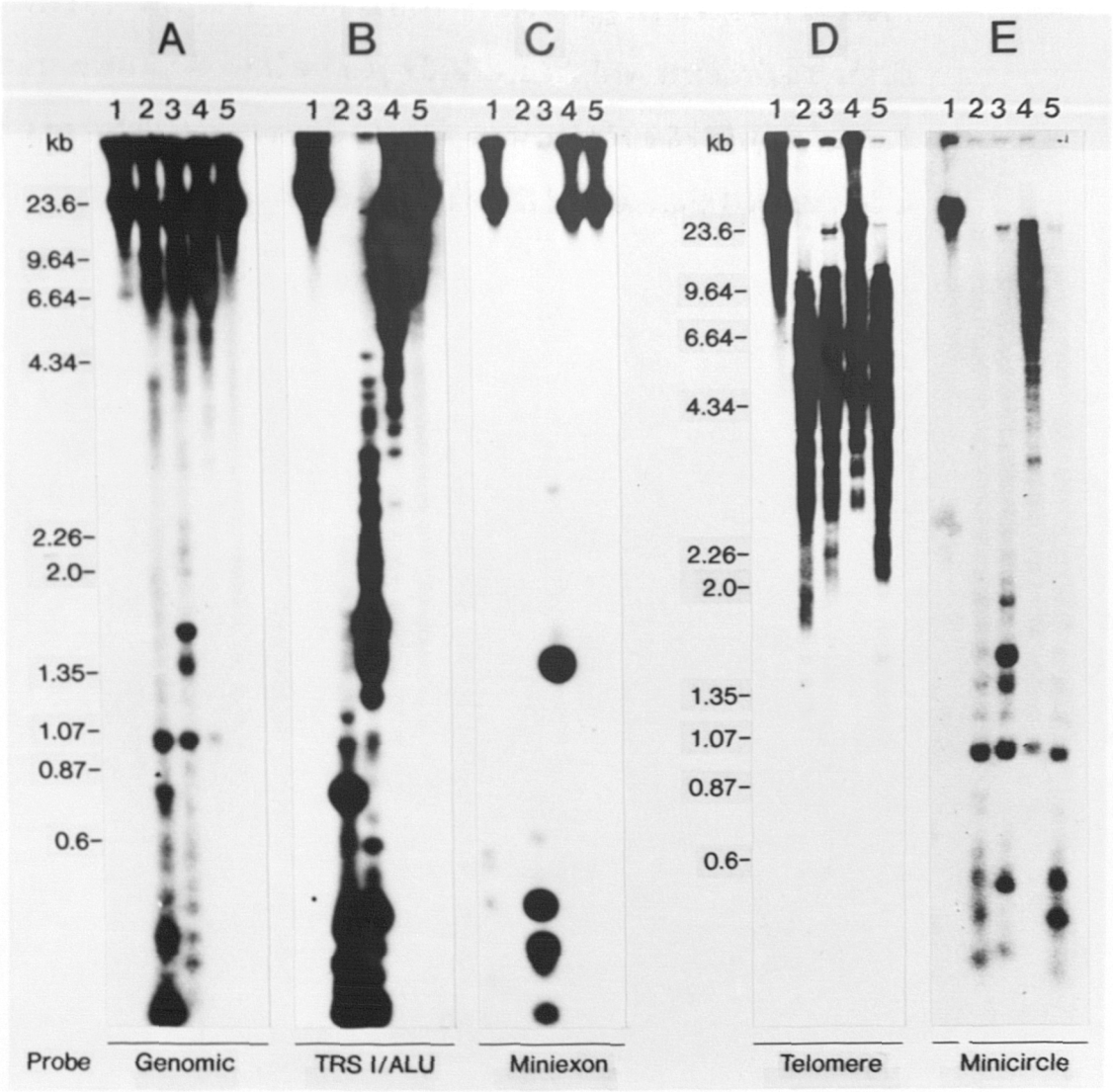
The problems encountered with the above-mentioned different approaches to clone sequences contained on the 6.6 kbp element highlighted that the very low recoverable amounts of gel-purified 6.6 kbp DNA and contaminating genomic DNA were the two major obstacles towards the characterisation of the element. Some of the fragments and clones generated from these efforts hybridised to repetitive sequences from the trypanosome genome, some of which corresponded to well characterised repeats. Therefore, to rule out the possibility that the 6.6 kbp element contains some of the well characterised repetitive sequences, Southern blots with lanes of both undigested total CP 547 DNA and lanes of DNA cut with some commonly used restriction enzymes (Figure 8) were hybridised with six well characterised repetitive sequence probes; namely TRS-1 (ingi), minicircles, the miniexon repeat, the 177 bp Alu-satellite repeat and a telomere repeat sequence. However, even at low stringency washes (4 x SSC at 65°C), none of these repetitive probes hybridised to the 6.6 kbp element, thus ruling out their presence on the 6.6 kbp element.

3.2.1 Instability of the copy number of the 6.6 kbp element

Large amounts of genomic DNA from CP 547 stock were constantly required for gel purification of sufficient quantities of the 6.6 kbp element, for labelling purposes, for the purification of the various selected restriction fragments (Section 3.1.2 b), and for the preparation of Southern blots required to test the various clones and restriction fragments. To meet this continuous demand the CP 547 stock was continuously passaged and then expanded in rodents over the first 6 months of this investigation. However, as DNA was prepared from the consecutive passaged isolations, problems were emerging in detecting the 6.6 kbp extrachromosomal element. This was initially attributed to faulty transfer and binding of the DNA to the nylon filters, as the batch of nylon had changed. To overcome

Figure 8

Southern blot analyses of CP 547 DNA using some of the well characterised *T. b. brucei* repetitive sequences as probes. Identical 1.4% agarose gels with lanes each containing 2 μg of total CP 547 DNA which was (1) undigested, and digested with (2) *Alu* I, (3) *Sau*3A I, (4) *Eco*R I and (5) *Taq* I were generated. Southern blots of these gels were hybridised with ^{32}P-labelled **(A)** total genomic DNA, and five other well characterised repetitive DNA sequences which are **(B)** TRS-1 (ingi), and the 177 bp Alu repeat, **(C)** the miniexon repeat sequence, **(D)** a telomere repeat sequence and **(E)** minicircles.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1.35–
1.07–
0.87–
0.6–
Probe
Genomic
TRS I/ALU
Miniexon
Telomere
Minicircle

this problem, nitrocellulose was used as the solid support and more caution was exercised in ensuring efficient transfer of all DNA from the gel to the filters. In addition, for clearer visualisation of the 6.6 kbp element on autoradiographs, it became apparent that it was essential to avoid mechanical shearing of the genomic DNA during its preparation. Nevertheless, although care was taken, some proportion of shearing was inevitable resulting in masking of the already weak signal corresponding to the 6.6 kbp element. The problem of shearing was however overcome by lysing the cells *in situ* through embedding of purified trypanosomes in 0.5% low melting point agarose and processing them as for PFGE (Section 2.5.2). The agarose blocks containing the embedded CP 547 trypanosomes were cut into strips sized 0.5 cm by 2.5 cm so that when required the strips could be aligned to fit the length of the preparative gel slot. The DNA in the agarose blocks was then resolved by electrophoresis in conventional agarose gels. Using this approach, not only was the 6.6 kbp element visualised more clearly but the problem of contaminating genomic sequences appeared to be reduced (Figure 9a). However, despite these precautions, the hybridisation signal corresponding to the 6.6 kbp element from the consecutive trypanosome isolations was still extremely faint and eventually un-detectable on Southern blots. The reduction and eventual loss of signal corresponding to the 6.6 kbp element indicated that the copy number of the 6.6 kbp element was unstable. Continuous passaging of the CP 547 stock for 6 months in mice, in the absence of selective drug pressure, resulted in the selection of a sub-population lacking the element. Subsequently, the CP 547 stock that had been passaged has been referred to as the 'passaged CP 547 stock'.

Similar reductions in the copy number of extrachromosomal elements have been observed in some drug-resistant *Leishmania* spp., whereby the instability is known to be directly associated to the removal of selective drug pressure (Beverley *et al.*, 1987). Since the CP 547 stock had been passaged for almost 6 months in the absence of drug pressure, as for *Leishmania* spp., the reduction and loss of the 6.6 kbp element could be associated with a change in the drug resistance profile of this population. Therefore, experiments were carried out to determine the sensitivity of the 'passaged CP 547 stock'

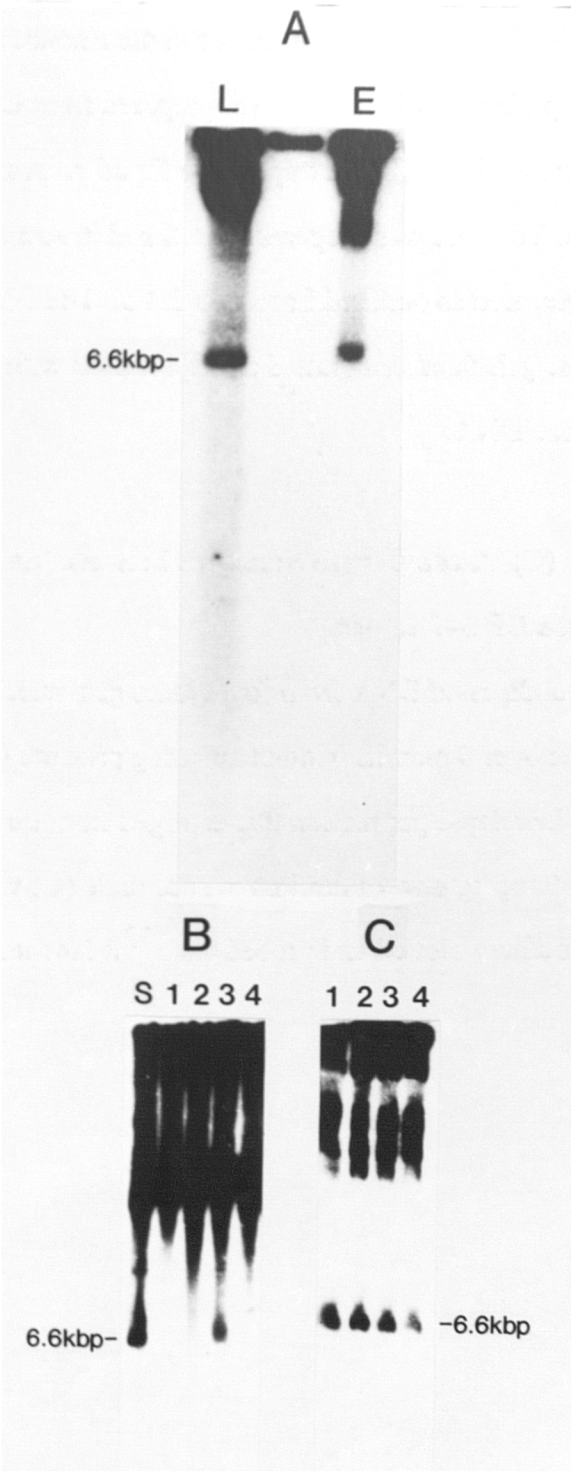
Figure 9

Southern blot analysis of CP 547 genomic DNA prepared in agarose blocks and in liquid.

(A) This blot shows the difference in the amount of shearing and masking of the 6.6 kbp element when DNA was prepared from CP 547 trypanosomes embedded in agarose blocks (E), as opposed to liquid preparation (2 μg) of DNA (L). The embedded DNA was prepared as outlined in section 2.5.2c, whereas liquid DNA was prepared as outlined in section 2.4.1. The DNA was resolved on a 1% agarose gel, Southern blotted and hybridised with ^{32}P-labelled CP 547 total genomic DNA.

(B) & (C) A comparative analysis between 'unselected' and diminazene selected CP 547 clones.

Total undigested DNA from four clones generated from a CP 547 stock passaged in mice over 6 months without any drug pressure (B), and four clones generated from the relapse population that emerged from treating the parental CP 547 stock with 60 mg/kg b.w. of diminazene aceturate (C) was resolved on a 1% agarose gel, Southern blotted and probed with ^{32}P-labelled total CP 547 genomic DNA.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C
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-6.6kbp

to diminazene aceturate and isometamidium chloride. These experiments disclosed that the drug sensitivity of the 'passaged CP 547' population had altered. The 'passaged CP 547' stock had become less resistant to the two trypanocides in comparison to the parental CP 547 population (Table 9). For instance, 60 mg per kg body weight (b.w.) of diminazene aceturate, the maximum tolerated dose in mice, now cured infections of the 'passaged CP 547 population', whereas the original isolate relapsed at this drug dosage. These findings, however, contradicted earlier studies by Kaminsky and Zweygarth (1989a), who showed no alteration in the drug-sensitivity profiles of CP 547 populations to diminazene aceturate and isometamidium chloride after *in vitro* propagation for up to 275 days in the absence of drug pressure.

Thus, as for *Leishmania* spp., the CP 547 population passaged without drug pressure expressed lower levels of resistance and also displayed a reduction and eventual loss of the 6.6 kbp element, suggesting a correlation or partial involvement of the element in resistance However, the original population of CP 547 was not cloned, it is possible that continuous passaging of the stock resulted in selection for a more sensitive population in the mixed population, similar to selection for pyrimethamine-sensitive populations in *P. falciparum* (Inselburg *et al.*, 1989).

3.2.2 Drug sensitivity of clones derived from the CP 547 stock

In order to determine the variation in sensitivity to diminazene aceturate of trypanosomes within the 'passaged CP 547' stock, four clones were generated from the population as described by Barry and Gathuo (1984). Since no drug pressure was applied during their production, these clones were designated as the 'unselected' clones. The sensitivity to diminazene aceturate of these four 'unselected' clones did not vary significantly from each other. It also appeared to be similar to the 'passaged CP 547' stock from which they were derived (Table 9). In comparison to the parental, un-passaged, population of CP 547, the four 'unselected' clones were significantly less resistant to diminazene aceturate (Table 9). However, although the 'unselected' clones were more sensitive to diminazene aceturate than the parental unpassaged CP 547

Table 9: Sensitivity of *T. brucei* CP 547 clones to trypanocides in mice, expressed as 50% curative dose (CD_{50}*) values

	Sensitive Control		Clones from passaged stock CP 547 (unselected)[a]					Parental Population (drug selected)[b]
Trypanocide	ILTat1.4	Passaged Stock	Cl 1	Cl 2	Cl 3	Cl 4	unpassaged Stock	Clones Cl 1-4
Isometamidium	N/D	<3.4	1.6	1.1	1.3	1.2	3.4	N/D
chloride			(1.5-1.8+)	(0.9-1.4)	(1.0-1.6)	(1.0-1.40)	(3.1-3.7)	N/D
Diminazene	1.19	<60	33.8	39.7	30.5	28.5	>60	>60
aceturate	(1.14-1.25)		(32.9-34.8)	(39.1-40.3)	(30.0-30.9)	(27.7-29.3)		

* CD_{50} = curative dose for 50% of the population (mg/kg b.w.).

\+ = 95% confidence interval of CD_{50} value (mg/kg b.w.).

\> = greater than; < = less than

N/D = not determined

a = no drug pressure applied

b = population that relapsed in mice following treatment with diminazene aceturate at 60 mg/kg b.w.

population, in comparison to the reference drug-sensitive population ILTat 1.4, the 'unselected' clones expressed significant levels of resistance to diminazene aceturate.

To evaluate the effect of selection with diminazene aceturate, four additional clones were generated from the parental population of CP 547. To generate these clones, six mice were each infected intraperitoneally with 10^3 CP 547 trypanosomes. After 24 h, the maximum tolerated dose of diminazene aceturate (60 mg/kg b.w.) was administered intraperitoneally. After a further six days a relapse population was detected in the tail blood smears. On purification of the relapsed parasites, four clones were generated and designated as the 'drug-selected clones'. These four selected clones were all characterised for their sensitivity to diminazene aceturate in mice and were found to be resistant to 60 mg/kg b.w. of diminazene aceturate. Since this was the maximum tolerated dose in mice, an absolute measure of the level of resistance displayed by these clones could not be established. However, from the evaluation of the clones' sensitivity to diminazene, it was evident that the application of diminazene aceturate selected for a population expressing a higher level of drug resistance whereas, in the absence of drug pressure, the population reverted to a lower level of resistance.

3.2.3 Southern blot analysis of the CP 547 clones.

Presence of the 6.6 kbp element in the diminazene-selected and 'unselected' CP 547 clones was determined in Southern blots of undigested DNA from each population. Hybridisations with ^{32}P-labelled CP 547 DNA showed that, of the four 'unselected' clones, only one (clone 3) had detectable amounts of the 6.6 kbp element (Figure 9b). In contrast, all four clones generated under diminazene pressure possessed detectable amounts of the 6.6 kbp element (Figure 9c). These experiments confirmed that in the absence of diminazene pressure, there was both loss of the 6.6 kbp element and a significant decrease in the level of resistance to diminazene. In contrast, if diminazene pressure was applied, the element reappeared and the maximum levels of resistance measurable in mice did not decrease. Although it was not possible to establish the

absolute levels of diminazene resistance that the populations expressed, the results suggest a correlation between presence of the element and expression of high levels of resistance. Having said this, a direct correlation between both observations is complicated by the presence of the 6.6 kbp element in 'unselected' clone 3. In addition, 'unselected' clone 2 expressed a higher level of diminazene resistance than 'unselected' clone 3 (Table 9). The results obtained from the Southern blots could thus be interpreted as: (1) the presence of the element in the multi-drug resistant isolate is fortuitous and not directly associated with diminazene resistance, (2) the element is associated with diminazene resistance but other unknown factors are also involved in expression of resistance, (3) the element is generated as a consequence of the drug but is not directly involved in the expression of drug resistance or (4) the higher hybridising forms of the element, and to some degree the 6.6 kbp element, are involved in diminazene resistance.

The unstable resistance characteristics of the 'passaged CP 547' population to diminazene aceturate, and the associated instability of the 6.6 kbp element, emphasised the requirement for generating uniform parasite material for informative analyses. Thus, generation of the diminazene selected clones containing the 6.6 kbp element helped overcome such problems. Henceforth, for all subsequent investigations, the diminazene selected "clone 2" was used as it displayed the strongest hybridisation signal for the element. Furthermore, in order to ensure maintenance of both a high level of diminazene-resistance and the associated 6.6 kbp element, "clone 2" parasites were passaged and expanded in mice treated with 55 mg/kg b.w. of diminazene aceturate. Whenever CP 547 trypanosomes were required, "clone 2" was expanded in mice which were each infected intraperitoneally with 10^3 CP 547 trypanosomes. After 24 h, 55 mg/kg b.w. diminazene aceturate was administered intraperitoneally and the relapse population harvested.

3.2.4 The 6.6 kbp element displays unusual mobilities

The drug-selected clone 2 was now expected to generate consistent and higher quantities of the element. Curiously however, when the region corresponding to the 6.6

kbp element was purified and labelled, it became evident that the material isolated from some electrophoretic runs did not incorporate the radio-isotope at all, whereas others labelled easily. This puzzling result was attributed to major losses still being incurred during purification of the element from the large agarose gel slices. Consequently, in order to improve the recovery, it was considered preferable to first localise the element and then excise the exact region of the agarose strip corresponding to it. To accomplish this, undigested CP 547 genomic DNA was first electrophoresed in a 0.8% w/v preparative agarose gel. To reduce co-migrating contaminating genomic DNA, as mentioned earlier, agarose blocks with parasites lysed *in situ* were used and the gels were also resolved slowly at 60 volts for a period of 18 h. On completing the electrophoretic run, a test agarose strip of about 3 cm was cut from one edge of the preparative gel and analysed by Southern blotting, while the remaining gel was stored at 4°C to reduce diffusion of the DNA. The test strip was probed with ^{32}P-labelled total CP 547 genomic DNA to localise the element. The hybridisation signal thus allowed accurate localisation of the region within the gel corresponding to the element which was then excised and purified by the geneclean®method. Alternatively, the DNA in the agarose strip was concentrated onto a DEAE-45 cellulose membrane from which it was eluted. This approach of purifying the element was an improvement because it reduced the amount of contaminating genomic DNA that was co-purified with the element from the wider agarose gel strips previously used. *It also gave a cleaner preparation* with less contaminating agarose material, and ensured consistent recovery of *the element. The* approach of first localising and then isolating the element also revealed that the element displayed differential mobility in different gel systems. This resulted in differences in the apparent size of the element with respect to linear DNA size markers in different percentage agarose gels, a property of circular DNA molecules (Beverley, 1988). This finding explained the lack of recovery of the element from some of the previous gel isolations in which the 6.6 kbp region was localised on the basis of linear DNA size markers.

3.3.1 PCR-amplification of membrane-bound 6.6 kbp element.

In order to obtain sequences specific for the 6.6 kbp element, efforts were first made to clone directly from the gel-purified 6.6 kbp element. Lack of success led to attempts at cloning the putative genomic copies of the element. However, these two approaches did not produce the desired results and were therefore abandoned. The major obstacles appeared to be the co-purified genomic contaminants which interfered in the identification and cloning of the required sequences. In addition, it was possible to purify only very small quantities of the 6.6 kbp element; from 500 µg of total genomic DNA, less than 20 ng of the 6.6 kbp element was isolated. Consequently, in order to overcome these two major problems, a PCR-based amplification method for sequences specific to the 6.6 kbp element was developed. This method involved the use of the 6.6 kbp element fixed on a nitrocellulose membrane as the target DNA in a PCR reaction. The localisation was achieved by conventional Southern blot analysis whereby the electrophoresed undigested CP 547 DNA was transferred onto a nitrocellulose membrane. The precise position of the 6.6 kbp element on this membrane was then determined by hybridisation with ^{32}P-labelled total CP 547 genomic DNA. The region encompassing the signal (approximately 0.5 cm x 0.1 cm) was excised and washed thoroughly in sterile water to remove salt and traces of genomic DNA. This was achieved by immersing the membrane in 50 ml of distilled water for 10 minutes and then soaking for an additional 20 minutes in 10 ml of warm distilled water maintained at 37°C. The washed and air dried membrane was then cut into pieces that were small enough for complete submersion in 100 µl of the PCR buffer. As there was no sequence information for the 6.6 kbp element, it was therefore not possible to generate specific primers required to initiate the amplification. Consequently, random hexanucleotide primers (Sigma) were used as non-specific primers. In order to achieve amplification of any sequences it was necessary to first optimise the concentrations of the $MgCl_2$ and the random hexanucleotide primers in the reaction mixture. On trying out varying concentrations in trial reactions it was found that 3 mM $MgCl_2$ and 10 to 20 µg of the random hexanucleotides in the PCR mixture resulted in amplification of membrane-

bound DNA. The temperature profile applied for 50 cycles involved denaturing at 94^{0}C for 1.5 min followed by primer annealing at 40^{0}C for 1 min, and primer extension at 72^{0}C for 3 min. The cycling was concluded with a final extension at 72^{0}C for 5 min. The resulting amplified sequences designated as the 'random-primed PCR product' consisted of heterogeneously sized fragments in the range of 7 kbp to about 200 bp. The excess primers and dNTPs were removed by centrifugation in a Sephadex G-50 column followed by ethanol precipitation of the flow-through DNA. Aliquots of this DNA were then used in standard random primer labelling reactions to generate a ^{32}P-labelled 'random-primed PCR probe' for Southern blot analysis.

3.3.2 Specificity of the random-primed PCR probe

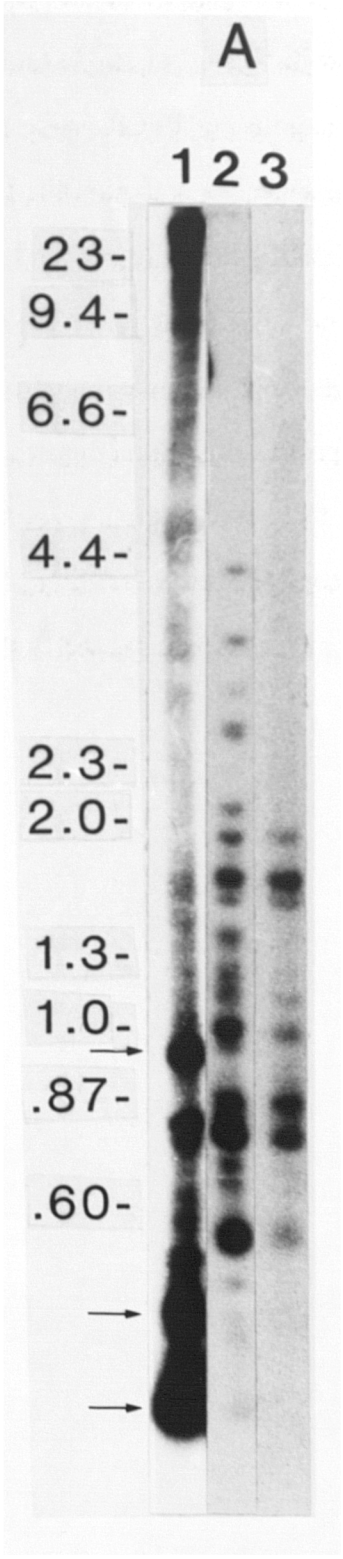
Southern blots of restriction enzyme digested CP 547 DNA hybridised with the random-primed PCR probe displayed profiles almost identical to those obtained when using a gel-purified 6.6 kbp DNA probe generated from the same CP 547 DNA (Figure 10a, 10b). Furthermore, as for the gel-purified 6.6 kbp probe, and the probes generated from putative genomic copies (Section 3.1.2b), the random-primed PCR probe also did not hybridise to the 6.6 kbp element (Figure 10a, 10b), except on very long exposures when only a faint signal was evident. The lack of hybridisation to the 6.6 kbp element by the random-primed PCR probe was similarly perplexing since judging from the lack of hybridisation of the random-primed PCR probe to any of the well characterised *T. brucei* repetitive sequences (Figure 10a lane 3) there was negligible contamination of genomic DNA. Thus it appeared that the random-primed PCR product originated from amplification of sequences present only on the excised membrane. The specificity of this method of PCR amplification was further tested by replacing the membrane-bound target DNA with approximately 2 ng of gel-purified 6.6 kbp DNA in solution. The amplification resulted in a product which, when used as a ^{32}P-labelled probe, produced a hybridisation profile similar to the one obtained by the random-primed PCR probe generated from membrane-bound 6.6 kbp (Figure 10b, lanes 2 and 3). The ability to generate random-primed PCR probes with identical specificity for the restriction

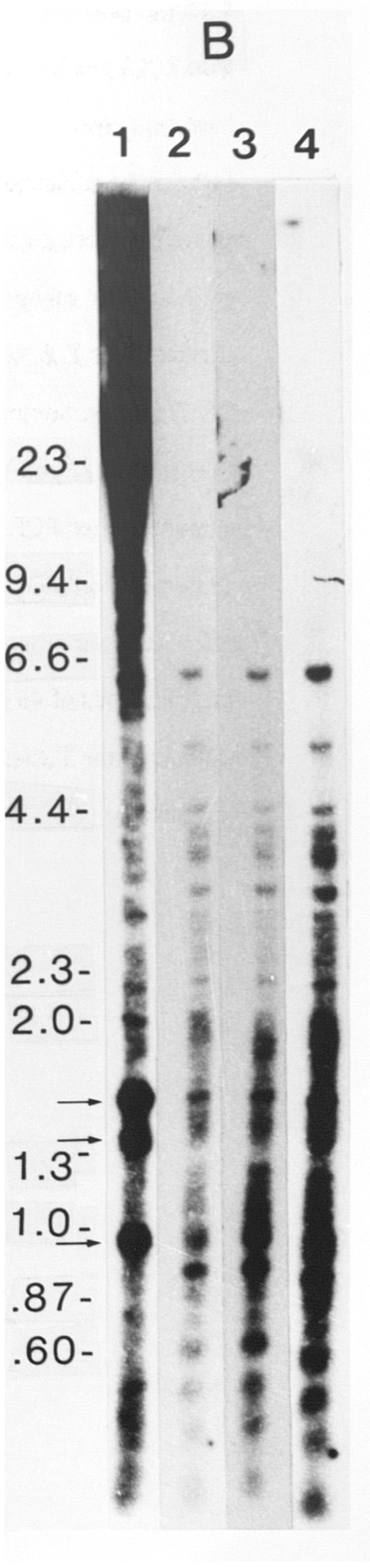
Figure 10

Comparison between the random primed PCR probe, the gel purified 6.6 kbp DNA probe and total genomic CP 547 DNA control probe by Southern blot analyses.

(A) Southern blots of *Alu* I digested CP 547 genomic DNA (2 µg) were generated, cut into strips and probed with ^{32}P-labelled (1) total CP 547 DNA, (2) gel purified 6.6 kbp DNA and (3) random primed PCR probe. In lane 1, the well characterised *T. b. brucei* Alu satellite repeat is evident at the bottom.

(B) Similarly, Southern blots of *Sau*3A I digested CP 547 genomic DNA (2 µg) were generated and hybridised with ^{32}P-labelled (1) total CP 547 DNA, (2) random primed PCR probe generated from membrane bound 6 6 kbp DNA, (3) random primed PCR probe generated from the liquid, gel purified 6 6 kbp DNA and (4) random primed PCR probe having gone through three more amplifications of 40 cycles each. The well characterised *T. b. brucei* repetitive sequences, the TRS-1 and the miniexon are evident in lane 1 at 1 6 kbp 1 35 kbp respectively.


A
1 2 3
23-
9.4-
6.6-
4.4-
2.3-
2.0-
1.3-
1.0-
.87-
.60-


B
1 2 3 4
23-
9.4-
6.6-
4.4-
2.3-
2.0-
1.3-
1.0-
.87-
.60-

fragments hybridising to the gel-purified 6.6 kbp probe using two separate 6.6 kbp target DNA sources, shows the high level of reproducibility and specificity of this approach. Furthermore, large quantities of random-primed PCR probe could be consistently generated by additional rounds of amplification resulting in little or no loss of specificity (Figure 10b, lane 4).

3.3.3 Analysis with the random-primed PCR probe

3.3.3a Screening of a lambda gt11 *T. b. brucei* genomic library

The availability of unlimited quantities of the random-primed PCR probe generated from the membrane-bound 6.6 kbp element now made it possible to screen a *T. b. brucei* CP 547 genomic library. The random-primed PCR probe was considered as a preferable alternative to the gel-purified 6.6 kbp probe for the following reasons:

(1) The random-primed PCR probe had a specificity for the same restriction fragments as the DNA probe generated from the gel-purified 6.6 kbp region.

(2) The generation of a random-primed PCR probe was more economical because the target DNA for the PCR amplification was obtained from only 2 µg of membrane-bound CP 547 DNA. From the resulting 100 µl PCR reaction, 10 radiolabelling experiments could be carried out, whereas only one radiolabelling was achieved with the gel-purified 6.6 kbp DNA isolated from 500 µg of total genomic CP 547 DNA. Additional rounds of amplification of the random-primed PCR product resulted in little or no loss of apparent specificity, thus generating an almost limitless supply of probe. Hence, the problems of having to isolate and work with low quantities of DNA were circumvented.

(3) Radiolabelling of the random-primed PCR product resulted in high specific activity probes, whereas there were persistent problems associated with labelling the gel-purified 6.6 kbp element as outlined in section 3.1.2.

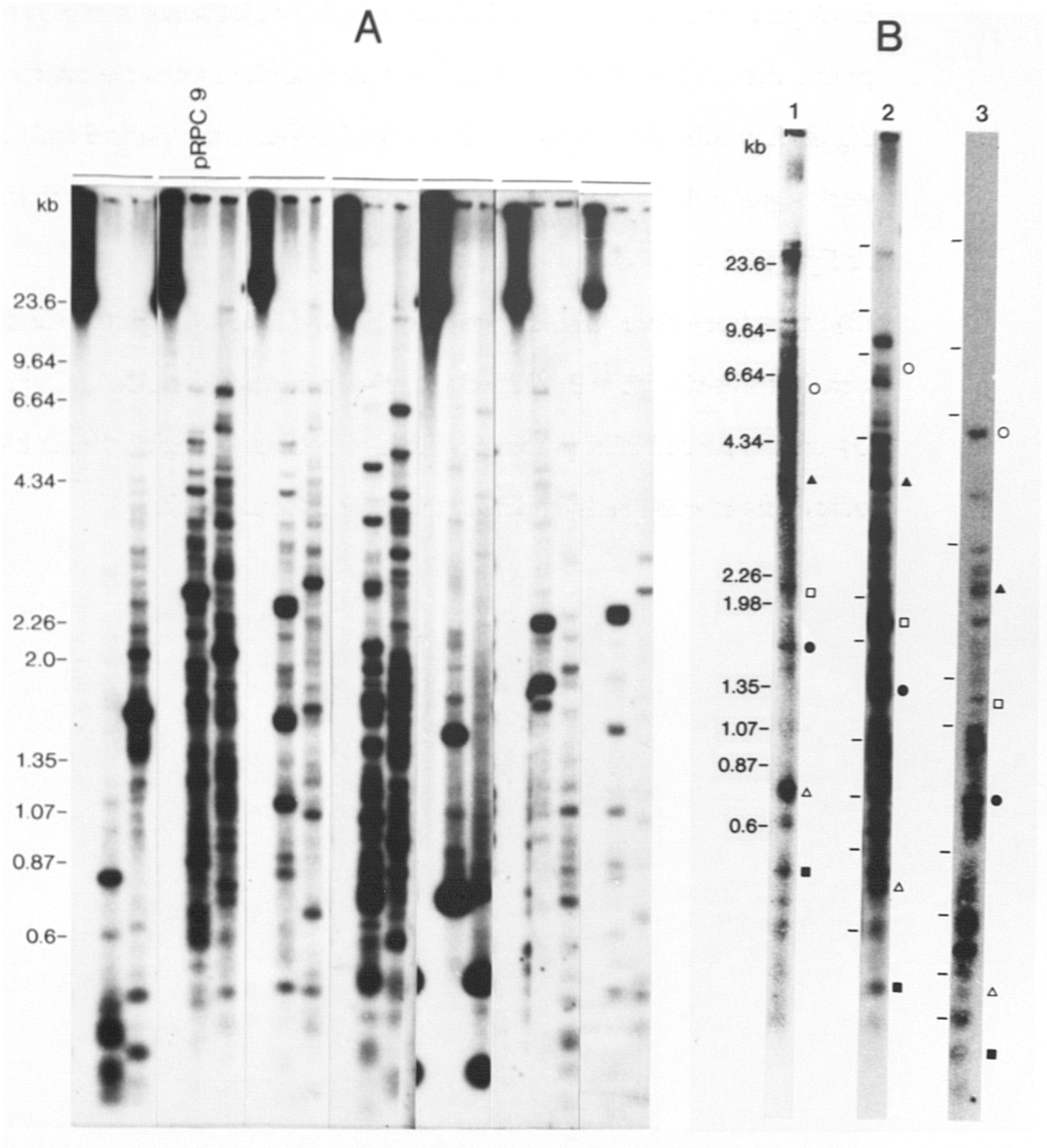
A lambda gt11 library of total genomic DNA from *T. b. brucei* CP 547 sheared to an average size of 4.0 kb was generated. The frequency of recombinants was estimated to be 70% on the basis of the number of blue and white plaques, and the total library represented approximately three *T. b. brucei* genomes. In order to screen the CP 547

Figure 11

Southern blot hybridisation profiles of randomly chosen genomic clones selected with the random primed PCR probe.

(A) This figure contains different Southern blots, each with three lanes (each blot is represented by the broken line on the top). Each blot is hybridised with DNA purified from one of the 7 clones selected with the random primed PCR probe. From left to right, the three lanes in each blot represents undigested, *Alu* I digested, and *Sau*3A I digested CP 547 total DNA. Only clone 9 (pRPC9) gave a weak signal of hybridisation to the 6.6 kbp element, as evident in the lane of undigested DNA.

(B) Strips from three different Southern blots of *Sau*3A I digested CP 547 DNA were hybridised with ^{32}P-labelled (1) gel-purified 6.6 kbp DNA, (2) pRPC9, and (3) random primed PCR probe. Similar symbols on the right of each blot indicate fragments that hybridise to the three different probes.


A
pRPC 9
kb
23.6-
9.64-
6.64-
4.34-
2.26-
2.0-
1.35-
1.07-
0.87-
0.6-
B
1
2
3
kb
23.6-
9.64-
6.64-
4.34-
2.26-
1.98-
1.35-
1.07-
0.87-
0.6-

genomic library with the random-primed PCR probe, about 50,000 recombinants were plated at a density of 5,000 per 150 cm^2 plate. Twenty randomly chosen positive clones were picked and the DNA prepared from each clone was used as a radiolabelled probe on test Southern blots of undigested and restriction enzyme digested CP 547 DNA. The DNA from all the selected clones hybridised to many restriction fragments (Figure 11a). However, only clone pRPC9 gave a weak signal of hybridisation to the 6.6 kbp element (Figure 11a set 2, lane 1). Since this clone hybridised to many restriction fragments, it was difficult to ascertain which of the restriction fragment(s), if any, was derived from the 6.6 kbp element. There were far too many differences between the hybridisation profiles of the clones not hybridising to the element in comparison to clone pRPC9, making identification of restriction fragments derived from the 6.6 kbp element difficult. The hybridisation profile of the pRPC9 disclosed that there were some restriction fragments that also hybridised to the gel-purified 6.6 kbp probe and the random-primed PCR probe. Figure 11b shows an example of such common hybridising sequences using the three different probes. Since analysis of the Southern blots with the random-primed PCR probe and clones selected with the random-primed PCR probe was complicated by the presence of many hybridising restriction fragments (Figure 10 and Figure 11a), alternative approaches to isolate sequences which were unequivocally derived from the 6.6 kbp element were required.

3.3.3b Northern blot analysis

Since there was no obvious genomic difference between the drug resistant and drug-sensitive isolates with the PCR probe and the clone pRPC9 which could account for the different phenotypes, it was decided to look for differences at the level of expression. Simpler profiles were expected in Northern blot analysis as hybridisation would be limited only to the transcribed sequences. Hence, poly(A)$^+$ RNA was purified from the bloodstream and procyclic forms of both the drug-resistant and the drug-sensitive populations. 2 µg of poly(A)$^+$ RNA from each of these was size-fractionated on a 1.4% agarose gel and transferred to a nylon membrane for Northern blot analysis.

Figure 12

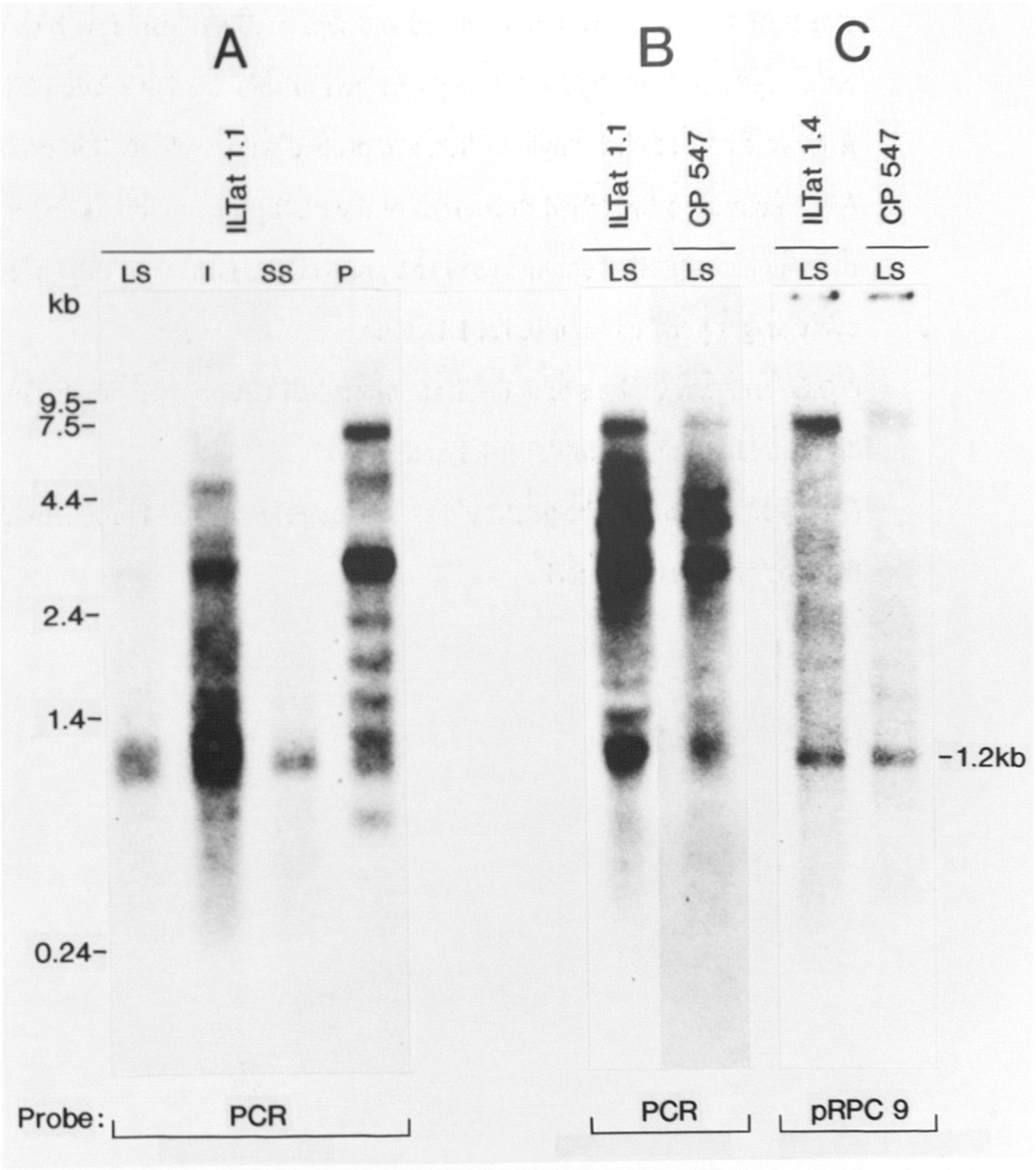
Northern blot analysis of transcripts prepared from different life cycle stages of ILTat 1.1 and long slender bloodstream forms of the drug-resistant CP 547 population.

Approximately 3 µg of poly(A)$^+$ enriched RNA from the different life cycle stages of *T. b. brucei* was glyoxylated and separated alongside with size markers (Promega RNA ladder) on 1.4% agarose gels as indicated in section 2.5.2g. The gels were blotted onto a nylon filter, and probed with ^{32}P-labelled probe.

(A) 3 hour exposure of hybridisations of the PCR probe to long slender (LS), intermediate (I), short stumpy (SS) and procyclic (P) forms of the different life cycle stages purified from ILTat 1.1 clone.

(B) Overnight exposure of hybridisations of the PCR probe to long slender (LS) bloodstream forms from ILTat 1.1 and CP 547.

(C) Hybridisations of the pRPC 9 probe to long slender (LS) bloodstream forms from ILTat 1.4 and CP 547.


A
B
C
ILTat 1.1
ILTat 1.1
CP 547
ILTat 1.4
CP 547
LS
I
SS
P
LS
LS
LS
LS
kb
9.5-
7.5-
4.4-
2.4-
1.4-
0.24-
~1.2kb
Probe:
PCR
PCR
pRPC 9

Hybridisation with the random-primed PCR probe revealed the presence of many transcripts (Figure 12b, PCR probe). At medium post-hybridisation stringency washing (50°C, 3 x SSC, 0.1% SDS for 45 min), no differences were apparent between the drug-resistant and the drug-sensitive populations (Figure 12b). Similarly, hybridisations to a Northern blot of poly(A)$^+$ RNA generated from four different life-cycle forms, the long slender, intermediate and short stumpy bloodstream, forms and *in vitro*-generated procyclic, cultured insect forms, of ILTat 1.1, disclosed that some of the transcripts hybridising to the random-primed PCR probe are developmentally regulated (Figure 12a). However, whether this was also true for the CP 547 population could not be established as the intermediate and short stumpy forms of this clone could not be produced as uniform populations. Clone pRPC9 was also used as a hybridisation probe because although it hybridised to the 6.6 kbp element weakly on Southern blots, the possibility existed that it might hybridise strongly to transcripts specific for the 6.6 kbp element. Hence, a Northern blot similar to the one in Figure 12b was probed with radiolabelled DNA from clone pRPC9. However, no differences were apparent between the drug-resistant CP 547 and the drug-sensitive 1LTat 1.4 populations (Figure 12c). Clone pRPC9 detected a transcript of 1.2 kb in both the populations. In addition to other transcripts, the random-primed PCR probe also hybridised to a 1.2 kb transcript in both the populations (Figure 12b). However, the transcriptional analysis did not reveal any apparent differences between the drug-resistant and drug-sensitive populations using either the random-primed PCR product or clone pRPC9 as hybridisation probes.

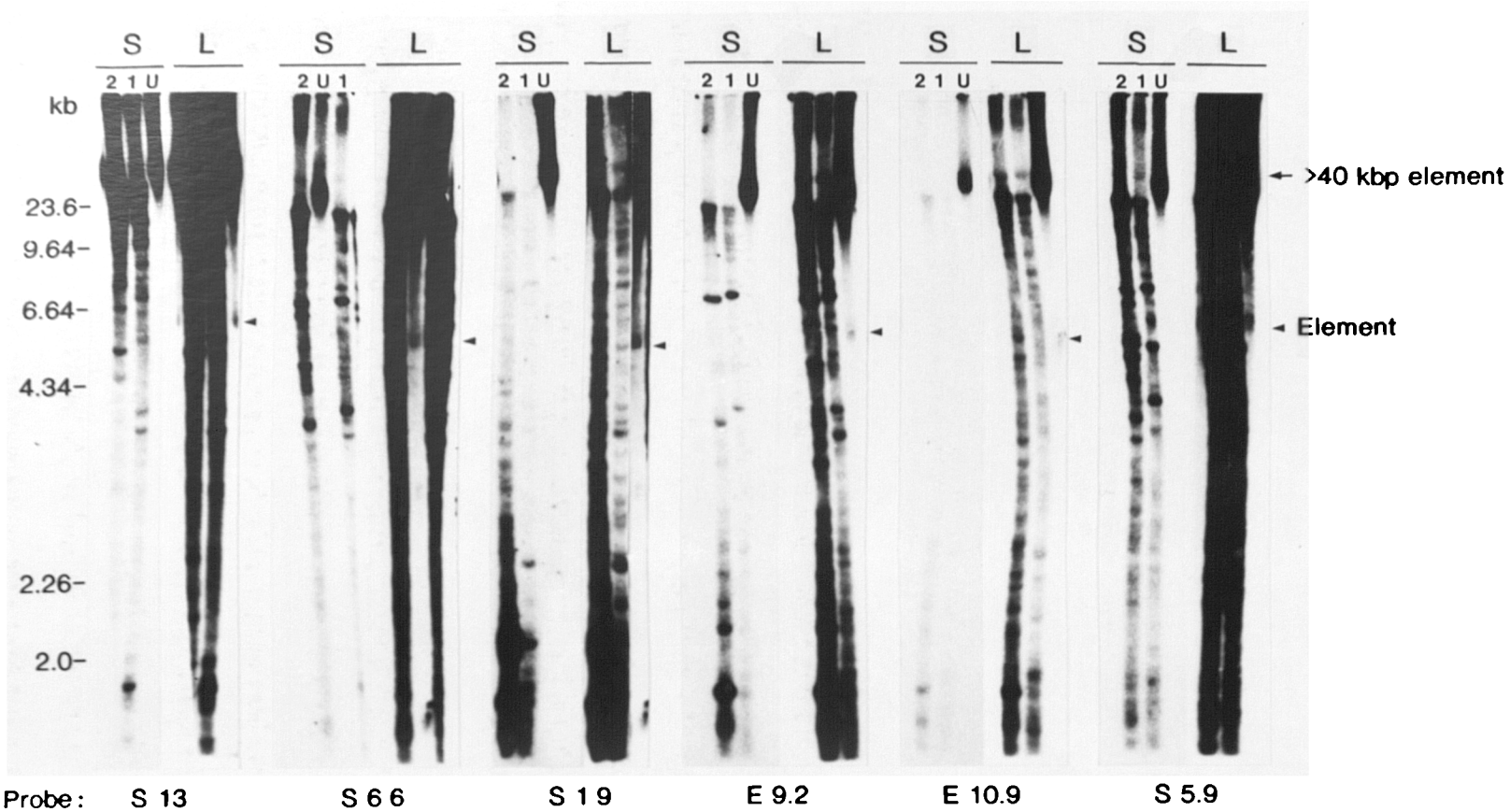
3.4.1 Collective analysis of results from the different Southern blots

Although the different approaches to identify sequences specific for the 6.6 kbp element should have resulted in the identification of such sequences, definitive results were not forthcoming. This prompted an overall re-evaluation of the results generated from four of the different approaches and a direct comparison between them. These four approaches had resulted in the generation of different Southern blot profiles produced by hybridisation with: (1) the gel-purified 6.6 kbp element (Section 3.1.2b), (2) the putative

genomic copies of the 6.6 kbp element (Section 3.1.2b), (3) the PCR product generated by random priming of the 6.6 kbp element (Section 3.3.1) and (4) DNA isolated from clone pRPC9 (Section 3.3.3). From the different Southern blots, a search was made for common restriction fragments that hybridised to the four different probes. DNA corresponding to some of these restriction fragments was isolated from size-fractionated DNA electrophoresed in preparative agarose gels and subsequently purified by the geneclean® method. The purified DNAs were ^{32}P-labelled and used as hybridisation probes on test Southern blots of undigested and *Alu* I and *Sau*3A I digested CP 547 genomic DNA. Of the 15 probes tested, the DNA purified from *Sau*3A I fragments of 1.9 kbp, 5.9 kbp, 6.6 kbp and 13 kbp, and *Eco*R I fragments of 9.2 kbp and 10.9 kbp regions hybridised weakly to the 6.6 kbp element in addition to other restriction fragments (Figure 13). On careful scrutiny of these blots (Figure 13), it became evident that when there was hybridisation to the 6.6 kbp element, then strong hybridisation was also apparent to a larger element that was higher than the 23 kbp fragment of the lambda *Hind* III size marker. However, when there was no hybridisation to the 6.6 kbp element, then there was either no hybridisation or a very faint hybridisation to the larger DNA size class. Although not shown, in the ethidium-bromide stained gels of the Southern blots from figure 13, this large element was visible as a faint band. Since it was difficult to determine the exact size of this large element from conventional agarose gel electrophoresis, it was referred to as the '> 40 kbp element'. Re-analysis of earlier Southern blots probed with putative genomic copies of the 6.6 kbp element also displayed that, although no hybridisation to the 6.6 kbp was evident, some of the putative genomic copies hybridised faintly to the > 40 kbp element (Figure 6, fragment *Sph* I 7.8 and *Eco*R I 9.2). Examination of some of the earlier ethidium-bromide stained gels revealed that the > 40 kbp element was not evident in all the preparations of CP 547, even though the gels were well resolved. Therefore, it appeared that the > 40 kbp element, like the 6.6 kbp element, also displayed a level of instability with respect to its copy number. These observations suggested a possible linkage between the 6.6 kbp element and the > 40 kbp element, thus prompting its

Figure 13

Southern blot analysis of DNA from the CP 547 population using as probe, size-selected genomic gel-purified fragments shown to have cross-hybridised with i) the gel purified 6.6 kbp element, ii) the putative genomic copies of the 6.6 kbp element, iii) the PCR-product generated by random priming of the 6.6 kbp element and (iv) DNA isolated from clone pRPC9. This figure shows six Southern blots (the blots labelled as S, represent short exposures carried out overnight and those labelled as L represent longer exposures of four nights at -80°C with intensifying screens). *The lanes contain (U) undigested total* CP 547 DNA, (1) *Alu* I and (2) *Sau*3A I digested total CP 547 DNA. The ^{32}P-labelled hybridisation probes are indicated at the bottom of the blots whereby, S=*Sau*3A I and E=*Eco*R I. For example, S 13 represents DNA purified from the 13 kbp region of *Sau*3A I digested CP 547 DNA, resolved by gel electrophoresis. The washing conditions were of high stringency (0.1 X SSC, 65°C). The arrow heads indicate the 6.6 kbp element which is more evident in the longer exposures. The arrow points to the > 40 kbp element evident in some of the hybridisations.


S
L
2 1 U
2 U 1
kb
23.6-
9.64-
6.64-
4.34-
2.26-
2.0-
>40 kbp element
Element
Probe: S 13
S 6 6
S 1 9
E 9.2
E 10.9
S 5.9

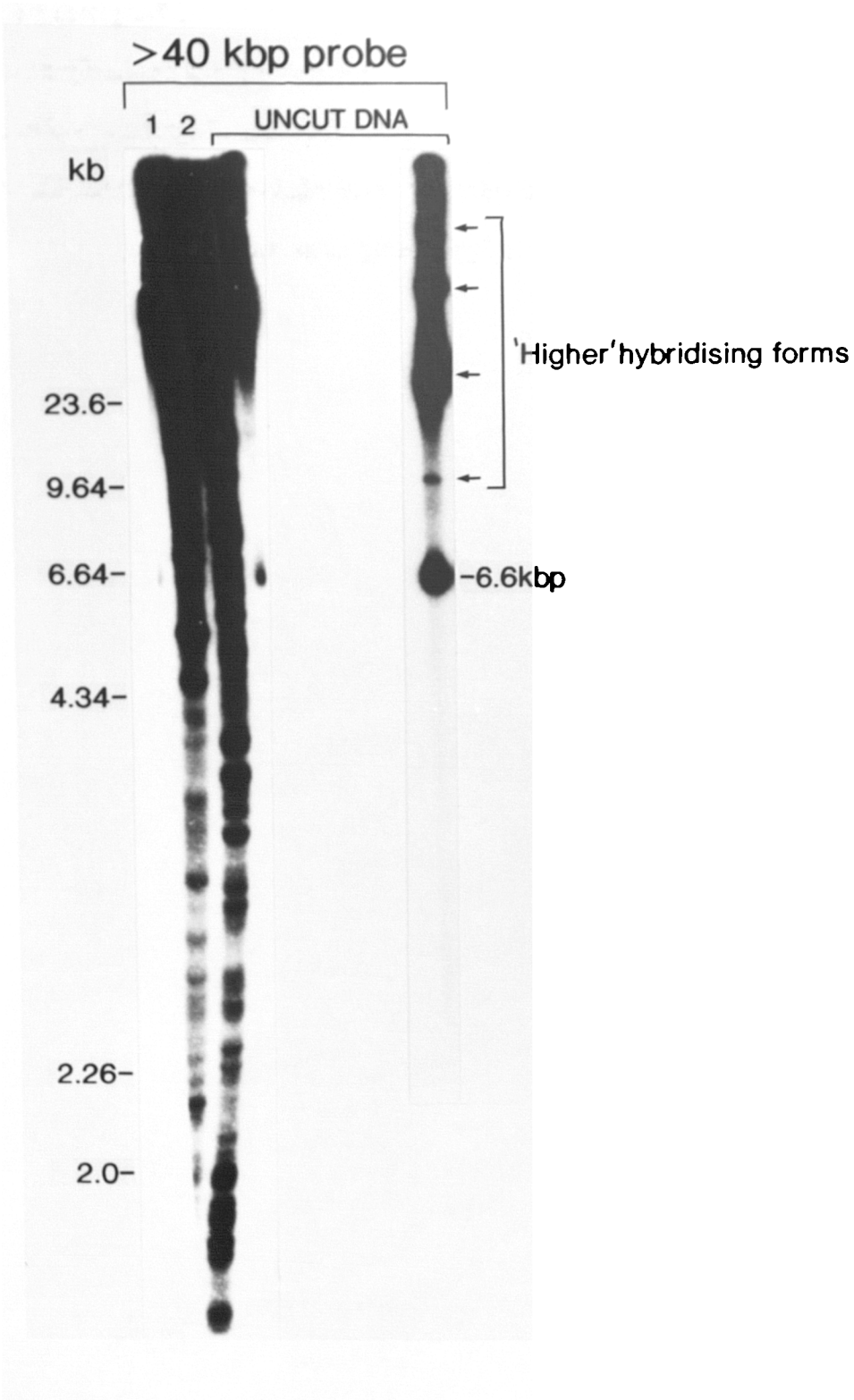
isolation. Purification of sufficient quantities was achieved from CP 547 clone 2 by electrophoresis of two preparative gels, each with 300 μg of total DNA. Following resolution of the region corresponding to the > 40 kbp element, the corresponding gel slice was excised and the DNA extracted by the geneclean® method. Approximately 20 ng of this DNA was then labelled with ^{32}P-dCTP and used as a probe on a test Southern blot of undigested and restricted CP 547 DNA (Figure 14). At high post-hybridisation stringency the > 40 kbp probe hybridised strongly not only to the 6.6 kbp element, but also to a 13 kbp fragment, a region corresponding to 23 kbp, a region corresponding to the > 40 kbp element and a region just below the well. The hybridising size classes larger than the 6.6 kbp element may represent its multimeric forms (Figure 14) and have hence forth been referred to as the 'higher hybridising forms'. In some cases the hybridisation was more evident on longer exposures of the filters to X-ray films.

3.4.2 Analysis with the > 40 kbp element

In order to identify clones containing sequences specific for the 6.6 kbp element, the remainder of the gel-purified > 40 kbp element was used as a ^{32}P-labelled hybridisation probe for screening the genomic and cDNA libraries generated from the CP 547 clone. For each library, about 50,000 recombinants were plated at a density of 5,000 plaques per 150 cm^2 plate. There were approximately 180 positive plaques per plate for the genomic library. From this value, it was estimated that approximately 5% of the genome is represented by this sequence. Screening the cDNA library displayed approximately seven positive plaques per plate, indicating that this sequence is highly transcribed. From each library, five positive clones were randomly chosen and DNA purified from each was used as a radiolabelled probe on test Southern blots containing undigested, *Sau*3A I and *Alu* I digested CP 547 genomic DNA. All these clones gave hybridisation to the 6.6 kbp element and the higher hybridising size classes. Most clones hybridised strongly, while others weakly to the 6.6 kbp and the 13 kbp elements. However, all the clones hybridised strongly to the other higher hybridising forms. In addition, all the clones hybridised to fragments of various sizes generated by digestion

Figure 14

Identification of the 6.6 kbp element in Southern blots of CP 547 DNA using ^{32}P-labelled > 40 kbp probe. DNA from CP 547 digested with (1) *Sau*3A I, (2) *Alu* I and undigested DNA was resolved in a 1.2% agarose gel and blotted onto a sheet of nitrocellulose filter paper. The blot on the right is one generated with only undigested CP 547 DNA. The autoradiogram was obtained when the blots were hybridised with the ^{32}P-labelled > 40 kbp probe. The arrows on the right point to the higher hybridising elements.

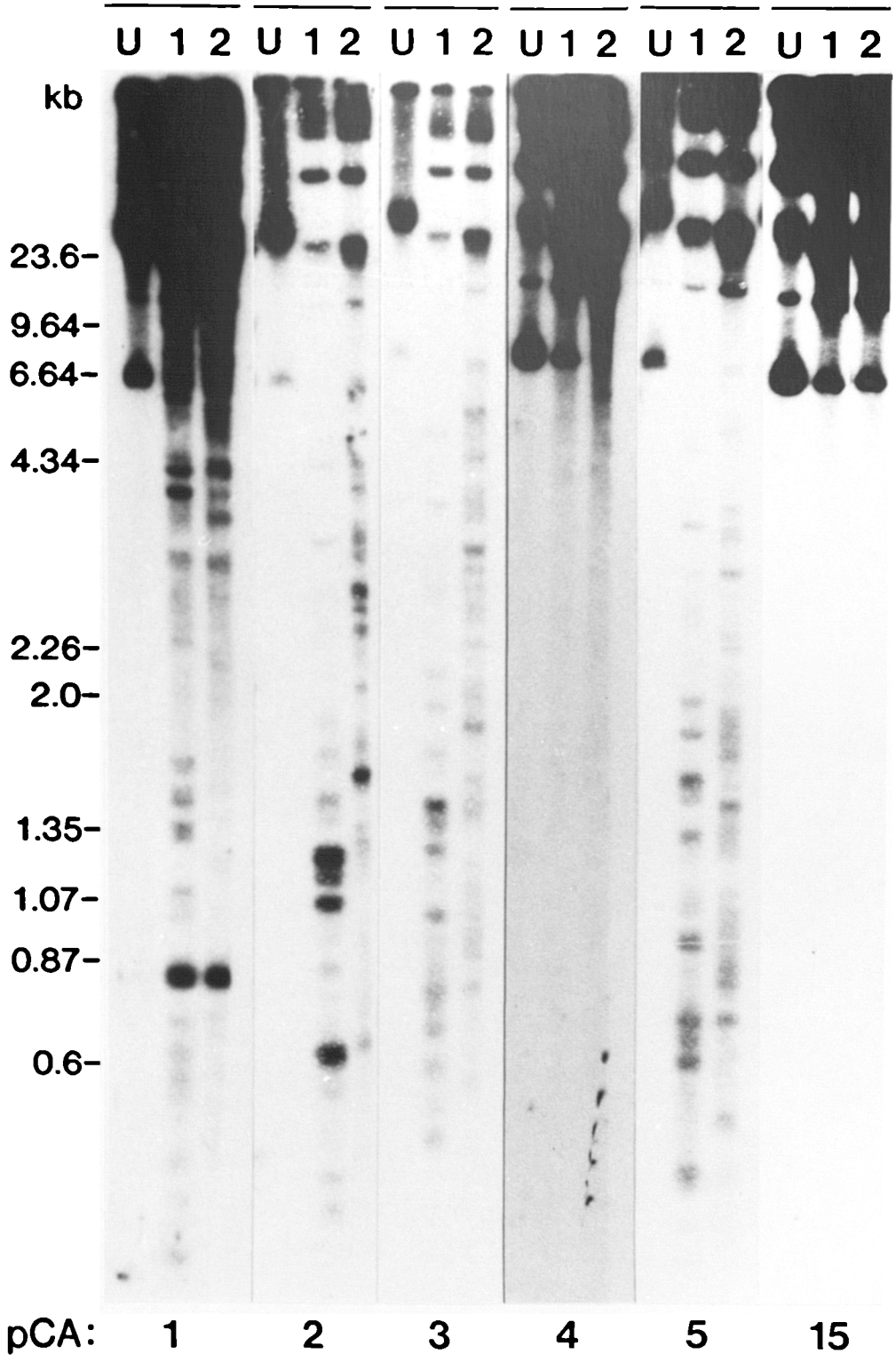

>40 kbp probe
1 2
UNCUT DNA
kb
23.6-
9.64-
6.64-
4.34-
2.26-
2.0-
'Higher' hybridising forms
-6.6kbp

with the two restriction enzymes (Figure 15). Curiously, however, although complete digestion of the genomic DNA was evident from the ethidium-bromide stained gels by the presence of the *Sau*3A I 1.6 kbp fragment representing the TRS-1 sequences (Murphy *et al.*, 1987), and the 177 bp satellite repeat in *Alu* I digested DNA (Sloof *et al*,. 1983), the sizes of the 13 kbp and the > 40 kbp elements were not altered in lanes containing the digested DNA. Similarly, the region corresponding to the 6.6 kbp element in the lanes with the digested DNA was not altered in size although it appeared to be of weaker intensity in the digested DNAs, especially *Alu* I digests of some blots (Figure 15 lanes 1). Various charateristics such as simple sequence repeats, base modifications, single stranded (ss)DNA, ssRNA or DNA/RNA hybrids, could account for lack of digestion of DNA with frequently cutting restriction enzymes such as the *Sau*3A I and *Alu* I. Since the 6.6 kbp element and some of the higher hybridising elements were not digested completely with the frequently cutting enzymes, they were expected to display some such characteristic(s).

From the hybridisation profiles obtained with the gel-purified > 40 kbp probe (Figure 14), it became evident that screening of the CP 547 libraries would result in selection of clones which originate not only from the 6.6 kbp element and the > 40 kbp DNA, but also from other size classes represented by the higher hybridising forms. Hence, in an attempt to isolate clones specific for only the > 40 kbp or the 6.6 kbp element, 10 additional clones were randomly selected (five from the genomic library and five from the cDNA library), and DNA purified from them tested as hybridisation probes on Southern blots of undigested and digested CP 547 DNA. All 10 clones also hybridised to the 6.6 kbp element, other size classes of elements, and to some restriction fragments generated by digestion of total genomic DNA with *Sau*3A I and *Alu* I. However, two clones, cDNA clone CA15 and cDNA clone CA4 hybridised very strongly to the 6.6 kbp and the higher hybridising elements (Figure 15, blot 4 and 15). Unlike the other clones, these two clones did not hybridise to any restriction fragments, although complete digestion of the genomic DNA was evident from the ethidium-bromide stained gels. Failure to obtain clones specific only for the > 40 kbp and the 6.6 kbp element

Figure 15

Southern blot analysis of clones isolated from CP 547 genomic and cDNA libraries containing sequences specific for the 6.6 kbp element. Identical Southern blots were generated with CP 547 DNA whereby each lane contains 2 μg of (U) undigested DNA, (1) *Alu* I and (2) *Sau*3A I digests. The blots were hybridised with ^{32}P-labelled DNA purified from clones picked from CP 547 libraries; genomic clones pCA 1, 2 and 3 and cDNA pCA clones 4, 5, and 15. Both pCA4 and 15 were used for sequence analysis.


U 1 2 U 1 2 U 1 2 U 1 2 U 1 2 U 1 2
kb
23.6-
9.64-
6.64-
4.34-
2.26-
2.0-
1.35-
1.07-
0.87-
0.6-
pCA: 1 2 3 4 5 15

prompted the sub-cloning of the entire insert of 408 bp from CA15 and 1.4 kbp from CA4 in the *Eco*R I site of pBluescript SK (+) to enable further analyses. These clones, subsequently referred to as pCA15 and pCA4 were selected due to their lack of hybridisation to restriction fragments, suggesting that the cloned sequences were limited to the 6.6 kbp element and the higher hybridising classes only, and not distributed throughout the genome.

3.5.1 Verification of the characteristics of the 6.6 kbp element

Earlier experiments (Sections 3.1.2 and 3.2.4) suggested that the 6.6 kbp element is a circular, double stranded DNA molecule. However, these experiments were not conclusive. The availability of a probe specific for the extrachromosomal element now made it possible to verify some of the previous observations regarding the 6.6 kbp element.

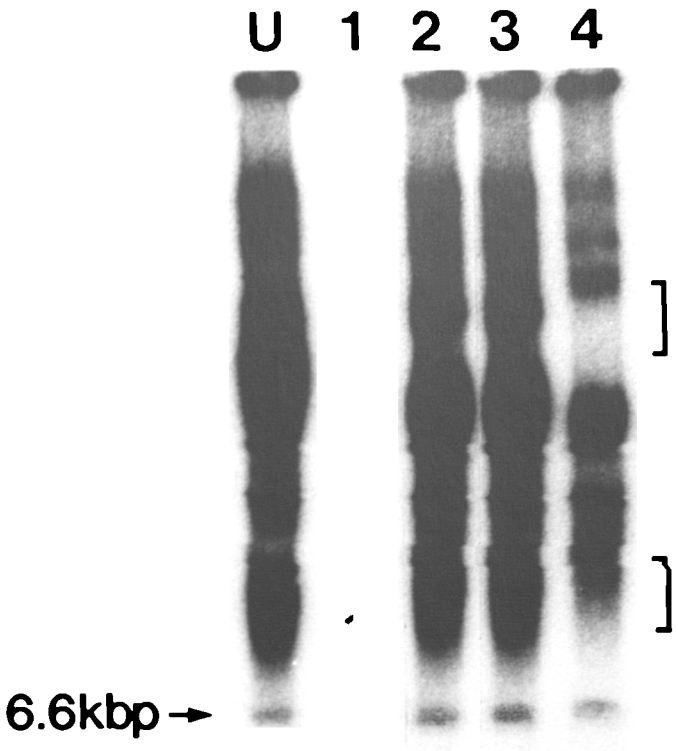
3.5.1a The 6.6 kbp element is DNA

To verify the chemical nature of the 6.6 kbp element, aliquots of CP 547 DNA were incubated with RNase free DNase I, (which digests double and single stranded DNAs), RNase A (that degrades RNAs into small oligoribonucleotides), RNace 'It^{TM} (Stratagene) (which is a cocktail mixture of a RNase A and a non-specific RNase from *Aspergillus olyzae* that also degrades RNAs into small oligoribonucleotides) and RNase H (which digests DNA-RNA hybrids). For DNase I digestion, 2 µg of total DNA was incubated in 5 units at 37°C for 30 min. 2 µg of DNA was incubated in 5 units of RNase A as for RNace 'It^{TM} at 37°C for 30 min. Similarly, for the RNase H digestion, 2 µg of DNA was incubated with 5 units at 37°C for 20 min. The enzymatic reactions were stopped by phenol-chloroform extraction followed by ethanol precipitation. The products were subjected to agarose gel electrophoresis, Southern blotted and hybridised with ^{32}P-labelled clone pCA4. The 6.6 kbp and the higher hybridising elements all appeared to be sensitive to DNase I, indicating that all the elements are either double stranded or single stranded DNA. On overnight exposures, all the hybridising size

Figure 16

The chemical nature of the 6.6 kbp element.

A Southern blot with each lane containing 2 μg of total CP 547 DNA treated with (1) RNase free DNase I, which digests double and single stranded DNAs, (2) RNase A that degrades RNAs into small oligoribonucleotides, (3) RNace 'It^{TM}' (Stratagene) which is a cocktail mixture of a RNase A and a non-specific RNase, (4) RNase H which digests DNA-RNA hybrids, and (U) undigested CP 547 total DNA. This blot was hybridised with ^{32}P-labelled pCA 15 at high stringency (0.1 X SSC, 65°C), and exposure was carried out for 3 h at room temperature with intensifying screens. The brackets indicate the sequences that are sensitive to RNase H.


U
1
2
3
4
6.6kbp

classes appeared to be resistant to the two RNase As and RNase H, indicating that all are DNA molecules. Curiously, however, shorter exposures ranging from 1-3 h revealed that in the region just above the 23 kbp DNA size marker fragment, some of the hybridising elements that are resistant to digestion with RNase A and RNace 'It™' are digested with RNase H (Figure 16). This suggests that these elements may be DNA-RNA hybrids. The existence of such molecules was confirmed by repeating the experiments with a different preparation of the CP 547 DNA. However, these molecules were not characterised further.

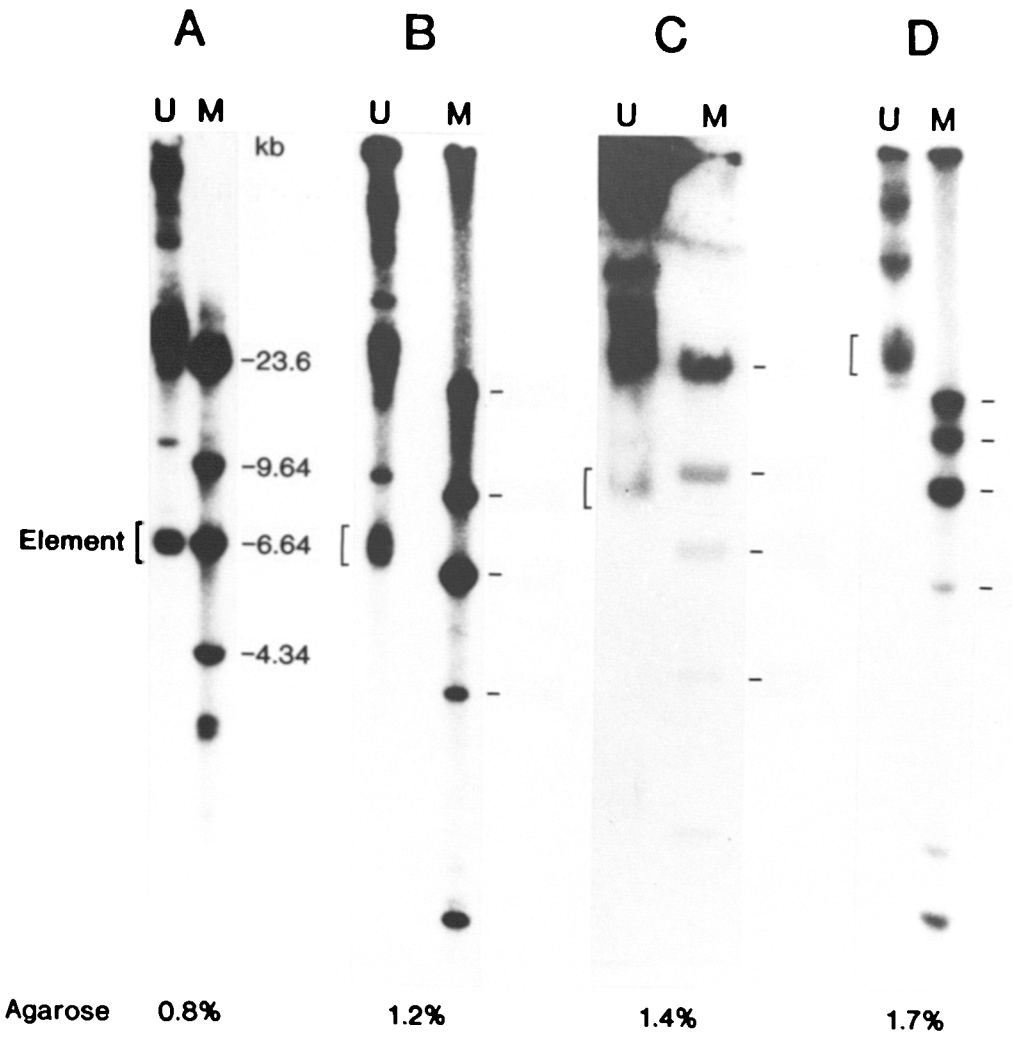
3.5.1b The 6.6 kbp element has characteristics of circular DNA molecules

In the earlier experiments it was curious that some preparations of the gel-purified 6.6 kbp element could not be ^{32}P-labelled (3.1.2b). At the time, this was attributed to insufficient quantities of gel-purified 6.6 kbp DNA on account of its low copy number and due to major losses incurred during its purification. Thereafter, it was realised that in different percentage agarose gels the mobility of the 6.6 kbp element varied relative to linear DNA size markers; for instance, in a 0.8% agarose gel, the 6.6 kbp element migrated at 6.6 kb, in a 1.2% gel, it migrated at 8 kb, in a 1.4% gel, it migrated at 9 kb and in a 1.7% gel it migrated at > than 23 kb (Figure 17). It was then realised that the lack of labelling of some 6.6 kbp DNA preparations was due to the absence of the element in the 6.6 kbp region excised from the agarose gel. Such gel migration characteristics have previously been shown to be typical of circular DNA elements (Beverley *et al.*, 1984). To verify the circular nature of the 6.6 kbp element, an attempt was made to enrich for it by an alkaline lysis method used in the isolation of double stranded circular plasmid DNA molecules from bacteria (Sambrook *et al.*, 1991). Approximately 5 x 10^8 CP 547 trypanosomes were suspended in 500 µl of solution I and the protocol was followed as outlined in section 2.4.5. Southern blot containing undigested CP 547 DNA was hybridised with one fifth of the alkaline lysis preparation. At high stringency washes, strong hybridisation to the 6.6 kbp element, and the elements of higher size classes (Figure not shown) were observed. It was estimated that from 5 x

Figure 17

The mobility of the 6.6 kbp element varies relative to linear DNA size markers in different percentage agarose gels.

Southern blots containing 2 μg of undigested CP 547 DNA (U) resolved in different percentage agarose gels were generated. Lambda DNA cut with *Hind* III was used as a size marker (M). The Southern blots of these gels were hybridised with a mixture of ^{32}P-labelled digested lambda size marker and ^{32}P-labelled total genomic CP 547 DNA. High stringency (0.1 X SSC, 65°C) washing conditions were used. The bracket on each panel is what has been referred to as the 6.6 kbp element (even when it is not localised to the 6.6 kb region).


A
B
C
D
U M
U M
U M
U M
kb
-23.6
-9.64
-6.64
-4.34
Element
Agarose 0.8%
1.2%
1.4%
1.7%

10^8 trypanosomes, approximately 200 ng of DNA specific for the 6.6 kbp element and the higher hybridising elements could be purified. The extent of enrichment by the alkaline lysis protocol was determined by concurrently size separating titrations of the DNA enriched by alkaline lysis in comparison to titrations of total CP 547 DNA by agarose electrophoresis. A Southern blot of this gel was probed with the ^{32}P-labelled clone pCA4 (Figure 18). This revealed that approximately 10 ng of DNA enriched by alkaline lysis gave a signal intensity corresponding to the 6.6 kbp element achieved by approximately 2 µg of total genomic DNA from CP 547 (Figure 18). Therefore, the alkaline lysis protocol enriched for the 6.6 kbp and the higher hybridising elements by a factor of 200 X. An attempt was made to digest 10 ng aliquots of the alkaline lysed preparation with 1 unit of frequently cutting restriction enzymes like *Alu* I, *Sau*3A I and *Taq* I. However, as for the 6.6 kbp and the higher hybridising elements in total CP 547 genomic DNA, the elements enriched by the alkaline lysis method also lacked sites for these restriction enzymes. Since the alkaline lysis protocol enriches for double stranded circular elements, the enrichment of the 6.6 kbp element and the higher hybridising elements using this protocol establishes their circular, and double stranded, nature.

The circular nature is also indicated by the lack of hybridisation to an oligonucleotide probe generated from a consensus telomeric sequence (5 CCCTGAACCCTGAA 3) (gift from Dr. Bishop). Even at medium stringency washes (5 X SSC, 0.1% SDS at 50°C for 40 min) (Figure 19), this probe did not hybridise to the 6.6 kbp element and the > 40 kbp element, indicating that either these elements do not have classical telomeric ends characteristic of chromosomes, or that they are circular in nature.

In order to further confirm the circular nature of the 6.6 kbp and the higher size class elements, an attempt was made to determine their sensitivity to *BAL* 31 exonuclease digestion. 10 µg of total CP 547 DNA was incubated at 30°C with 2 units of *BAL* 31 in a volume of 1 ml of the enzyme specific buffer (one unit was previously determined to delete 400 base pairs per minute). At time points 0.5, 1, 5, 10 and 15 min, 200 µl aliquots were withdrawn from the reaction and added to 100 µl of phenol, 10 mM EDTA to stop the reaction. The DNA was phenol-chloroform extracted and isopropanol precipitated.

Figure 18

The 6.6 kbp and the higher hybridising elements are enriched by an alkaline lysis protocol for the isolation of circular DNA molecules.

This Southern blot shows the extent of enrichment of the 6.6 kbp element from total genomic CP 547 DNA by the alkaline lysis protocol (Section 2.4.5) in comparison to total genomic DNA prepared by conventional protocols (Section 2.4.1). Lanes 1-5 of the alkaline lysis DNA preparation represent 10 ng, 20 ng, 30 ng, 40 ng and 50 ng of DNA respectively. For the total CP 547 DNA lanes 1-5 represent 100 ng, 200 ng, 300 ng, 400 ng and 500 ng, respectively. To ensure that complete transfer of the resolved DNA was achieved, a 0.7% agarose gel was used and the transfer on to nylon membrane was carried out over 2 days. The blot was then hyridised with ^{32}P-labelled clone pCA4 with washing conditions of high stringency (0.1 X SSC, 65°C) and exposed overnight at -80°C with intensifying screens.

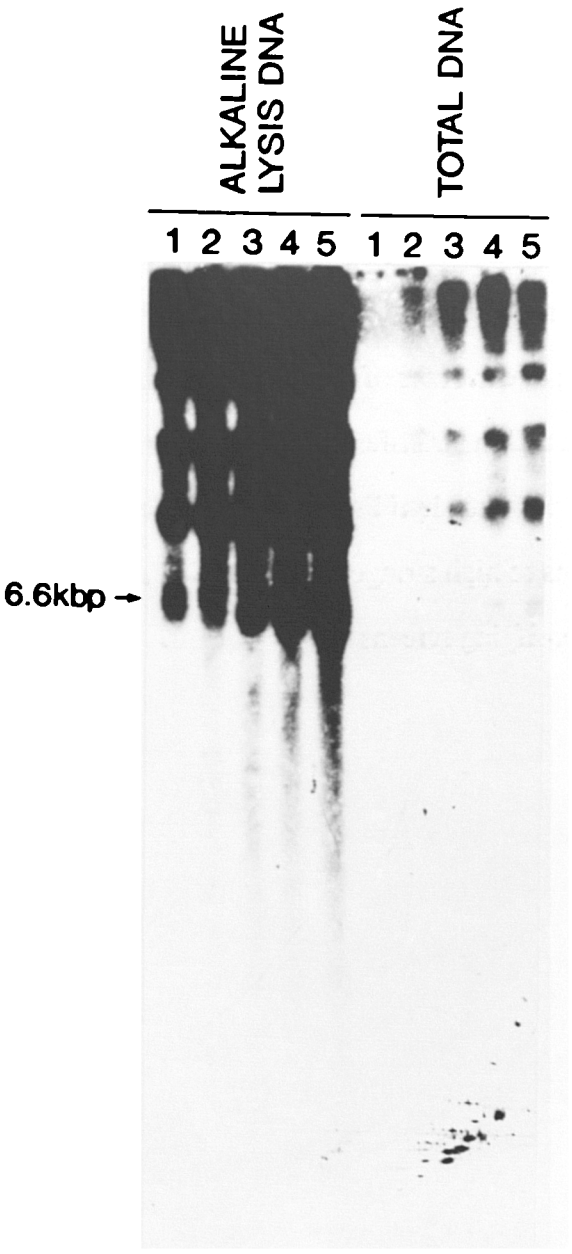
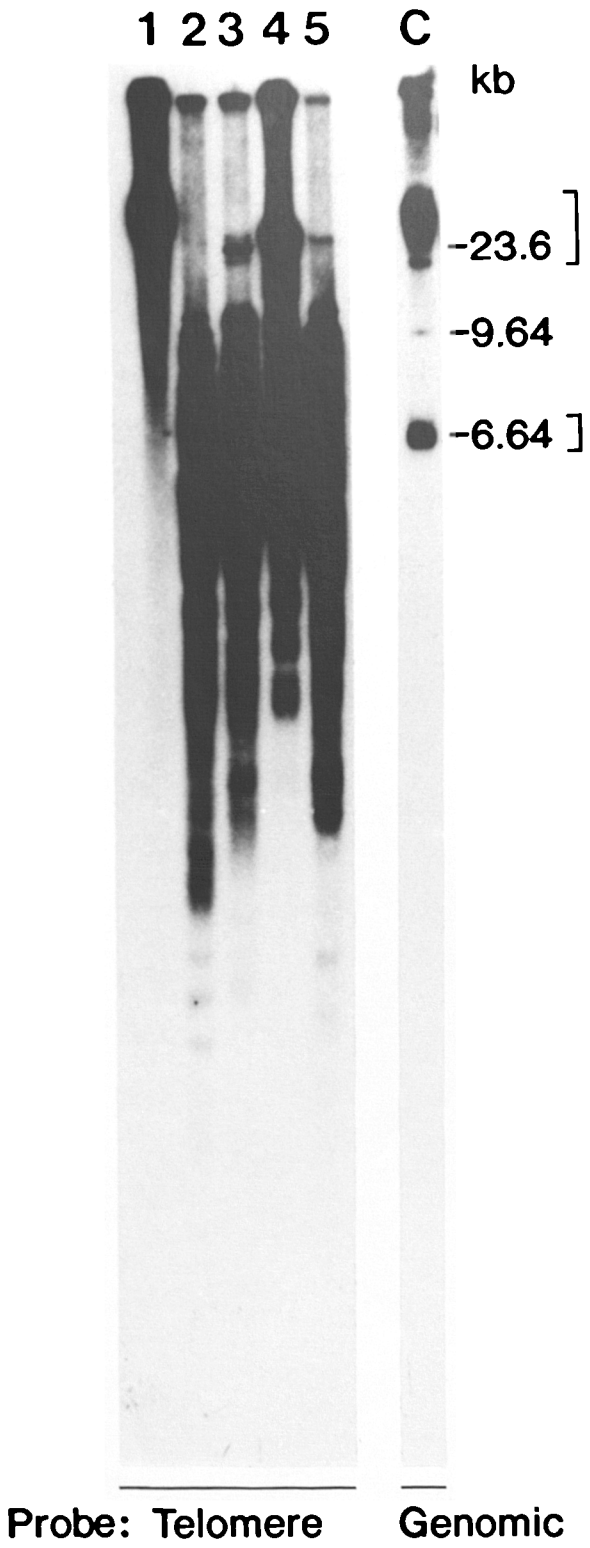

ALKALINE LYSIS DNA
TOTAL DNA
1 2 3 4 5 1 2 3 4 5
6.6kbp

Figure 19

The 6.6 kbp and the > 40 kbp element do not hybridise to telomeric sequences.

A Southern blot generated from a gel of (1) undigested DNA, (2) *Alu* I, (3) *Sau*3A I, (4) *Eco*RI and (5) *Taq* I digested CP 547 DNA. The blot was hybridised with a ^{32}P-labelled consensus telomeric sequence. Even at medium stringency washes (5 X SSC, 0.1% SDS at 50°C for 40 min) this probe did not hybridise to the 6.6 kbp and the > 40 kbp element; in lanes 2-5 the > 40 kbp element is not visible. (C) represents a control blot of undigested CP 547 DNA. This strip was cut from the same Southern blot as the one probed with the telomere probe, but was hybridised with ^{32}P-labelled total CP 547 DNA. From lane C, the position of the 6.6 kbp element and the > 40 kbp element (in brackets) is evident.


1 2 3 4 5
C
kb
-23.6
-9.64
-6.64
Probe: Telomere
Genomic

All the *BAL* 31-digested DNA aliquots and a control sample of undigested CP 547 DNA were resolved by agarose gel electrophoresis and analysed by Southern blot hybridisation with ^{32}P-labelled clone pCA4. The hybridisations revealed that the region corresponding to the 6.6 kbp element was consistently masked by sequences generated from the degradation of genomic DNA and possibly some of the higher hybridising elements sensitive to the exonuclease activity of *BAL* 31. However, on short exposures it was clearly evident that the 13 kbp and some regions corresponding to the 23 kbp of the lambda DNA size marker were *BAL* 31 sensitive, indicating their linear nature (Figure 20a). Attempts to confirm these results using the DNA enriched by alkaline lysis were unsuccessful, most likely due to the presence of impurities that inhibited the *BAL* 31 exonuclease. Although it was difficult to establish the sensitivity of the 6.6 kbp element to *BAL* 31 exonuclease, it became apparent that the > 40 kbp element is resistant to *BAL* 31 digestion and is thus most likely a circular molecule (Figure 20a marked with an asterisk). Efforts to visualise the 6.6 kbp region involved doubling the number of units of *BAL* 31 in order to increase the rate of digestion and therefore clear the fragments masking the 6.6 kbp region. This approach, however, did not accomplish the desired results because of apparent nicking of the circular DNAs as evidenced by the apparent linearising of the > 40 kbp molecule (Figure 20b, marked with an asterisk). Although the 6.6 kbp element could not be clearly visualised, there are indications of it being resistant to the exonuclease (Figure 20b, marked with an arrow). Size-fractionation of the *BAL* 31-digested total CP 547 DNA revealed far more higher hybridising extrachromosomal elements than is evident by electrophoresis of undigested CP 547 DNA. From their response to *BAL* 31 digestion it became evident that some of these elements are linear while others are circular (Figure 20b).

3.5.1c Plasticity within the 6.6 kbp element

i) Unstable copy number

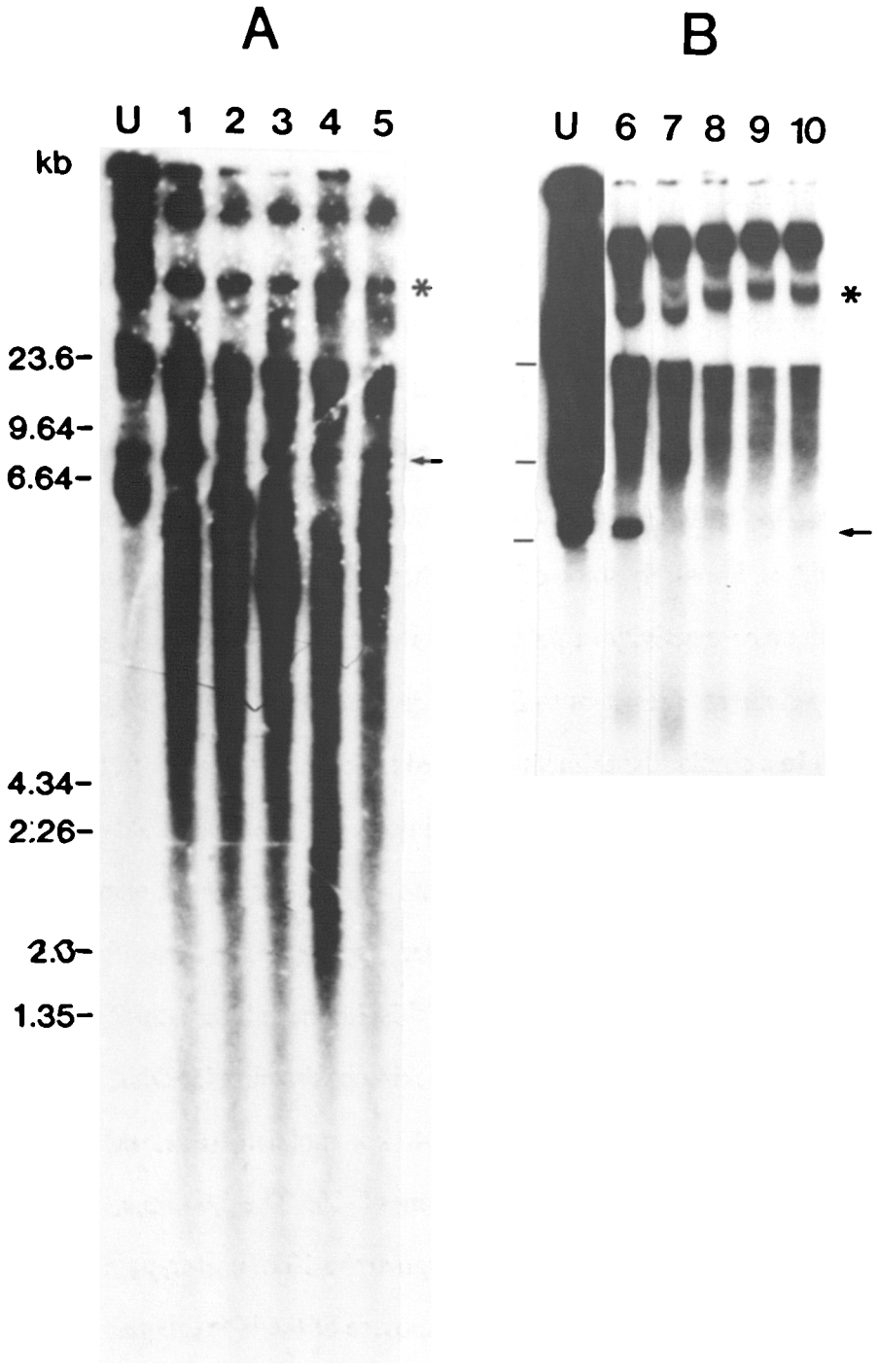
The availability of a specific probe for the 6.6 kbp element enabled the unequivocal demonstration of instability of its copy number. In Southern blots of

Figure 20

The sensitivity of the 6.6 kbp and the higher size class-elements to *BAL* 31 exonuclease digestion.

(A) 10 μg aliquots of total CP 547 DNA were treated with 2 units of *BAL* 31 (one unit was previously determined to delete 400 base pairs per minute) and samples removed at different time points. Lanes 1-5 represent time points 0.5, 1, 5, 10 and 15 min and U is untreated, undigested DNA . The DNA was resolved by agarose gel electrophoresis and analysed by Southern blot hybridisation with ^{32}P-labeled clone pCA4. High stringency washes (0.1 X SSC, 65°C) revealed that the region corresponding to the 6.6 kbp element (indicated by arrow) was consistently masked by sequences generated from the degradation of genomic DNA and possibly some of the higher hybridising elements sensitive to the exonuclease activity of *BAL* 31. However, it was clearly apparent that the > 40 kbp element is resistant to *BAL* 31 digestion (marked with an asterisk).

(B) In a similar experiment designed to increase the rate of digestion, and therefore clear the fragments masking the 6.6 kbp region, 4 units of *BAL* 31 were used for time points 8, 10, 30, 40 and 45 min (lanes 6-10 respectively). In addition, this gel was electrophoresed longer so as to better resolve the region around > 40 kbp. Hybridisation of this Southern blot with ^{32}P-labeled clone pCA4 however showed apparent nicking of the circular DNAs. This is evident by the possible linearising of the > 40 kbp molecule (asterisk) in lane 7 which shows relative shift in mobility in lanes 8-10. The 6.6 kbp element was clearly seen at the 8 min time point, although at the 10 min time-point the 6.6 kbp element was degraded. In longer exposure of the blots, there are indications of some copies of it being resistant to the exonuclease (at the position indicated by arrow).


A
B
U 1 2 3 4 5
U 6 7 8 9 10
kb
23.6-
9.64-
6.64-
4.34-
2.26-
2.0-
1.35-
*
*

undigested CP 547 DNA purified from different CP 547 populations it was possible to illustrate that the copy number of the element is directly associated with the application of diminazene aceturate drug pressure (Figure 21). The response to passaging diminazene-selected clone 2 (figure 21, lane 3) in the absence of diminazene pressure is evident by the reduction in the copy number of the 6.6 kbp element (Figure 21, lanes 4). Hence, as previously mentioned in section 3.2.2, Figure 21 verifies the instability of the copy number of the 6.6 kbp element in the absence of selective drug pressure. That the DNA loading was uniform in all the lanes was confirmed by reprobing the Southern blot with a ^{32}P-labelled ß-tubulin gene probe isolated from *T. b. brucei* (Figure 21).

ii) Size and sequence heterogeneity of the 6.6 kbp element

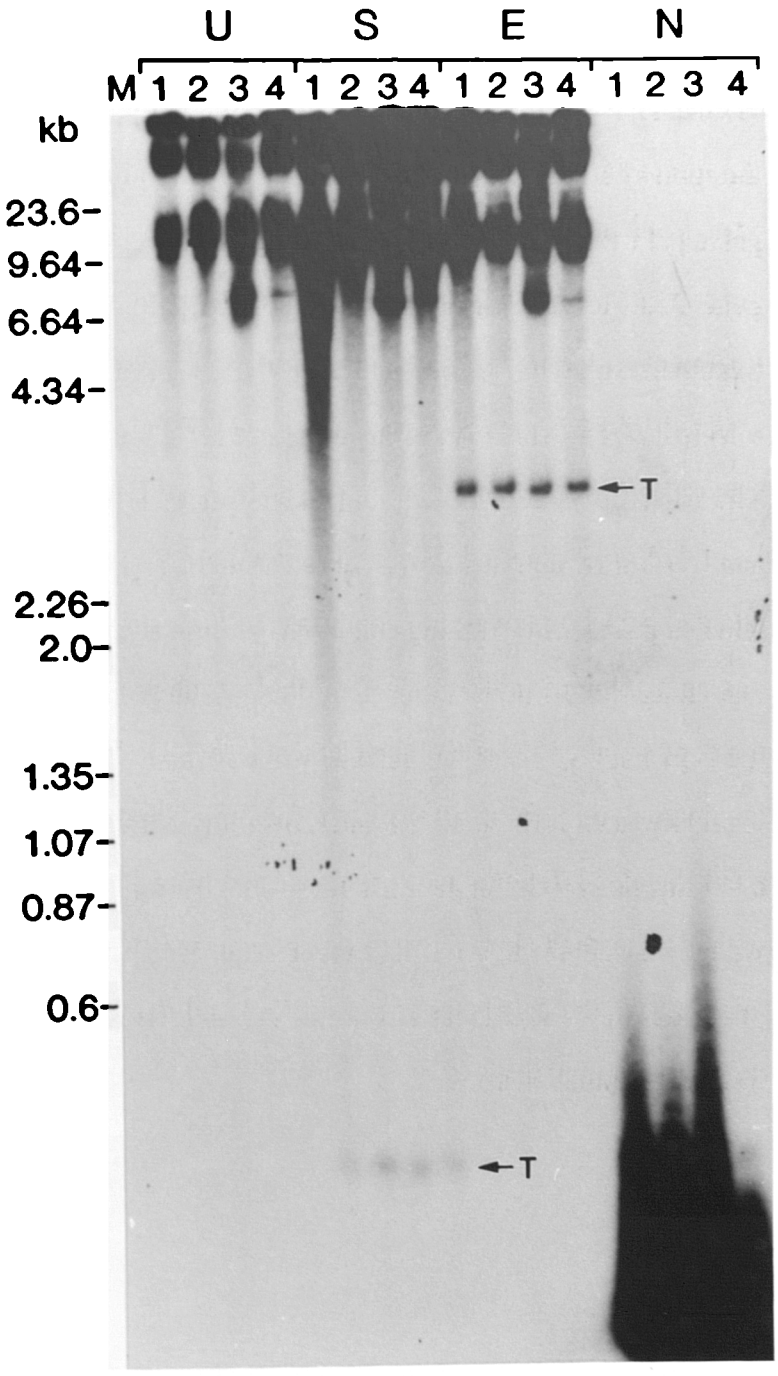
Southern blots generated from different isolations of CP 547 total genomic DNA showed that the 6.6 kbp element varied not only in size and copy number, but also in appearance (Figure 22a). In most preparations of the CP 547 DNA, the signal corresponding to the 6.6 kbp element appeared as a tight band, although in some preparations it appeared as a smeared, fuzzy region. Furthermore, in some DNA isolations from the CP 547 stock, the 6.6 kbp element appeared as a doublet, whereas clones generated from this stock gave a single band corresponding to the element (Figure 22a). This variability in the 6.6 kbp element implies that there may be rapid reorganisation occurring within the population(s) of the 6.6 kbp element, giving rise to a heterogeneously sized species of the element. In addition, it could be possible that the CP 547 stock is a mixed population.

During the course of these investigations, evidence was obtained which suggested that in addition to size heterogeneity, the 6.6 kbp element also displays a level of sequence heterogeneity. The first indication was obtained from earlier results presented in section 3.1.2b whereby, although infrequently some gel-purified 6.6 kbp probes hybridised to restriction fragments that were not recognised by other 6.6 kbp probes. These results suggested that there may be some level of sequence heterogeneity within the 6.6 kbp region, either due to the presence of different sheared, comigrating genomic

Figure 21

The copy number of the 6.6 kbp element is directly influenced by the application of diminazene aceturate drug pressure.

A Southern blot was generated containing DNA from different CP 547 populations resolved on a 1.2% agarose gel. Each set of 4 lanes is represented by (1) the CP 547 stock passaged in mice, without any drug pressure over the duration of 6 months, (2) the unselected CP 547 clone 1 generated from the passaged CP 547 stock in lane 1, (3) the diminazene selected CP 547 clone 2 generated from the relapse population of the parental CP 547 stock after treatment with diminazene aceturate (60 mg/kg b.w.) and (4) the diminazene selected CP 547 clone 2, passaged in mice for 2 months without drug pressure. The sets of lanes contain 2 µg of (U) undigested DNA, (S) *Sau*3A 1, (E) *EcoR* I and (N) *Nla* III digested DNAs. The Southern blot was hybridised with ^{32}P-labelled pCA4 with washing conditions of high stringency (0.1 X SSC, 65°C). Passaging clone 2 in the absence of diminazene pressure results in a reduction in the copy number of the 6.6 kbp element (lanes 4). To verify that the DNA loading was uniform in all the lanes, the same Southern blot was re-probed with a ^{32}P-labeled ß-tubulin gene probe isolated from *T. b. brucei*. The arrow marked with T indicate the hybridising tubulin sequences which show equal signal intensities in the four lanes of the *Sau*3A I and *EcoR* I digested DNAs from the different populations.


U
S
E
N
M 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
kb
23.6-
9.64-
6.64-
4.34-
2.26-
2.0-
1.35-
1.07-
0.87-
0.6-
T
T

contaminants, or heterogeneity within the 6.6 kbp element itself. Subsequently, verification that not all 6.6 kbp species are homogeneous in sequence was established from one CP 547 clone generated without selective drug pressure. Total DNA purified from this clone was digested with selected frequently cutting restriction enzymes and analysed by Southern blot analysis through hybridisation with ^{32}P-labelled clone pCA4. The 6.6 kbp element from this CP 547 population was completely digested by *Sau*3A I and *Alu* I, but not *Eco*R I (Figure 22b, lane 2), suggesting that unlike most 6.6 kbp elements, this population of the 6.6 kbp element possessed sites for *Sau*3A I and *Alu* I but not for *Eco*R I. However, after digestion of the 6.6 kbp element no hybridisation to restriction fragments smaller than 6.6 kbp element was evident. This observation could be explained by the possibility that in the 6.6 kbp element the sequence specific for the pCA4 was digested to such small sized fragments, that they were not evident because they ran off the agarose gel. Due to lack of specific probes, sequences other than those present on pCA4 can not be detected. This CP 547 clone appeared to have the ideal species of the 6.6 kbp element, since the presence of the different restriction enzyme sites could allow one to clone sequences directly from the element. Therefore, in an attempt to isolate sufficient quantities of this 6.6 kbp species, the clone of CP 547 was expanded in mice and DNA isolated from the parasites. Curiously, however, the 6.6 kbp element from the expanded population reverted to being the typical form of 6.6 kbp element, devoid of restriction enzyme sites that could have permitted its cloning into an *E. coli* plasmid vector. These puzzling observations indicated that a high level of sequence reorganisation may be occurring within the 6.6 kbp element or that selection of a sub-population from a heterogeneous 6.6 kbp population is taking place. Further evidence to support the presence of heterogeneous 6.6 kbp species was obtained from Southern blots in which the total CP 547 DNA was digested with frequently cutting restriction enzymes. Some of these blots disclosed that in some isolations of CP 547 DNA, digestion with *Alu* I resulted in a decrease in signal intensity corresponding to the 6.6 kbp element in comparison to the signal intensity corresponding to the 6.6 kbp element from the undigested DNA (Figure 22c). However, in some isolations of total CP 547 DNA, no

Figure 22

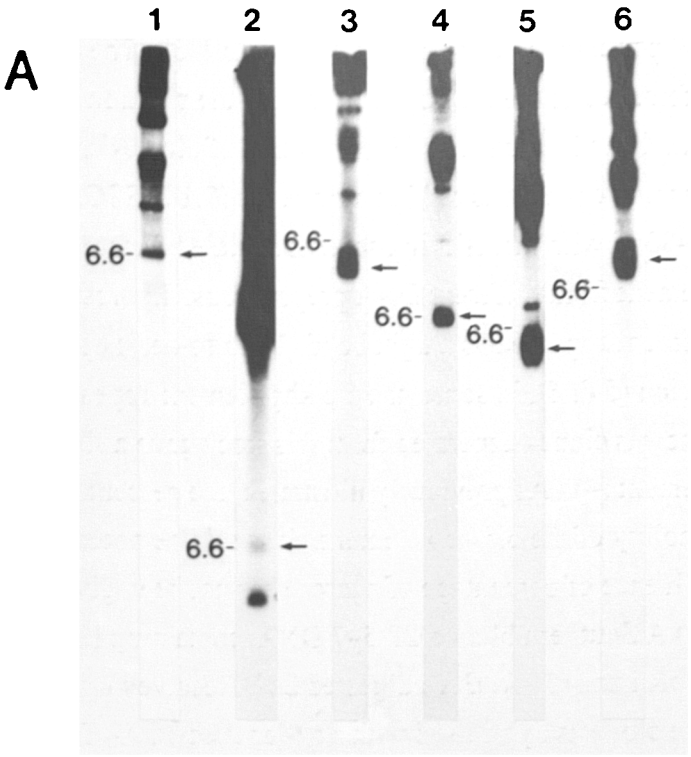
Size and sequence heterogeneity of the 6.6 kbp element in different populations of the same *T. b. brucei* isolate.

(A) Southern blot containing 2 µg of undigested CP 547 DNA from different CP 547 populations were generated (tracks 1-6). The arrows depict what has been referred to as the 6.6 kbp element and the position of the linear 6.6 kb size marker is indicated on the left. Hybridisation of blots with ^{32}P-labelled pCA4 with washing conditions of high stringency (0.1 X SSC, 65°C) and overnight exposure at -80°C with intensifying screens revealed differences in the appearance of the element from different DNA preparations. In most preparations, the 6.6 kbp element appeared as a tight band (1), in some, it appeared as a fuzzy, smeared region (5 & 6), in some, the 6.6 kbp element appeared as a doublet (2 & 3), whereas clones generated from this stock gave a single band corresponding to the element (4). As previously illustrated, the percentage of the agarose affects the mobility of the 6.6 kbp element. It should be noted that in panel A, the possible influence of percentage of agarose has not been given any consideration.

(B) A Southern blot of CP 547 DNA containing (U) undigested, (1) *Alu* I, (2) *Eco*R I and (3) *Sau*3A I digested DNA resolved by agarose gel electrophoresis. The blot was hybridised with ^{32}P-labelled pCA4 DNA. For this CP 547 clone, the 6.6 kbp element was un-typical as it was completely digested by *Alu* I (1) and *Sau*3A I (3). However, on expanding this clone in mice, in addition to *Eco*R I, the 6.6 kbp element could not be digested by *Alu* I and *Sau*3A I.

(C) The 6.6 kbp element from the diminazene selected clone 2 also displayed some heterogeneity. The lanes in panel C are as in panel B, except DNA from the diminazene selected clone was used. This and similar blots disclosed that in some preparations of CP 547 DNA, digestion with *Alu* I resulted in a decrease in signal intensity corresponding to the 6.6 kbp element (1) in comparison to the signal intensity corresponding to the 6.6 kbp element from the undigested DNA (lane U). However, in some isolations of total CP 547 DNA, no such decrease in signai intensity was apparent in *Alu* I digested DNA (Figures 15 & 36b). That this is not a result of unequal loading is evident from the equal signal intensities of the higher hybridising elements.

The observations in panels A, B and C show that the 6.6 kbp element is composed of heterogeneous populations whereby minor populations of the 6.6 kbp species, displaying 'un-typical' characteristics, are sometimes selected for.


A
1
2
3
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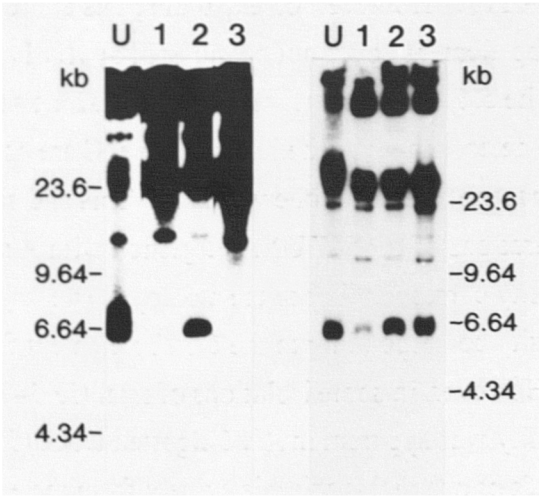
5
6
6.6-
6.6-
6.6-
6.6-
6.6-
6.6-


B
U 1 2 3
kb
23.6-
9.64-
6.64-
4.34-
U 1 2 3
kb
C
-23.6
-9.64
-6.64
-4.34

such decrease in signal intensity was apparent in *Alu* I digested DNA. These observations indicate that the 6.6 kbp element is composed of heterogeneous populations, and in some total DNA isolations, minor populations of 6.6 kbp species contain site(s) for *Alu* I and are digested by this enzyme. Since the major species lack an *Alu* I site and remains undigested, the digestion of some of the elements results in a decrease in hybridisation signal intensity. However, in other isolations, for unknown reasons, the species containing sites for *Alu* I are selected against. If the estimation of copy numbers in section 3.1.2a is reasonably accurate, then the minor species containing the *Alu* I sites must occur at less than one copy per genome in a mixed population of parasites once they have been expanded for analysis.

3.5.2 Sequence analysis of clones hybridising to the 6.6 kbp element

In order to identify the precise sequences responsible for hybridisation to the 6.6 kbp and the higher hybridising elements, cDNA clones pCA15 and pCA4 were sequenced. To localise the sequences within these clones responsible for the hybridisation to the 6.6 kbp element, restriction enzyme analysis of both these clones was performed. Digestions of 2 µg aliquots of each clone were attempted with some frequently cutting enzymes, and some restriction enzymes with sites present in the pBluescript polylinker sequence. However, since neither of the inserts contained sites for any of the tested restriction enzymes, the entire inserts of pCA 15 and pCA 4 were sequenced by the di-deoxy method developed by Sanger *et al.* (1977).

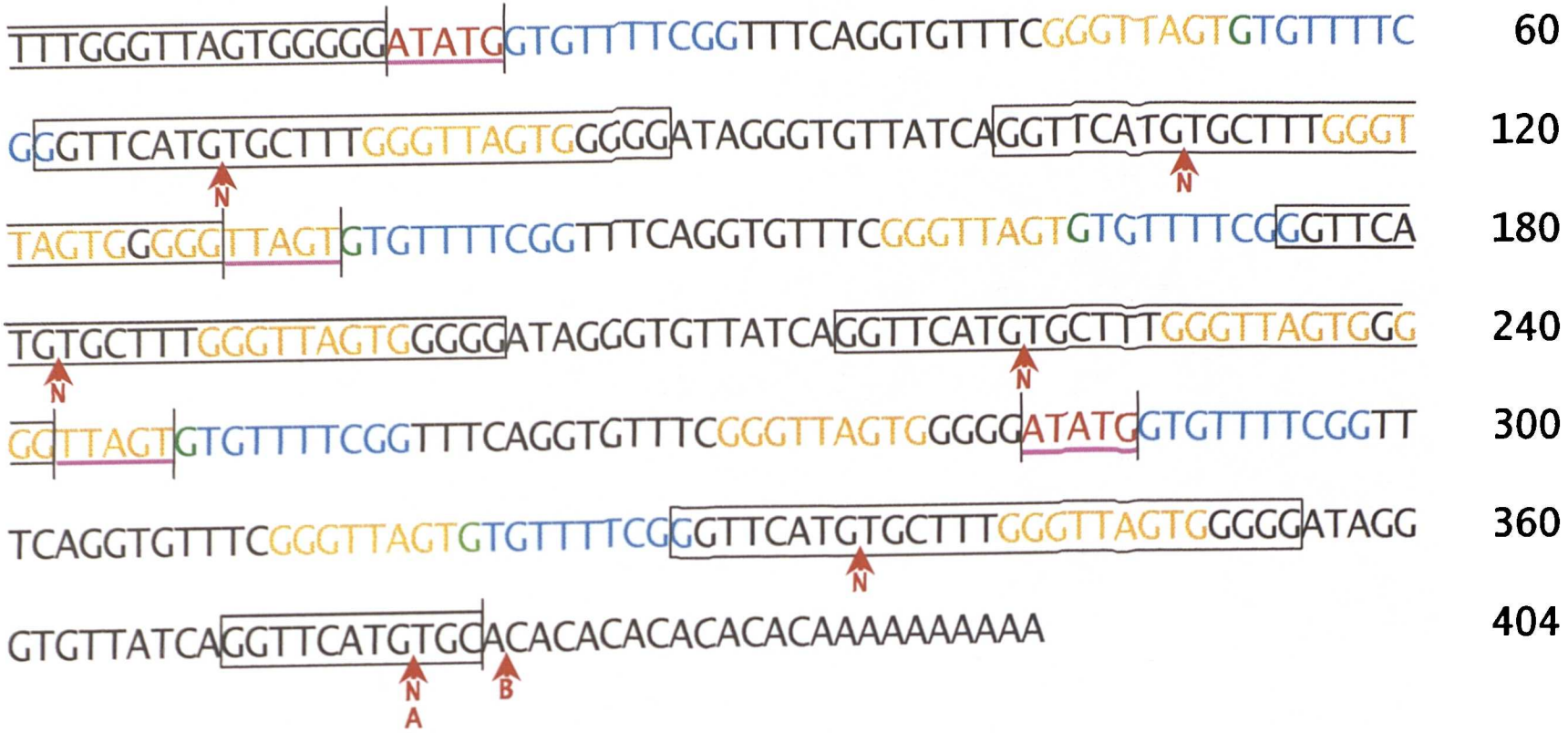
Nucleotide sequence analysis of pCA15 disclosed the presence of a short poly A tail composed of 9 A's (Figure 23). In addition, it contained 2 complete and 2 partial copies of a 108 bp tandem repeat sequence, whereby, each repeat unit is interspersed with a 5 bp spacer sequence (Figure 23). A direct 5 bp repeat sequence flanks copy 1 and partial copy 3. These 5 bp direct repeats are unique to pCA15 as they are and not present in other sequenced clones. Upstream of the poly A tail is a small microsatellite sequence of seven CA dimeric repeats. From the sequence generated, *Nla* III is the only restriction enzyme that cuts within the 108 bp repeat, thus explaining the lack of digestion of the

Figure 23

Nucleotide sequence of pCA15.

Using a ^{32}P-labelled >40 kbp probe, this clone was isolated from a cDNA library generated from CP 547. By the dideoxy sequencing method, this clone was found to contain two complete copies of a 108 bp repeat (subsequently referred to as the *Nla* III repeat) and two incomplete copies of the 108 bp repeats. The complete and partial copies of the 108 bp repeat lying consecutive to each other are defined within the vertical lines. Each copy is flanked by a 5 bp spacer sequence (underlined in purple, and lying between the two vertical lines). In most, but not all clones, the ends of the 108 bp repeat is followed by the TTAGT sequence. However, the 5 bp ATATG sequence represented in red possibly represents the duplicated ends of a transposed segment containing two complete and one partial copy of the 108 bp repeat. Each 108 bp repeat contains overlapping copies of smaller sub-repeats. These are represented by three copies of a 9 bp repeat (shaded yellow), two copies of a 10 bp repeat (shaded blue) and two copies of a 27 bp repeat (boxed). The nucleotide overlapping the yellow and the blue repeats is represented in green.

At the 3' end of the 108 bp repeats, a short poly-A tail composed of 9 A's is present. Upstream to the poly-A tail is a small microsatellite sequence of seven CA dimeric repeats. The restriction sites identified in this clone are (N) *Nla* III, (A) *Apa*L I and (B) *Bsp*1286.


TTTGGGTTAGTGGGGGATATGGTGTTTTCGGTTTCAGGTGTTTCGGGTTAGTGTGTTTTC
60
GGGTTCATGTGCTTTGGGTTAGTGGGGGATAGGGTGTTATCAGGTTCATGTGCTTTGGGT
120
N
N
TAGTGGGGGTTAGTGTGTTTTCGGTTTCAGGTGTTTCGGGTTAGTGTGTTTTCGGGTTCA
180
TGTGCTTTGGGTTAGTGGGGGATAGGGTGTTATCAGGTTCATGTGCTTTGGGTTAGTGGG
240
N
N
GGTTAGTGTGTTTTCGGTTTCAGGTGTTTCGGGTTAGTGGGGGATATGGTGTTTTCGGTT
300
TCAGGTGTTTCGGGTTAGTGTGTTTTCGGGTTCATGTGCTTTGGGTTAGTGGGGGATAGG
360
N
GTGTTATCAGGTTCATGTGCACACACACACACACAAAAAAAAAA
404
N
A
B

inserts with all the tested restriction enzymes. Immediately 5' to the microsatellite sequences exists a site for *Apa*L I and *Bsp*1286 I restriction enzymes. As discussed later, the 5 bp direct repeats flanking the two complete and one partial 108 bp repeats in pCA15 may represent a transposed segment. The 108 bp repeat unit contains no open reading frames and is itself made up of several overlapping smaller repeat sequences. These are represented by three copies of a 9 bp sub-repeat, 2 copies of a 10 bp sub-repeat and 2 copies of a 27 bp sub-repeat (Figure 23). In expectation of additional sequence information, pCA4, the larger of the clones, was also sequenced. Nested deletions of this clone were generated using an Exonuclease III/ Mung bean-nuclease kit (Stratagene) (Figure 24a,b). Sequencing ends of six consecutive size deletions suggested that the entire pCA4 clone is made of 108 bp-derived repeats arranged in tandem. Various copies of different *Nla* III repeats sequenced from independent clones are shown in Figure 24c. Examination of these copies revealed that the repeat can be bigger than 108 bp due to insertion of parts of the same repeat sequence within the basic repeat unit; the repeat could be smaller than 108 bp due to deletion of some nucleotides. The 5 bp spacer sequence was most frequently, but not always, represented by the sequence TTAGT, a potion of 9 bp subrepeat. Sequence analysis of the ends of three additional genomic clones that also hybridised to the 6.6 kbp element and the larger elements revealed only the presence of 108 bp-derived repeats.

Database searches (Genbank database release 77) disclosed that the 108 bp repeats show the highest homology (87.5% in a 40 bp overlap) with a subtelomeric sequence in a subset of minichromosomes of *T. b. brucei* (Figure 25) (Weiden *et al.*, 1991). The maximum homology of the 108 bp repeat was with a 29 bp subtelomeric sequence repeated 6 X in the minichromosome.

3.5.3 The 6.6 kbp element contains the *Nla* III repeat.

The hybridisation of clones pCA15 and pCA4 at high stringency to the 6.6 kbp element and the higher hybridising elements indicates the presence of the 108 bp repeat, or a part its sequence, on these elements. To test this possibility, attempts were made to

Figure 24

(A) Nested deletions of cDNA clone pCA4 generated using an Exonuclease III/ Mung bean-nuclease kit (Stratagene).

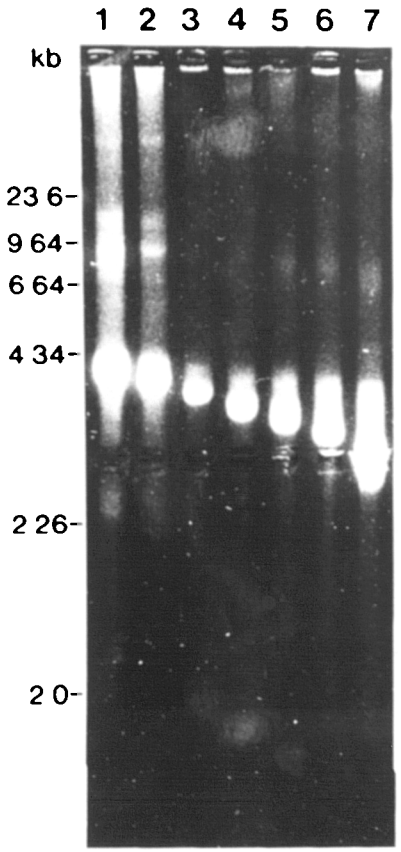
Panel A is an ethidium-bromide stained gel showing the progressive nested deletions of pCA4 in pBluescript (KS). Lanes 1-7 represent aliquots from the digestion removed for each increasing time point (1 min intervals). *Kpn* I was used to generate the 3' overhang and *Cla* I to generate the 5' overhang between the insert and the 3' overhang site (Section 2.8.1).

(B) Following digestion with Exonuclease III/ Mung bean-nuclease, the plasmids were re-circularised. The insert sizes from plasmids (lanes 1-13) digested for different times points was examined by digestion with *Pvu* II (a restriction enzyme with sites flanking the insert cloned in the *Eco*R I site).

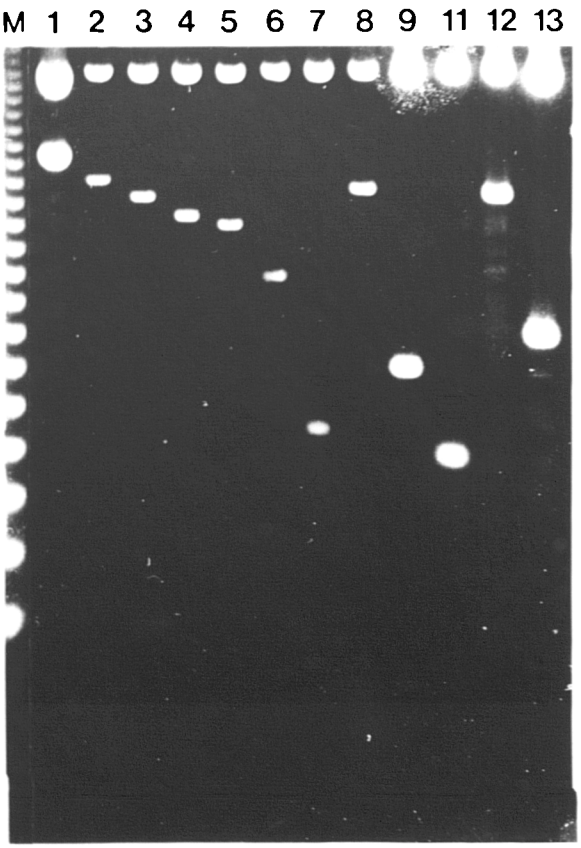
(C) Comparison of *Nla* III repeats sequenced from independent clones.

This figure shows alignment of copies of the *Nla* III repeats obtained from sequencing independent clones C1, C3, C4, C5, C6, C7, C8, C9, C10, C11 and C12. A, B and C represent the non overlapping copies from one clone. The purple hatched line represents the positions of deletions in some copies of the repeat, whereas the nucleotides in purple represent the inserted sequences. The nucleotides shown in blue represent the differences from the consensus 108 bp repeat sequence represented by C1A. The asterisks indicate the nucleotides that are conserved in all the copies.

A



B



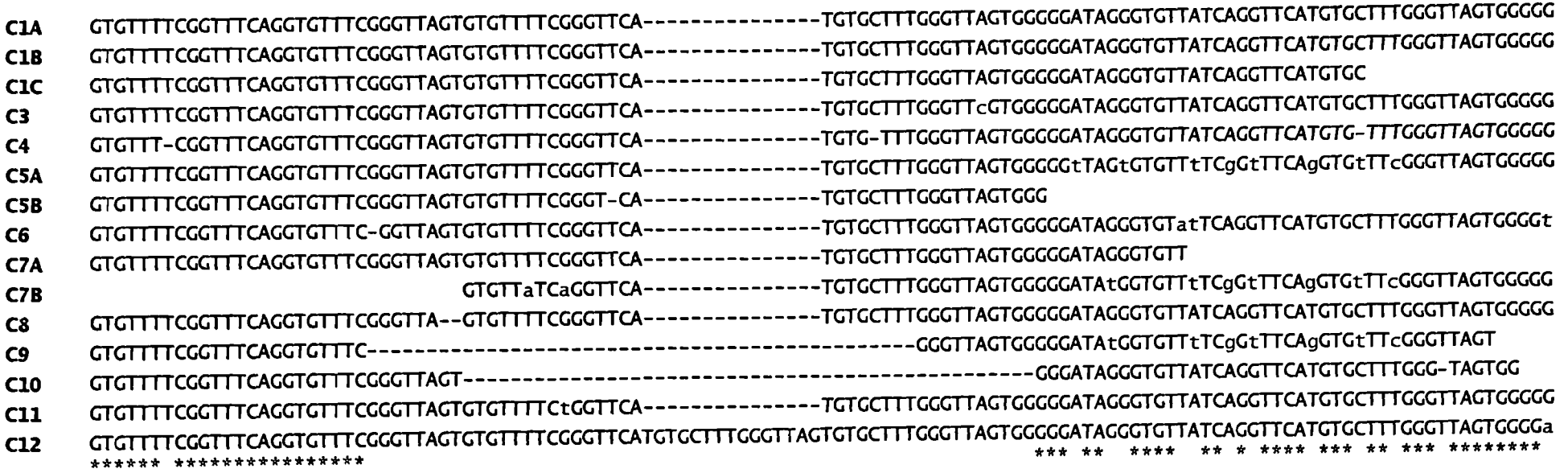
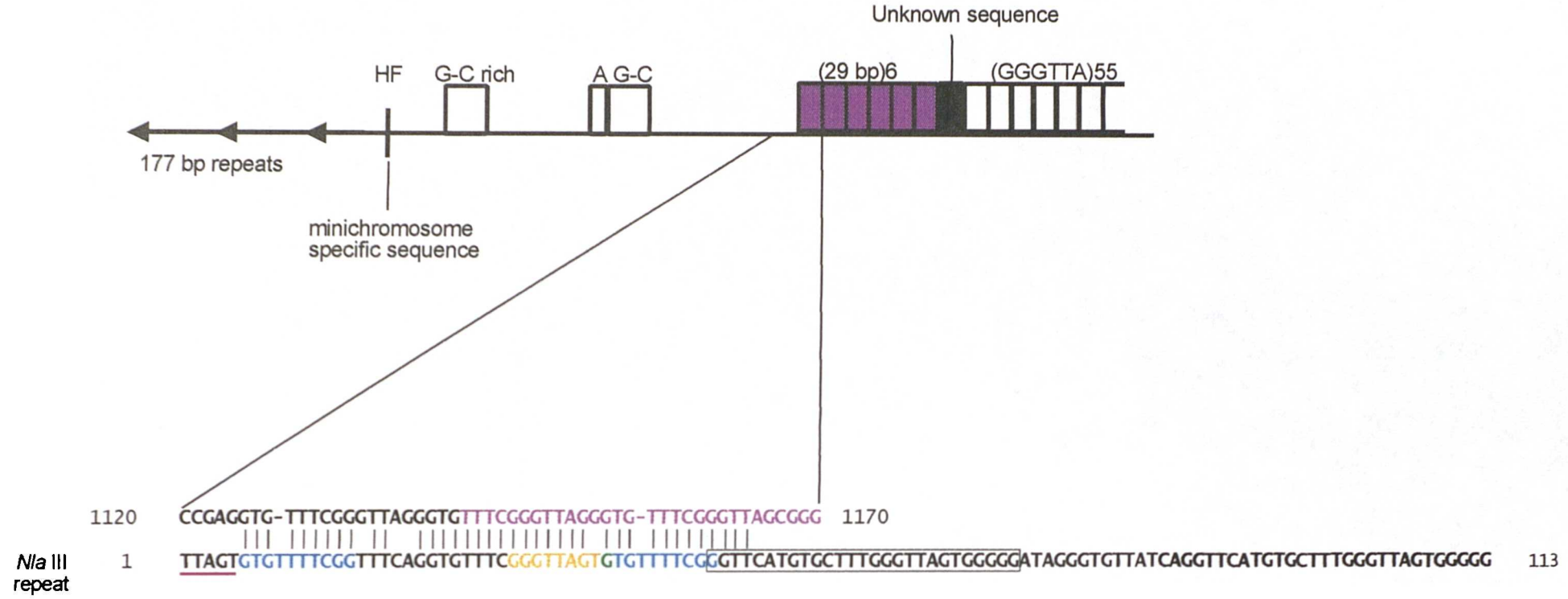

C1A GTGTTTTCGGTTTCAGGTGTTTCGGGTTAGTGTGTTTTCGGGTTCA----------TGTGCTTTGGGTTAGTGGGGGATAGGGTGTTATCAGGTTCATGTGCTTTGGGTTAGTGGGGG
C1B GTGTTTTCGGTTTCAGGTGTTTCGGGTTAGTGTGTTTTCGGGTTCA----------TGTGCTTTGGGTTAGTGGGGGATAGGGTGTTATCAGGTTCATGTGCTTTGGGTTAGTGGGGG
C1C GTGTTTTCGGTTTCAGGTGTTTCGGGTTAGTGTGTTTTCGGGTTCA----------TGTGCTTTGGGTTAGTGGGGGATAGGGTGTTATCAGGTTCATGTGC
C3 GTGTTTTCGGTTTCAGGTGTTTCGGGTTAGTGTGTTTTCGGGTTCA----------TGTGCTTTGGGTTcGTGGGGGATAGGGTGTTATCAGGTTCATGTGCTTTGGGTTAGTGGGGG
C4 GTGTTT-CGGTTTCAGGTGTTTCGGGTTAGTGTGTTTTCGGGTTCA----------TGTG-TTTGGGTTAGTGGGGGATAGGGTGTTATCAGGTTCATGTG-TTTGGGTTAGTGGGGG
C5A GTGTTTTCGGTTTCAGGTGTTTCGGGTTAGTGTGTTTTCGGGTTCA----------TGTGCTTTGGGTTAGTGGGGGtTAGtGTGTTtTCgGtTTCAgGTGtTTcGGGTTAGTGGGGG
C5B GTGTTTTCGGTTTCAGGTGTTTCGGGTTAGTGTGTTTTCGGGT-CA----------TGTGCTTTGGGTTAGTGGG
C6 GTGTTTTCGGTTTCAGGTGTTTC-GGTTAGTGTGTTTTCGGGTTCA----------TGTGCTTTGGGTTAGTGGGGGATAGGGTGTatTCAGGTTCATGTGCTTTGGGTTAGTGGGGt
C7A GTGTTTTCGGTTTCAGGTGTTTCGGGTTAGTGTGTTTTCGGGTTCA----------TGTGCTTTGGGTTAGTGGGGGATAGGGTGTT
C7B GTGTTaTCaGGTTCA----------TGTGCTTTGGGTTAGTGGGGGATAtGGTGTTtTCgGtTTCAgGTGtTTcGGGTTAGTGGGGG
C8 GTGTTTTCGGTTTCAGGTGTTTCGGGTTA--GTGTTTTCGGGTTCA----------TGTGCTTTGGGTTAGTGGGGGATAGGGTGTTATCAGGTTCATGTGCTTTGGGTTAGTGGGGG
C9 GTGTTTTCGGTTTCAGGTGTTTC----------GGGTTAGTGGGGGATAtGGTGTTtTCgGtTTCAgGTGtTTcGGGTTAGT
C10 GTGTTTTCGGTTTCAGGTGTTTCGGGTTAGT----------GGGATAGGGTGTTATCAGGTTCATGTGCTTTGGG-TAGTGG
C11 GTGTTTTCGGTTTCAGGTGTTTCGGGTTAGTGTGTTTTCtGGTTCA----------TGTGCTTTGGGTTAGTGGGGGATAGGGTGTTATCAGGTTCATGTGCTTTGGGTTAGTGGGGG
C12 GTGTTTTCGGTTTCAGGTGTTTCGGGTTAGTGTGTTTTCGGGTTCATGTGCTTTGGGTTAGTGTGCTTTGGGTTAGTGGGGGATAGGGTGTTATCAGGTTCATGTGCTTTGGGTTAGTGGGGa
*** ** **** ** * **** *** ** *** ********
****** **********

Figure 25

Database searches (Genbank database release 77) disclosed that the 108 bp repeats shows the highest homology (87.5% in a 40 bp overlap) with a subtelomeric sequence in a subset of minichromosomes of *T. b. brucei* (Weiden *et al.*, 1991).

This schematic figure shows the combined information of the homology between the 108 bp repeat and the region within the subset of minichromosomes described by Weiden *et al*, (1991). One end of the minichromosome (MC) is orientated with the telomere sequence to the right. The purple hatched box represents the subtelomeric 29 bp repeat, the open hatched box represents the telomeric repeat sequence and the three open boxes represent the G+C-rich subtelomere repeats. The sequence in purple in the MC sequence represents the 29 bp subtelomeric repeat. In the *Nla* III repeat, the yellow represents the 9 bp, the blue the 10 bp repeat and the boxed region the 27 bp repeat sequences. The regions of homology between the MC and the *Nla* III repeat are indicated by the vertical lines between the two sequences.


Unknown sequence
HF
G-C rich
A G-C
(29 bp)6
(GGGTTA)55
177 bp repeats
minichromosome
specific sequence
1120 CCGAGGTG-TTTCGGGTTAGGGTGTTTCGGGTTAGGGTG-TTTCGGGTTAGCGGG 1170
NIa III repeat 1 TTAGTGTGTTTTCGGTTTCAGGTGTTTCGGGTTAGTGTGTTTTCGGGTTCATGTGCTTTGGGTTAGTGGGGGATAGGGTGTTATCAGGTTCATGTGCTTTGGGTTAGTGGGGG 113

amplify sequences from gel-purified 6.6 kbp DNA in a polymerase chain reaction using primers ILO 1148 and ILO 1149 designed from the ends of the 108 bp repeat. Approximately 2 ng of gel-purified 6.6 kbp element in solution, and membrane-bound 6.6 kbp element derived from 2 µg of total DNA were used as target DNAs in the PCR. 10 ng of pCA4 insert was used as a positive target control DNA, and 10 ng of pBluescript DNA was used as the negative target control DNA. The PCR was carried out in a total volume of 100 µl at an annealing temperature of 55°C for 40 cycles. On Southern blots, the amplified PCR products of both the 6.6 kbp DNAs and the pCA4 insert appeared as a faint uneven ladder which ran into a smear (Figure 26a, lanes 2, 3 and 4, respectively), whereas no amplified product was generated from the pBluescript (Figure 26a, lane 1). The amplified PCR products generated from the 6.6 kbp target DNA were size-seperated from the un-incorporated primers by agarose gel electrophoresis. The PCR products were then excised from the gel and purified by the geneclean® method and used as a ^{32}P-labelled probe on a test Southern blot of undigested CP 547 DNA. At high stringency washes the products hybridised to the 6.6 kbp and higher hybridising elements, verifying the presence of the 108 bp repeat sequence on them. Furthermore, since both the 6.6 kbp element and pCA4 generated a PCR product which appeared as a ladder (Figure 26a), these results suggest that the 6.6 kbp element also contains tandem repeats of sequences similar to pCA4. It is evident from sequencing independent clones that not all copies of the repeats are always 108 bp (Figure 24c). Insertions and deletions may vary the size of the basic repeat. Therefore, an uneven ladder of the PCR products can be expected.

Nla III is the only restriction enzyme site identified from sequence analysis as cutting within the 108 bp repeat. Now that a specific probe was available, experiments were performed to verify whether this was also true for the 6.6 kbp element and the higher hybridising elements. 2 µg aliquots of total genomic CP 547 DNA from diminazene selected clone 2 were digested overnight with restriction enzymes such as *Nla* III, *Alu* I, *Eco*R I and *Sau*3A I. The digested DNA was resolved by agarose gel electrophoresis and the Southern blots generated were probed with ^{32}P-labelled pCA4 (Figure 26b). Only *Nla* III (Figure 26b, lane 4) completely digested the 6.6 kbp element

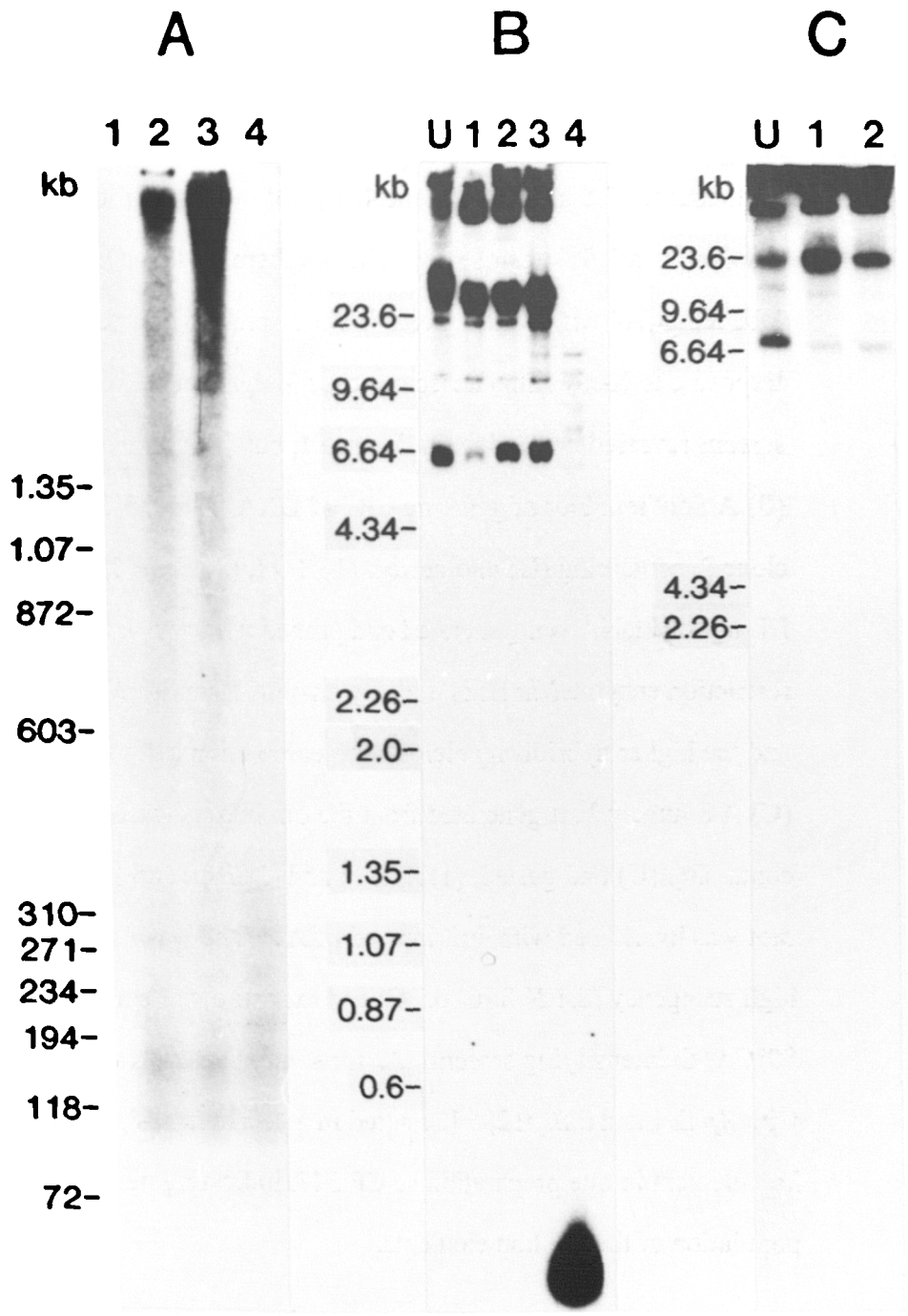
Figure 26

The 6.6 kbp element is composed of *Nla* III repeats.

(A) Primers ILO 1148 and ILO 1149 designed from the ends of the 108 bp repeat were used in a PCR to amplify sequences from pBluescript DNA as the negative control DNA (1), gel-purified membrane bound 6.6 kbp DNA (2), gel-purified liquid 6.6 kbp DNA (3), and pCA4 as a positive control DNA (4). In lanes 1-3, 75% of the PCR product (from a total volume of 100 µl) was ethanol precipitated and resuspended in 15 µl; in lane 4, 1 µl of the PCR product was directly resolved in a 1.5% agarose gel. The Southern blot was hybridised with ^{32}P-labelled pCA4 with washing conditions of high stringency (0.1 X SSC, 65°C). Exposure to X-ray film carried out over 3 nights at -80°C with intensifying screens revealed no product in the lane 1, but faint, uneven ladders in lanes 2-4.

(B) A Southern blot of genomic CP 547 DNA from the diminazene selected clone 2, containing (U) undigested, (1) *Alu* I, (2) *Eco*R I, (3) *Sau*3A I and (4) *Nla* III digested DNA was generated and probed with ^{32}P-labeled pCA4. The restriction enzyme *Nla* III is the only enzyme found to digest the 6.6 kbp element and the higher hybridising elements to completion (4).

(C) A Southern blot generated from the diminazene-selected CP 547 clone 2 containing (U) undigested, (1) *Apa*L I and (2) *Bsp*1286 I digested DNA. The blot was hybridised with ^{32}P-labeled pCA4. The washing conditions were at high stringency (0.1 X SSC, 65°C) and exposure was carried out overnight at -80°C with intensifying screens. As for some digestions with *Alu* I, digestion with *Apa*L I and a *Bsp*1286 I resulted in a decrease in signal intensity of the 6.6 kbp element in one preparation of CP 547, indicating heterogeneity in this population of the 6.6 kbp elements.


A
1 2 3 4
kb
1.35-
1.07-
872-
603-
310-
271-
234-
194-
118-
72-
B
U 1 2 3 4
kb
23.6-
9.64-
6.64-
4.34-
2.26-
2.0-
1.35-
1.07-
0.87-
0.6-
C
U 1 2
kb
23.6-
9.64-
6.64-
4.34-
2.26-

and the higher hybridising forms into DNA that migrated predominantly to the region corresponding to 113 bp (Figure 26b). Henceforth, the 108 bp repeat sequence was referred to as the '*Nla* III repeat'. As mentioned earlier, in some CP 547 preparations digestion with *Alu* I resulted in decrease in signal intensity corresponding to the 6.6 kbp element, whereas in others such a decrease in signal intensity was not evident. This experiment confirmed this and an earlier suspicion that the 6.6 kbp element lacked the sites for commonly used restriction enzymes (Figure 26b, lanes 1, 2, 3). The absence of these sites offered an explanation for the lack of success in attempts to directly clone sequences from the gel-purified 6.6 kbp DNA digested with these restriction enzymes (Section 3.1.1b).

Sequence analysis of pCA15 displayed the presence of an *Apa*L I and a *Bsp*1286 I restriction enzyme site downstream from the *Nla* III repeat sequence (Figure 23). To test whether the 6.6 kbp and the higher hybridising elements contain sites for these restriction enzymes, 2 µg aliquots of total DNA from one preparation of CP 547 were digested overnight with these enzymes. The digested DNA and undigested CP 547 DNA was resolved by agarose gel electrophoresis and the Southern blots generated were probed with ^{32}P-labelled pCA4. As for some digestions with *Alu* I, a decrease in signal intensity of the 6.6 kbp element was observed (Figure 26c, lanes 1, 2), suggesting that only some species of the element contain sites for *Apa*L I and a *Bsp*1286 I.

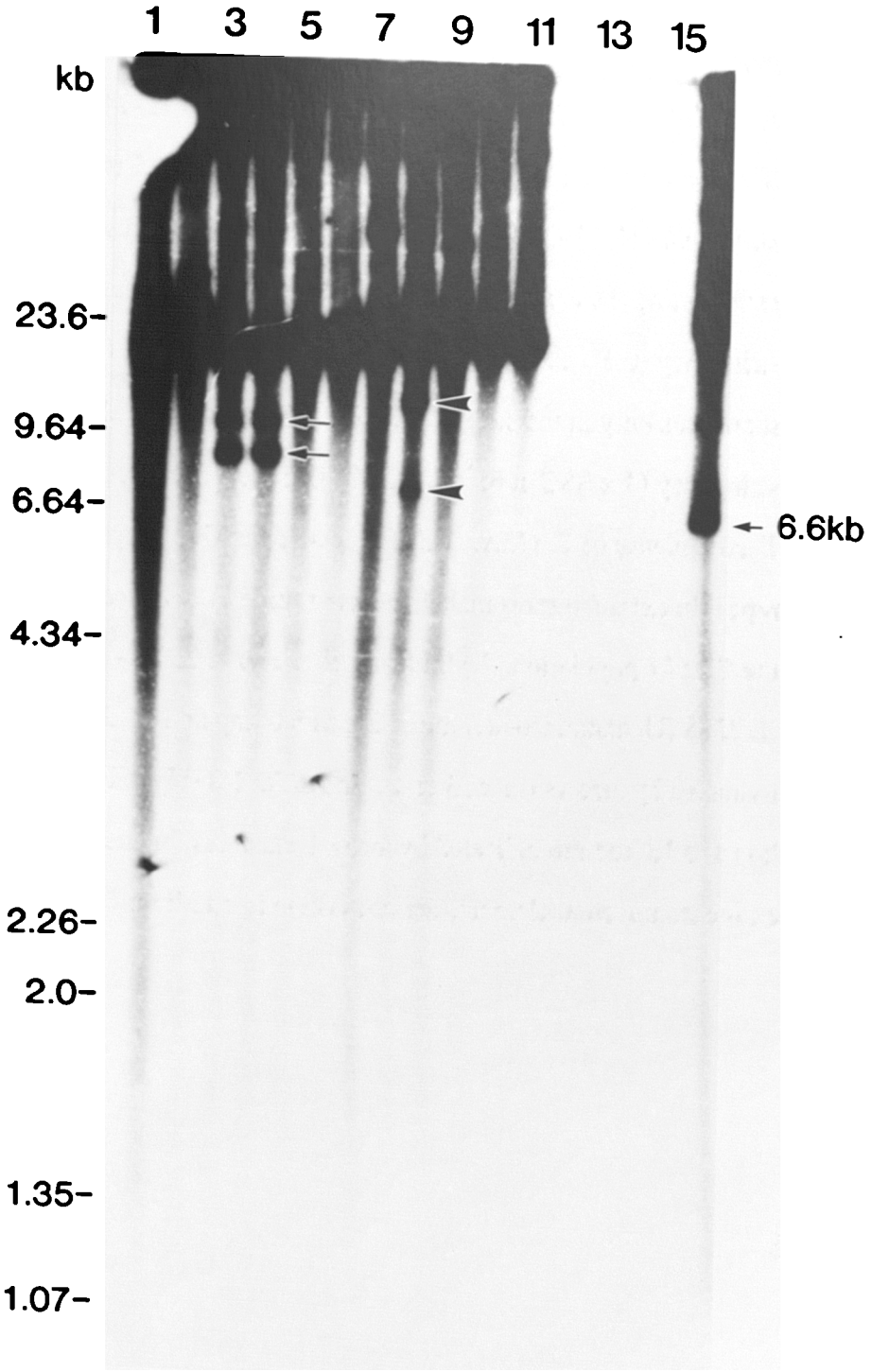
3.6.1 Specificity of the *Nla* III repeat for the subgenus *trypanozoon*

To test for the presence of the *Nla* III repeat sequence among the different subgenera of trypanosomes, Southern blots of undigested DNA from different trypanosome species were probed with ^{32}P-labelled pCA4. At high stringency, hybridisation to large DNA only in the subgenus *Trypanozoon* was observed (Figure 27). Even at low stringency (4 x SSC at 65°C), the *Nla* III repeat did not hybridise to DNA from *T. congolense* or *T. vivax*. For the 10 populations tested belonging to the subgenus *Trypanozoon*, strong hybridisation signals were apparent in the regions just below the slot

Figure 27

Specificity of the *Nla* III repeat for the subgenus *Trypanozoon*.

2 μg of undigested DNAs purified from different trypanosome species were resolved in a 1.2% agarose gel. Lanes 1-11 are represented by *Trypanozoon* populations ILTat 1.1, IL 16EI, IL B24, IL 770, IL 3400, GUTat 3.1, IL 3397, AnTat 1.8, ILTar 2, IL 3398 and ETAt 1.8. Lanes 12-14 are represented by *Nannomonas*, IL 1180, IL 3000 and IL 2985. Lane 15 is represented by a *Duttonella*, IL 1392. Lane 16 contains the multidrug-resistant CP 547, representing the control DNA. A Southern blot of this gel was hybridised at high stringency (0.1 x SSC at 65°C) with a ^{32}P-labelled *Nla* III repeat. Signals to sequences only in the subgenus *Trypanozoon* were revealed; even at low stringency (4 x SSC at 65°C), the *Nla* III repeat does not hybridise to DNA from *T. congolense* or *T. vivax*. This figure also verifies the presence of the *Nla* III repeat in extrachromosomal elements of three *T. b. brucei* populations other than the CP 547 population. Hybridising elements in IL B24 (Ugandan isolate) and IL 3388 (Ugandan isolate) migrating as 8.8 kbp and 13.5 kbp fragments are indicated by arrows (lanes 3 & 4). In AnTat 1.8 (Uganda) (lane 8) elements of 7 kbp and 15 kbp are indicated by arrow heads. As for the 6.6 kbp element, these extrachromosomal elements are not visible in ethidium-bromide stained gels.


1
3
5
7
9
11
13
15
kb
23.6-
9.64-
6.64-
4.34-
2.26-
2.0-
1.35-
1.07-
6.6kb

and at the main genomic band around the 23 kbp marker region (Figure 27). These experiments showed the presence of the *Nla* III repeat sequence in populations belonging to the subgenus *Trypanozoon*. In addition to the 6.6 kbp and the higher hybridising extrachromosomal elements in the CP 547 clone, extrachromosomal elements were identified in three additional *T. b. brucei* populations. In IL B24 (Ugandan population) and IL 3388 (Ugandan population) hybridisation to sequences migrating as 8.8 kbp and 13.5 kbp fragments (Figure 27 lanes 3 and 4) were identified. In AnTat 1.8 (Uganda) (Figure 27 lane 8,) elements of 7 kbp and 15 kbp were identified. As for the 6.6 kbp element, these extrachromosomal elements were not visible in ethidium-bromide stained gels. Furthermore, these elements also lacked sites for frequently used restriction enzymes, suggesting the presence of a sequence arrangement similar to that of the 6.6 kbp element. However, since the drug-sensitivity profiles of these three trypanosome populations was un-determined, further analysis of these extrachomosomal elements was not pursued.

3.6.2 Organisation of the *Nla* III repeat sequence in the trypanosome genome

In order to determine the genomic organisation of the *Nla* III repeat, CP 547 DNA and DNA form different trypanosome populations embedded in agarose plugs was subjected to PFGE or orthogonal-field-alternation gel electrophoresis (OFAGE). The pulse intervals indicated in the figure legends (Figures 28 and 29) were used to size separate the chromosome-sized DNA molecules from the different populations. To determine the genomic distribution of the *Nla* III repeats, the gels were Southern blotted and hybridised with several different probes. The blots were first probed with a ^{32}P-labelled consensus telomeric sequence to identify all the chromosomes (Figures 28b and 29b). This blot was subsequently stripped and re-probed with the ^{32}P-labelled *Nla* III repeat (pCA4) (Figures 28c and 29c). The resolved chromosomes as apparent from the ethidium-bromide stained gels (Figures 28a and 29a), ranged from 50 to greater than 1,500 kbp, when compared with size standards (oligomers of bacteriophage lambda DNA or yeast chromosomes). Depending on the pulse time, there was also a region of

Figure 28

Chromosomal location of the *Nla* III repeats using Orthogonal-field-alternation gel electrophoresis (OFAGE).

(A) An ethidium-bromide stained OFAGE gel In this figure, chromosome-sized DNA from seven different trypanosome populations was resolved by OFAGE. The conditions for separation were 50 V for 4.5 h at 40 sec pulses followed by 14 h at 300 V at 40 sec pulses. The 1.5% agarose gel contained embedded DNA prepared from diminazene-selected CP 547 clone 2 (1), CP 2469, a drug resistant *T. b. brucei* field isolate (2), a *T. b. brucei* (IL 3509) (3), and two clones generated from the CP 2469 stock (4 & 5); lanes 6 & 7 contain DNA from a *T. congolense* clone IL 1180 and a quinapyramine-resistant clone derived from the IL 1180 clone, respectively.

(B) Chromosome profiles identified by hybridisation with a ^{32}P-labelled consensus telomere sequence.

A Southern blot of the gel in (A) was hybridised with a ^{32}P-labelled consensus telomeric sequence. The arrow heads in panel B are representative examples of intermediate chromosomes that do not hybridise with the *Nla* III repeat (panel C).

(C) Distribution of the Nla III repeats.

Following removal of the telomere probe the blot in (B) was hybridised with the ^{32}P-labelled *Nla* III repeat at high stringency conditions.

(D) A longer exposure of lane 1 in panel C representing hybridisation of the *Nla* III repeat was carried out. The arrows in panel C and panel D represent curious molecules containing the *Nla* III repeat sequence that do not hybridise to the telomere sequence and are not visible in the ethidium-bromide stained gel.

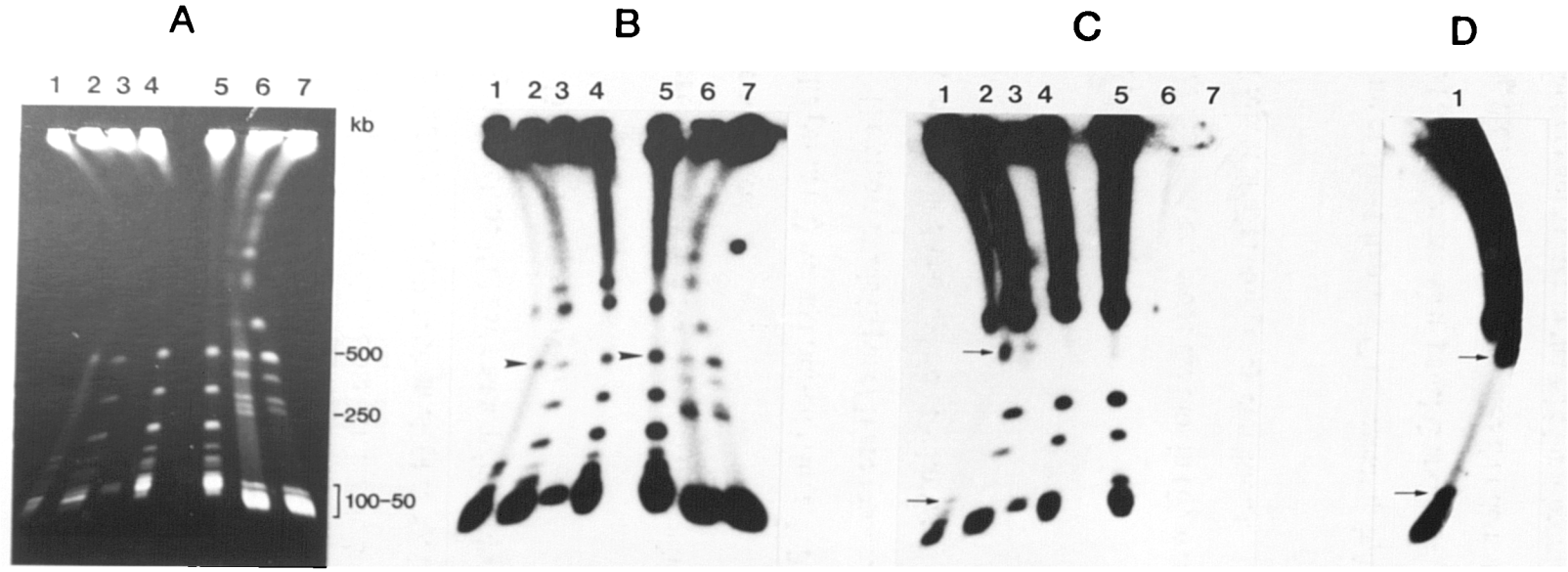

A
B
C
D
1 2 3 4 5 6 7
kb
-500
-250
100-50
1 2 3 4 5 6 7
1 2 3 4 5 6 7
1
Probe:
Telomere
Nla III
Nla III

Figure 29

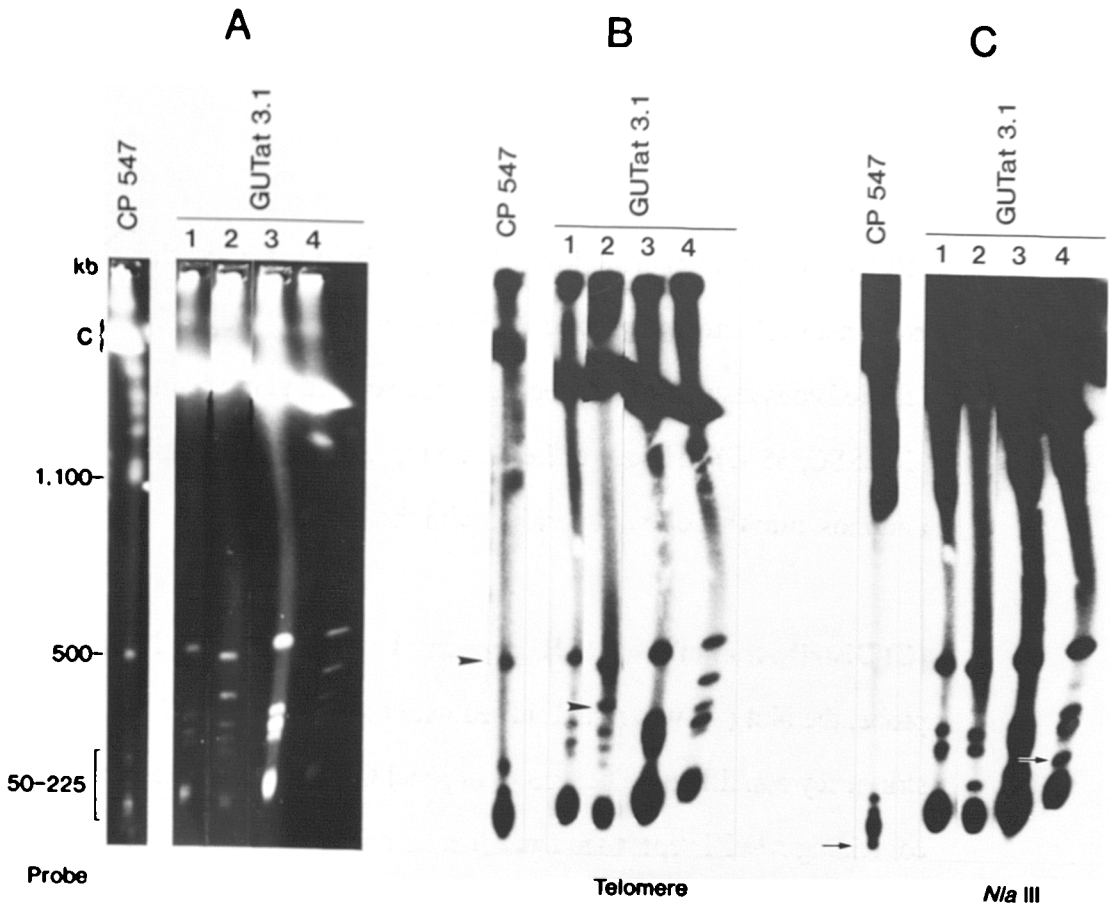
Chromosomal location of the *Nla* III repeats in CP 547 and subclones of GUTat 3.1 using pulsed field gel-electrophoresis (PFGE).

(A) A ethidium-bromide stained PFG.

This is a figure of a 1.5% PFG containing DNA prepared from parental CP 547 stock and GUTat subclones 1, 2, 3 and 4 (cultured *in vitro* under different culture conditions by Dr. Hirumi). The chromosome DNA was resolved by PFGE using a pulse times of 250 sec for 30 h, followed by 180 sec for 20 h and 120 sec for 12 h.

(B) Chromosome profiles identified by hybridisation with ^{32}P-labelled consensus telomere sequence. A Southern blot (A) was hybridised with a ^{32}P-labelled consensus telomere sequence and washed at moderately high stringency (1 X SSC, 65°C). The arrow heads are representative examples of intermediate chromosomes that do not hybridise with the *Nla* III repeat.

(C) Distribution of the Nla III repeats. Following removal of the telomere probe, the blot (A) was re-hybridised with the ^{32}P-labelled *Nla* III repeat at high stringency conditions. The arrows in panel C show the 'curious' molecules containing *Nla* III repeat sequence that do not hybridise to the telomere sequence, and are not visible in the ethidium-bromide stained gel.


A
B
C
CP 547
GUTat 3.1
1 2 3 4
kb
C
1.100-
500-
50-225
Probe
Telomere
Nla III

compression in which larger sized DNA migrated (marked as 'c' in Figure 29a). The ^{32}P-labelled consensus telomeric probe hybridised to all the size separated chromosomes that were visible on the ethidium-bromide stained gels (Figure 28b and 29b). The telomeric probe also disclosed the marked heterogeneity in the overall molecular karyotypes of the different populations of trypanosomes. Following the removal of the telomere probe, the Southern blots were reprobed with the *Nla* III repeat sequence (Figure 28c and 29c). High post hybridisation stringency showed that the *Nla* III repetitive sequence is dispersed in the genome of different populations of the subgenus *Trypanozoon*. Under these stringency conditions, the *Nla* III repeat sequence was not detected in populations of *T. congolense* or *T. vivax*, which belong to different subgenera. For members of the *Trypanozoon* subgenus, the majority of the *Nla* III repeat hybridising sequences were on chromosomes larger than 1 Mb. Not all the intermediate chromosomes (120 to 1,000 kbp) contained *Nla* III repeats (Figure 28b, arrows pointing to the intermediate chromosomes which do not hybridise with the *Nla* III repeat). However, the intermediate-sized chromosomes in most populations contained the *Nla* III repeat sequence, but the ratio of the hybridisation signal to the ethidium-bromide fluorescence showed substantial variation in the numbers the *Nla* III repeat units carried in a given length of chromosome-sized DNA.

Superimposing the hybridisation results obtained with the telomeric and the *Nla* III repeat probes (Figure 28b, 28c and 29b, 29c) to the profiles of the ethidium-bromide stained gels (Figure 28a and 29a) showed that the *Nla* III repeat hybridises to some chromosome-sized DNA molecules which are not chromosomal since they are not detectable by staining with ethidium-bromide or by hybridisation with the telomeric probe (Figure 28c, d and 29c, marked with arrows). These molecules have subsequently been referred to as 'curious' DNA molecules. Since the curious molecules are present in size classes in which the haploid chromosomes stain with ethidium-bromide, these molecules apparently exist in copy numbers of less than one per genome. Alternatively, these molecules are not seen in ethidium-bromide stained gel because they are considerably smaller than their migration would suggest. In PFGE, altered migration of

circular DNAs is a recognised characteristic (Beverley, 1988). These curious chromosome-sized molecules differ even in the subclones derived from a cloned parental population of GUTat 3.1 (Figure 29c). Although the subclones have similar karyotypes, in two of the subclones, a new intensely hybridising DNA molecule is evident (Figure 29c, lane 2 & 4). Similarly, in the lane with CP 547 DNA, no hybridisation was evident to the telomere probe in the region below the 50 kbp marker, whereas this region had strong hybridisation to the *Nla* III repeat (Figure 29c, indicated by an arrow in lane containing CP 547 DNA). This hybridising region in CP 547 DNA may represent the 6.6 kbp element and possibly some higher hybridising elements evident by conventional agarose gel electrophoresis.

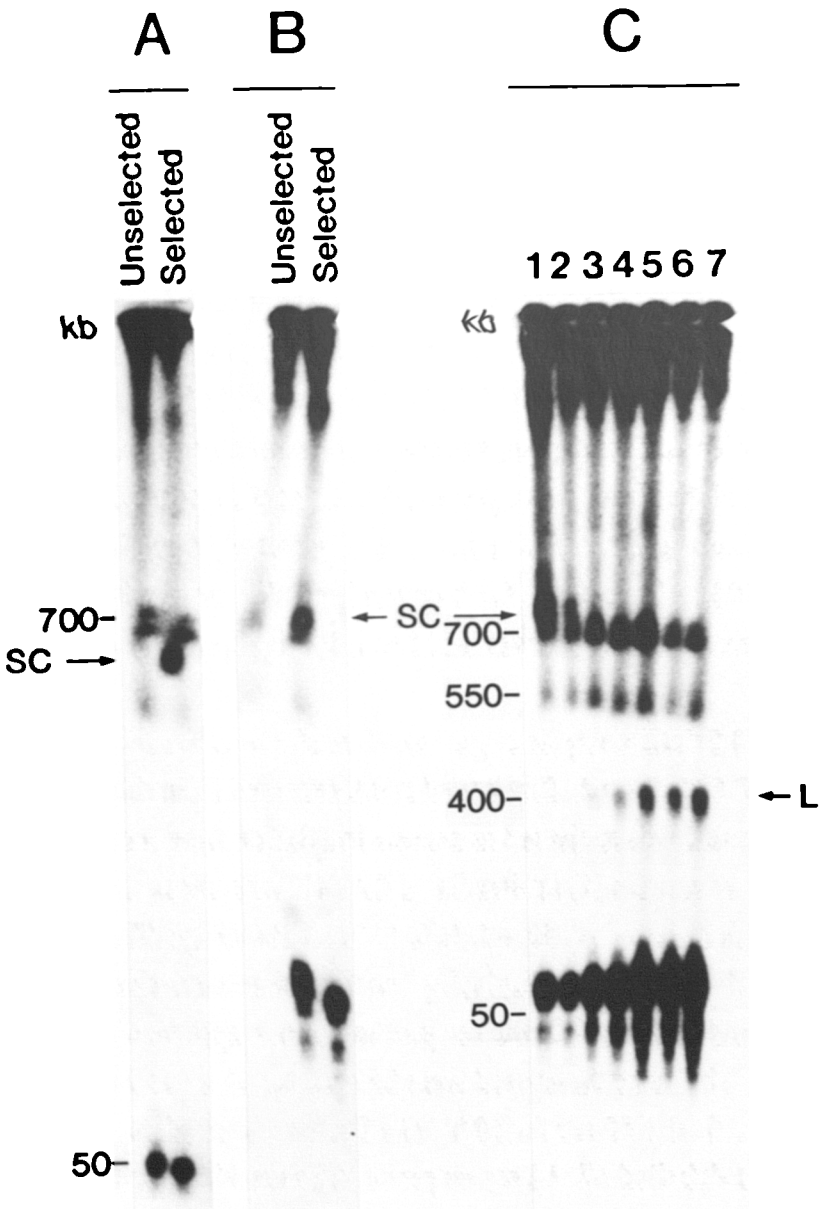
The PFGE gels disclosed that the diminazene-selected multidrug-resistant CP 547 clone 2 contains a non-chromosomal curious element, which does not hybridise to the telomere probe also is not visualised in ethidium-bromide stained gels (Figure 30a, lane 2, indicated as SC). This curious molecule exhibits pulse time-dependent mobility and a migration path slightly displaced from the consensus "track" of the linear DNAs (Figure 30a and 30b marked as SC). This intermediate sized molecule, at one set of pulse times, is approximately 600 kbp (Figure 30a, lane 2), and at another set of pulse times, is 750 kbp (Figure 30b, lane 2). Such unusual mobility properties are known to be characteristic of circular DNAs (Garvey and Santi, 1986; Hightower *et al.*, 1987; Beverley, 1988). Hence, to examine whether this intermediate-sized molecule in CP 547 is circular, CP 547 DNA samples embedded in agarose plugs were subjected to different doses of gamma irradiation (Figure 30c). Gamma irradiation introduces a spectrum of structural lesions into DNA, including base modifications and strand breaks, with single-stranded breaks occurring 10 to 20 times more frequently than double-stranded breaks (Hutchinson, 1985). After an appropriate dose of gamma irradiation, linear molecules yield a heterogeneous collection of fragments of various lengths because of the introduction of random double-stranded breaks. In contrast, a circular molecule initially gives rise to a discrete linear molecule whose length corresponds to that of the original

Figure 30

PFGE analysis of DNA from unselected and diminazene-selected CP 547 show that the 'curious' DNA molecules have characteristics of circular DNA molecules.

(A & B) Agarose embedded DNA from unselected CP 547 clone 1 and diminazene selected clone 2 was resolved in a 1% agarose gel by PFGE using two different sets of pulse times. The pulse times in panel A were 250 sec for 40 h, 180 sec for 20 h, 10 sec for 20 h and finally 5 sec for 10 h; the pulse times in panel B were 250 sec for 40 h; 150 sec for 20 h; 60 sec for 20 h and finally 50 sec for 10 h. Southern blots of these gels were hybridised with a ^{32}P-labelled *Nla* III repeat probe. The washing conditions were of high stringency (0.1 X SSC, 65°C) and exposure was carried out overnight at -80°C with intensifying screens. SC in the lanes with diminazene selected clone 2 represents the curious molecule absent from the unselected CP 547 clone. As verified from panel A and B, this curious molecule exhibits pulse time-dependent mobility and a migration path slightly displaced from the consensus "track" of the linear DNAs. In panel A the curious molecule has a relative size of approximately 600 kbp whereas it is approximately 750 kbp in panel B. Such unusual mobility properties are characteristic of circular DNAs (Garvey and Santi, 1986; Hightower *et al.*, 1987; Beverley, 1988).

(C) PFGE analysis of gamma-irradiated DNA from the diminazene-selected CP 547 clone 2. Embedded DNA prepared from the diminazene selected CP 547 clone 2 was subjected to gamma irradiation from a caesium source. The different lanes contain (1) un-irradiated CP 547, whereas lanes 2-7 contain DNA irradiated with doses of 10, 30, 60, 100, 150 and 180 Gray. The position of the curious molecular species displaying mobility characteristics of a circular DNA molecule is indicated by SC, and the position of the apparently linearised SC molecule by L. The pulse times used were 250 sec for 40 h, 150 sec for 20 h, 60 sec for 20 h and finally 50 sec for 10 h. The Southern blot of this gel was hybridised with a ^{32}P-labelled *Nla* III repeat probe. The washing conditions were of high stringency (0.1 X SSC, 65°C) and exposure was carried out overnight at -80°C with intensifying screens.


A
B
C
Unselected
Selected
Unselected
Selected
1 2 3 4 5 6 7
kb
kb
700-
SC
SC
700-
550-
400-
L
50-
50-

circular form. The appearance of a new molecule is therefore diagnostic for circular DNA (Beverley, 1989).

Titrations of gamma irradiation were performed using an irradiator which employs a caesium source. CP 547 DNA embedded in agarose sample plugs were placed in a standard 1.5 ml polypropylene microcentrifuge tube and then irradiated at ambient temperatures for different times. Samples then remained at room temperature for at least 30 min prior to separating the chromosomes by pulsed-field electrophoresis (Figure 30c). Southern blots of these gels were generated, and hybridised with the ^{32}P-labelled *Nla* III repeat. The increasing doses of gamma irradiation resulted in successive decreases in hybridisation intensity of the molecule migrating as a 750 kbp DNA molecule (marked as SC in Figure 30c), and the successive increase of a new component of about 400 kbp (Figure 30c marked as "L"). The appearance of this new molecule is likely to be the result of linearisation of the curious circular molecule of 750 kbp. A specific probe for this molecule would verify this result. A dose of 60-100 Gray converted about 50% of the circular DNA to linear forms, while 100-150 Gray results in complete conversion to the linear form. Therefore, from these gamma irradiation experiments and experiments showing its pulse-time dependent mobility, it is established that the intermediate chromosome-sized curious molecule in the CP 547 clone is a circular molecule. One of the physical properties of circular DNA molecules is aberrantly slow migration through pulsed field gels, leading to a larger apparent size than is deduced from either sequence analysis or from migration of the same DNA when linearised (Beverley, 1989). This offers a possible explanation for the lack of staining of the 750 kbp molecule by ethidium-bromide. Since it is approximately 400 kbp in size, and migrated at 750 kbp in PFGs, its relative staining is expected to be much fainter in comparison to a larger, linear DNA molecule migrating at the 750 kbp position in a gel (Figure 30c). The curious, *Nla* III repeat-containing elements in the GUTat 3.1 sub-clones (Figure 29c) may also be a circular molecule migrating among molecules larger in size, explaining its lack of staining by ethidium-bromide. This result therefore shows that in addition to the drug

resistant CP 547 isolate, other *Trypanozoon* populations may also contain large circular *Nla* III repeat containing extrachromosomal elements.

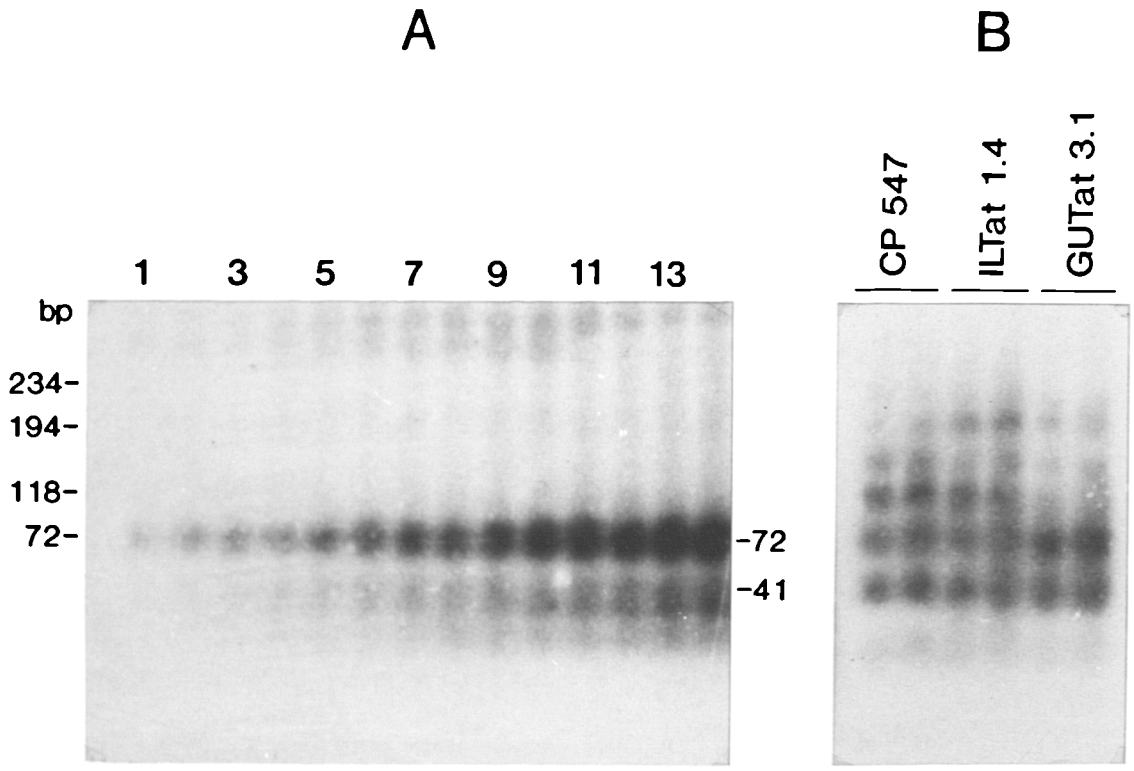
3.6.3 Copy number of the *Nla* III repeats

The revelation that *Nla* III restricted once within the *Nla* III repeat, and that pCA15 insert contains 3.4 copies of the *Nla* III repeat, provided a means to estimate the copy number of this newly identified repeat family in three different *T. b. brucei* populations; the multidrug-resistant CP 547 clone 2, the drug-sensitive ILTat 1.4 clone and the GUTat 3.1 clone. To estimate the copy number, 1 µg and 2 µg aliquots of total genomic DNA from the three *T. b. brucei* populations and different concentrations of pCA15 control DNA, accounting for approximately 9,500 to 71,000 copies of genomic equivalents of the *Nla* III repeats were digested with excess *Nla* III restriction enzyme. The reactions were carried out overnight to ensure complete digestion. In order to resolve the small-sized digestion products, all the digested samples were electrophoresed slowly in 2% agarose gels at 40 volts for a period of 18 h. Southern blots of these gels were generated and DNA was transferred onto nylon membranes for a minimum of 38 h to ensure complete transfer. The blots were subsequently hybridised with ^{32}P-labelled *Nla* III repeat (Figure 31). The densitometric scanning of the hybridisation signals using a Molecular Dynamics Laser Densitometer was used to compare the signal intensity between the different concentrations of the pCA15 DNA and the genomic DNA. Such densitometric analyses allowed the determination of the number of copies of the *Nla* III repeat in the different genomic DNA samples. These experiments established that in *T. b. brucei* the *Nla* III repeat is represented by approximately 40,000 copies per diploid genome with no significant differences between the CP 547 drug-resistant and the ILTat 1.4 and GUTat 3.1 drug-sensitive populations. Since the *T. b. brucei* diploid genome size is estimated to be 8.6 x 10^7 bp (Borst *et al.*, 1982), the *Nla* III repeat sequence or the *Nla* III repeat family could therefore account for about 5% of the parasite genome. Although these experiments suggested that there are no significant differences between the drug-resistant CP 547 clone in comparison to the drug-sensitive populations, direct

Figure 31

Copy number of the *Nla* III repeat sequence in different *T. b. brucei* populations.

Digestion of tandem repeats of the 113 bp sequence (108 bp repeat and one of the 5 bp flanking sequences) with *Nla* III generates fragments sized 41 bp and 72 bp. If this restriction site is lost, a fragment of 113 bp is generated. Genomic DNA (1 and 2 μg each) from the drug-resistant CP 547 population and drug-sensitive clones ILTat 1.4 and GUTat 3.1 was digested overnight with excess of *Nla* III (**Panel B**). This was resolved in a 2.5% agarose gel alongside carefully calibrated amounts of the purified cloned pCA15 insert, also digested with *Nla* III; the amount of DNA of the pCA15 insert, lanes 1-13, was 0.4 to 3.0 μg in increments of 0.2 μg (**Panel A**). These represented 9,500 to 71,000 copies of the 108 bp repeat relative to the *T. brucei* genome content. To ensure complete transfer of the DNA, blotting onto nylon membranes was performed for a minimum of 38 hours. The Southern blot was hybridised with a ^{32}P-labelled *Nla* III repeat. The washing conditions were of high stringency (0.1 X SSC, 65°C) and exposure was carried out overnight at -80°C with intensifying screens. Densitometric scanning of the area encompassing the hybridisation signals was performed using a Molecular Dynamics Laser Densitometer to compare the signal intensity between the different concentrations of the pCA15 DNA and the genomic DNAs. The area encompassing lane 7, representing approximately 40,000 copies, gave an equal signal intensity to that for 1 μg of each of the genomic DNAs.


A
B
1
3
5
7
9
11
13
bp
234-
194-
118-
72-
-72
-41
CP 547
ILTat 1.4
GUTat 3.1

comparisons between the copy number of different clones of CP 547 expressing varying drug sensitivities was not carried out.

3.6.4 Transcription of the *Nla* III repeat sequence

To ascertain whether the *Nla* III repetitive sequence is transcribed, poly(A)$^+$ RNA was isolated from long slender and procyclic forms of the drug-resistant CP 547 and the drug-sensitive ILTat 1.4 populations and analysed by Northern blot analysis as previously explained in section 3.3.3. ^{32}P-labelled pCA4 was used as the hybridisation probe because sequence analysis showed it to consist of only the *Nla* III repeats. Medium stringency post-hybridisations did not reveal any transcriptional differences between the long slender bloodstream forms of the drug-resistant CP 547 and drug-sensitive ILTat 1.4 clones (Figure 32a). Similarly, no differences were observed between the procyclic forms of CP 547 and ILTat 1.4 (Figure 33b). For both these parasite populations, in both long slender bloodstream forms and procyclic insect forms, the *Nla* III repetitive probe did not hybridise to transcripts of any discrete size-class, but to a region constituting heterogeneous transcripts ranging between 300 bp and over 10 kb (Figure 32a, b). To test whether these hybridisations represented true transcripts, the poly(A)$^+$ RNA was pre-treated with 5 units of ribonuclease free DNase I for 1 hour at 37°C. Northern blot hybridisations of the pre-treated samples showed that the heterogeneous RNA transcripts contained DNA contamination only in the higher regions (Figure 32b). Similar hybridisations of a Northern blot generated from poly(A)$^+$ RNA isolated from the different life-cycle stages of ILTat 1.1 (gift from Dr. Murphy) displayed that the *Nla* III repeat is differentially expressed. Heterogeneous transcripts were identified in the actively dividing long slender and procyclic forms of the parasite. No hybridisation was evident in the poly(A)$^+$ RNAs of short-stumpy and intermediate forms (Figure 33a). Whether this is also true for the CP 547 clone was not determined because of the difficulty in obtaining a uniform-stumpy form parasite population. Since most of the Northern blot analyses were performed using poly(A)$^+$ RNA, experiments were carried out to determine if there were *Nla* III repeat transcripts that were not polyadenylated. To

Figure 32

Northern blot analysis of the *Nla* III repeat.

(A) Approximately 3 μg of poly(A)$^+$ enriched RNA from the long slender bloodstream form of the drug-resistant CP 547 population and drug-sensitive ILTat 1.4 clone was glyoxylated and separated on a 1.4% agarose gel. The RNA was blotted onto a Nytran filter and hybridised with a ^{32}P-labelled *Nla* III repeat. The integrity of the RNA preparation was verified by reprobing the stripped filters with a ^{32}P-labelled ß-tubulin hybridisation probe; the signals corresponding to the ß-tubulin are shown in the lower panels.

(B) The extent of DNA contamination in RNA preparations. An aliquot of about 10 μg of total RNA from the long slender bloodstream form of the drug-resistant CP 547 population was untreated (U) and one aliquot was pre-treated with 5 units of ribonuclease free DNase I (D) for 1 hour at 37°C. This RNA was subjected to Northern blotting and hybridisations as in panel A. The DNA contaminating the preparation is indicated by the bracket.

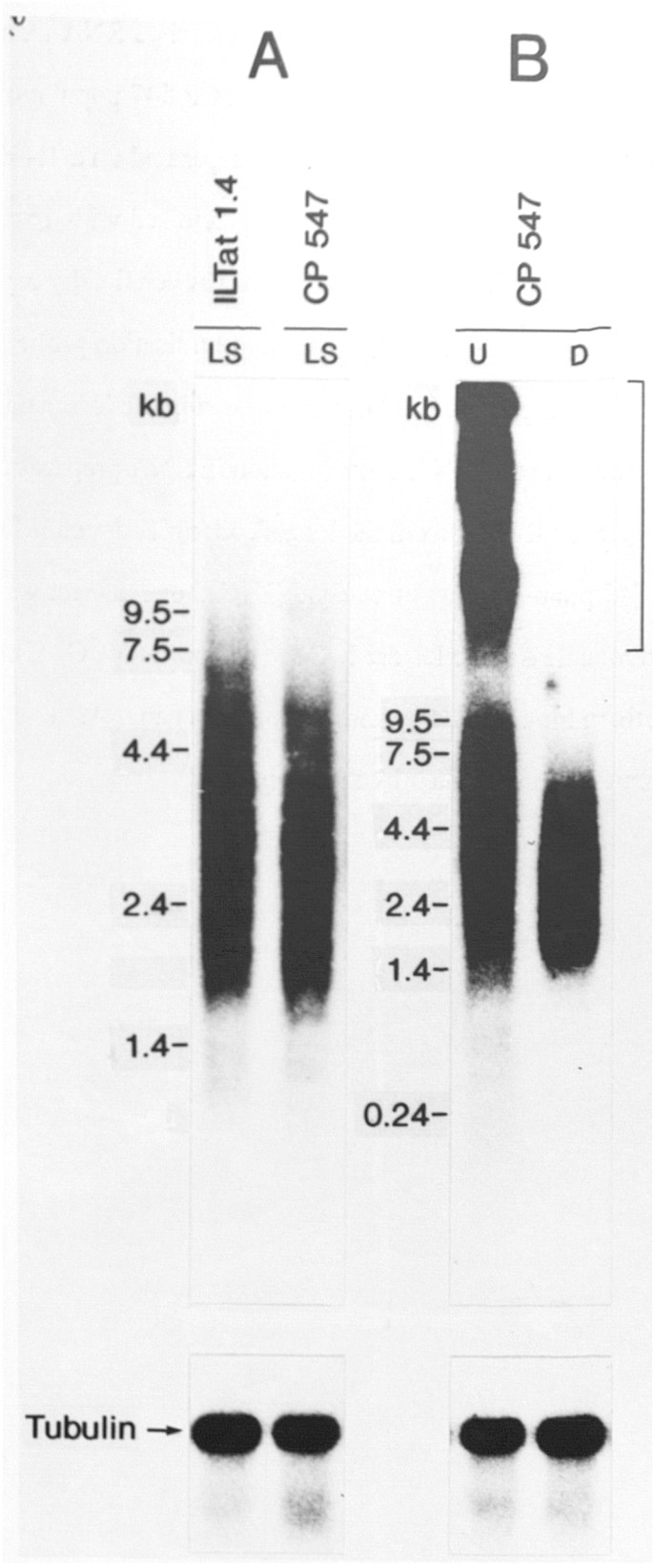
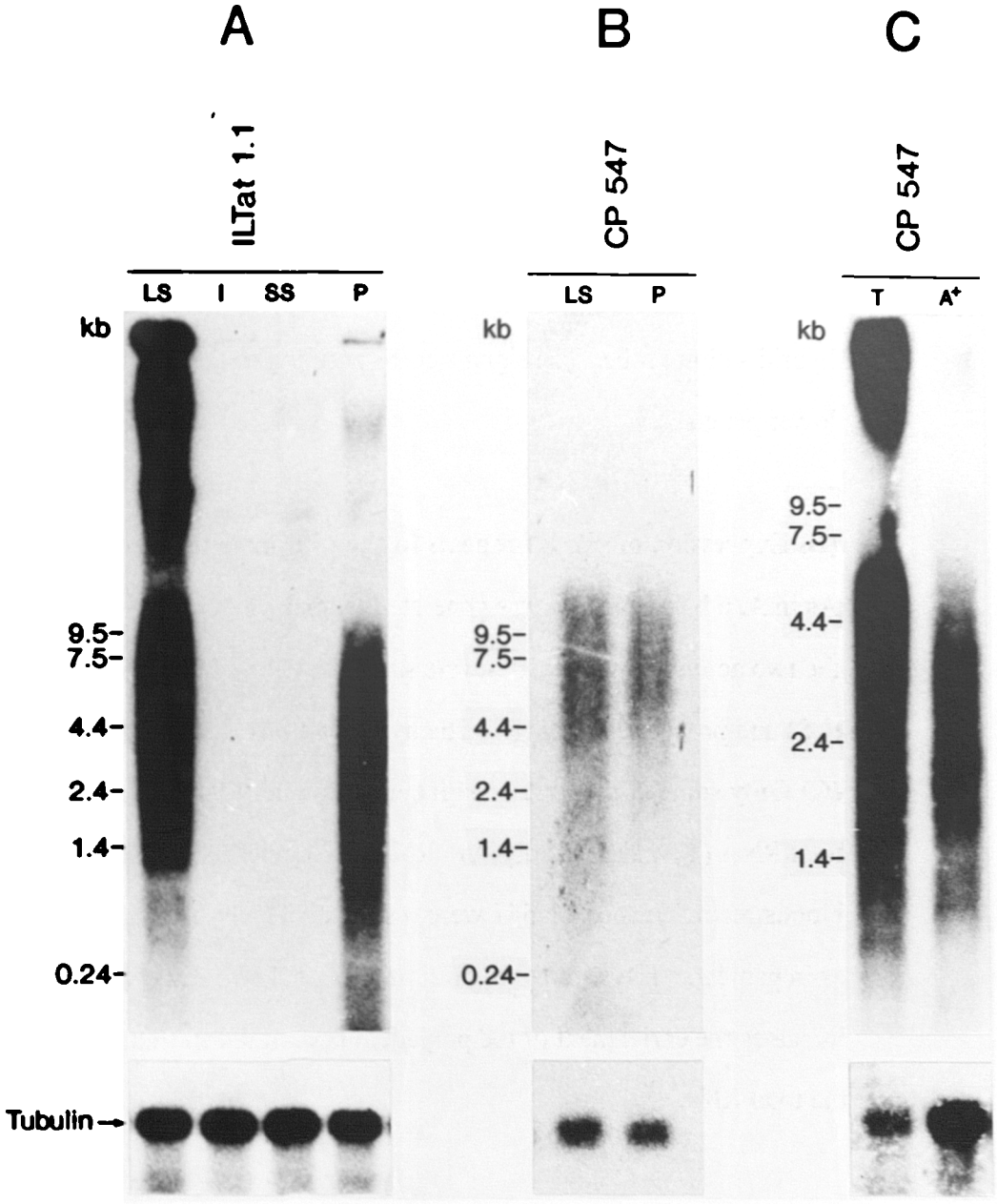

A
B
ILTat 1.4
CP 547
CP 547
LS
LS
U
D
kb
kb
9.5-
7.5-
4.4-
2.4-
1.4-
9.5-
7.5-
4.4-
2.4-
1.4-
0.24-
Tubulin

Figure 33

(A) Differential expression of the *Nla* III repeats. As outlined in Figure 12, a Northern blot was generated from 3 μg of poly(A)$^+$ RNA isolated from the different life cycle stages of ILTat 1.1; the actively-dividing long slender (LS) and procyclic forms (P) of the parasite and the non-dividing short-stumpy (SS) and intermediate forms (I). The blot was hybridised at high stringency with a ^{32}P-labelled *Nla* III repeat. Transcripts were only detected in the long slender and procyclic actively-dividing forms. The integrity of the RNA preparations was verified by reprobing the stripped filters with a ^{32}P-labelled ß-tubulin hybridisation probe. The signals corresponding to the ß-tubulin are shown in the lower panels.

(B) Expression of *Nla* III repeats in the diminazene selected CP 547 clone 2. As in A, a Northern blot was generated from 3 μg poly(A)$^+$ RNA isolated from the two actively dividing life-cycle stages of CP 547 clone 2; the long slender (LS) and procyclic forms (P). The hybridisations were performed as in panel A.

(C) Only some *Nla* III transcripts are polyadenylated. In this gel, 10 μg of total RNA (T) and 2 μg poly(A)$^+$ RNA (A$^+$) purified from long slender bloodstream forms of CP 547 were resolved. The hybridisations were performed as in panel A. The stronger hybridisation signal with ß-tubulin in lane A$^+$ indicates the enrichment of the polyadenylated RNA in this lane in comparison to the total RNA.


A
B
C
ILTat 1.1
CP 547
CP 547
LS
I
SS
P
LS
P
T
A+
kb
kb
kb
9.5-
7.5-
4.4-
2.4-
1.4-
0.24-
9.5-
7.5-
4.4-
2.4-
1.4-
0.24-
9.5-
7.5-
4.4-
2.4-
1.4-
Tubulin

establish this, 10 µg of total RNA and 2 µg poly(A)$^+$ RNA purified from long slender bloodstream forms of CP 547 was size separated in an agarose gel, blotted onto a nitrocellulose filter and probed with ^{32}P-labelled pCA4. Hybridisations revealed a strong signal in the track with total RNA in comparison to the track with the poly(A)$^+$ RNA. Hybridisation of the same Northern filter with control a ß-tubulin probe (Figure 33c) showed stronger hybridisation in the track with the poly(A)$^+$ RNA, confirming its enrichment. This experiment therefore indicated that the majority of the transcripts specific for the *Nla* III repeat are not polyadenylated. For all the Northern blots, the integrity of the RNA preparation was verified by reprobing the stripped filters with a ^{32}P-labelled ß-tubulin probe.

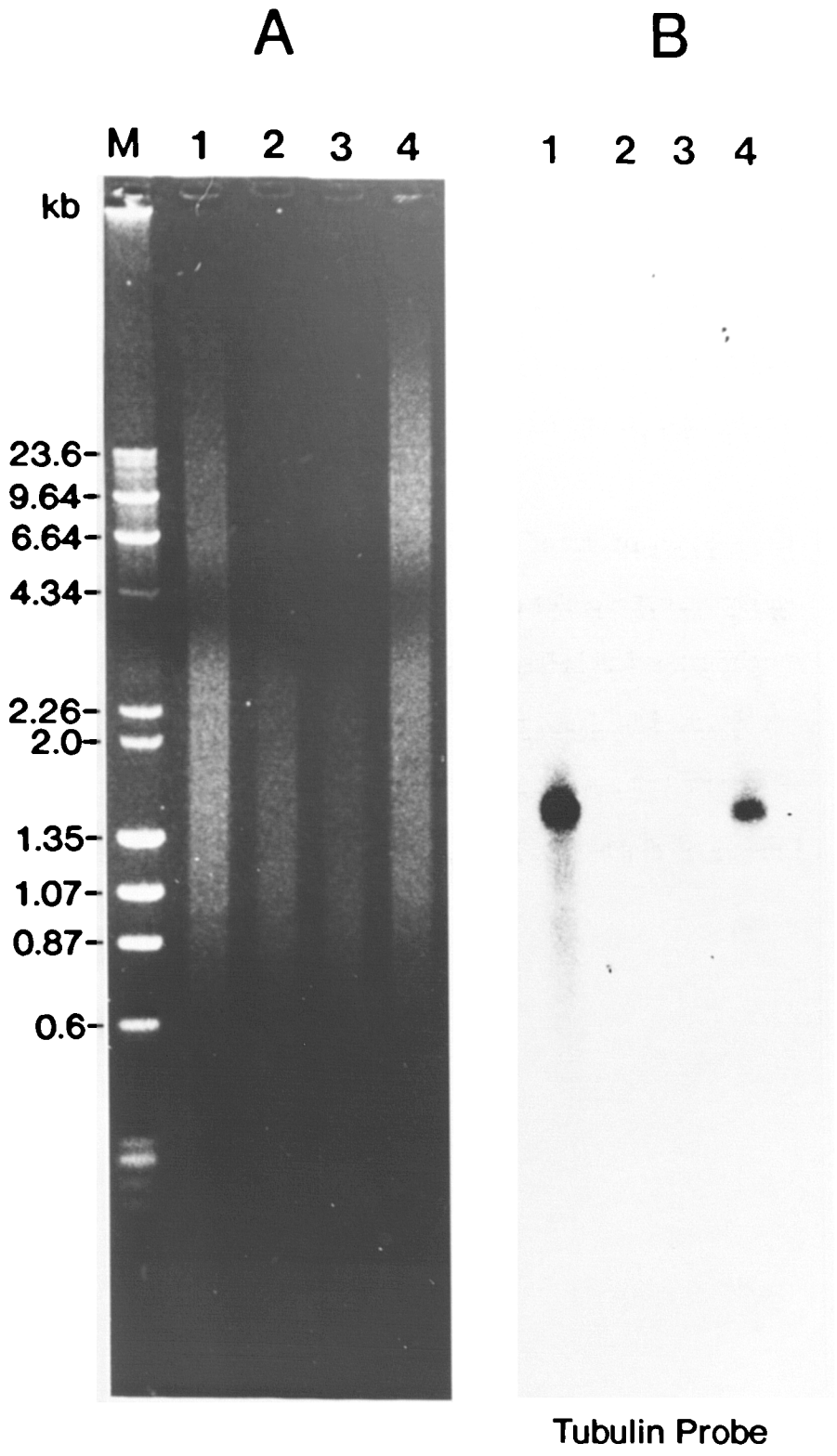
An attempt was also made to establish whether the transcripts of the *Nla* III repeats have a miniexon or spliced leader sequence, as has been reported for all protein coding genes in trypanosomes (Borst, 1986). Single stranded cDNA was generated by reverse transcribing CP 547 RNA using a cDNA synthesis kit (Stratagene). To amplify transcripts specific for the *Nla* III repeat, 5 ng of the single stranded cDNA was used as the target DNA in a PCR-amplification using miniexon-specific primers and *Nla* III repeat-specific primers. To control the PCR, oligo(dT) and miniexon-specific primers were used to amplify full length transcripts from the CP 547 cDNA. The PCR products were size separated by gel electrophoresis and the Southern blot generated was probed with the ^{32}P-labelled pCA4 (Figure 34a, b). At high stringency washes, no hybridisation was evident with the *Nla* III repeat. To ensure and verify that the PCR amplification had indeed worked, the same Southern blot was re-probed with the ß-tubulin probe which hybridised to a DNA band of 1.5 kb in the control PCR sample (Figure 34b). This confirmed that the miniexon and oligo(dT) primers amplified full length cDNA molecules, among which the tubulin transcripts were represented (Figure 34b). Therefore, the lack of hybridisation with the *Nla* III repeat probe indicates that transcripts specific for the *Nla* III repeat sequence were not amplified and most likely do not have a 5' miniexon sequence. This experiment therefore suggests that the *Nla* III repeats are

Figure 34

Determining whether the *Nla* III repeat transcripts contain a miniexon sequence by PCR analysis.

(A) 5 ng of single stranded cDNA generated from total CP 547 RNA was used as the target DNA in a PCR-amplification using miniexon-specific primers (ILO 2235) together with primers specific for the *Nla* III repeat, ILO 1148 (2) and ILO 1148 (3). To control the PCR, oligo(dT) and miniexon-specific primers were used to amplify full-length transcripts from 5 ng of single stranded (1) and 5 ng of double stranded (4) CP 547 cDNA. The PCR products were separated on a 1% agarose gel and stained with ethidium-bromide.

(B) A Southern blot of the gel in panel A was hybridised with a ^{32}P-labelled *Nla* III repeat, but revealed no signals. To ensure and verify that the PCR amplification had indeed worked, the same Southern blot was re-probed with the ^{32}P-labelled ß-tubulin probe. At high stringency washing conditions (0.1 X SSC, 65°C) and overnight exposure at -80°C with intensifying screens, a DNA band of 1.5 kbp corresponding to full length ß-tubulin cDNA was detected.


A
B
M 1 2 3 4
1 2 3 4
kb
23.6-
9.64-
6.64-
4.34-
2.26-
2.0-
1.35-
1.07-
0.87-
0.6-
Tubulin Probe

most likely not translated, as was also suggested by the lack of an open reading frame in the DNA sequence.

3.7 Search for unique "non-repetitive" sequences within the 6.6 kbp element

It is conceivable that the 6.6 kbp element is made up entirely of the *Nla* III repeats since it was completely digested by only *Nla* III. However, this possibility became questionable because when using two different probes generated from the 6.6 kbp region, both the gel-purified 6.6 kbp DNA and the random-primed PCR product (generated from membrane-bound 6.6 kbp) gave identical profiles of hybridisation to many restriction enzyme digested DNA fragments (Section 3.1.2 and 3.3.2). The identical profiles strongly suggested that the 6.6 kbp element contains within it copies of genomic sequences other than the *Nla* III repeats. Yet, when some of these hybridising restriction fragments were gel purified and used as radioactive probes, none hybridised back to the 6.6 kbp element, although some hybridised to the > 40 kbp element (section 3.1.2 and 3.4.1). The likely explanation for the lack of a hybridisation signal to the 6.6 kbp element is that these sequences are probably represented in only some species of the 6.6 kbp element which are present at a copy number that is below the level of detection by hybridisation. Thereafter, a further search for such unique "non-repetitive" sequences on the 6.6 kbp element was carried out since such sequences could contribute to discerning the function of the 6.6 kbp element and possibly the larger elements. In order to amplify these putative unique sequences, an approach using arbitrary primers (AP) in a PCR amplification was used. DNA fingerprints of three different target DNAs (total genomic CP 547 DNA, the alkaline lysis DNA preparation from CP 547 trypanosomes, and the gel-purified 6.6 DNA) were generated in a PCR amplification using arbitrary primers. The arbitrary primers were expected to prime, and thus amplify, many sequences in the total genomic DNA. However, it was anticipated that some sequences specific for the 6.6 kbp element would also be amplified. Similarly, in the alkaline lysis DNA preparation, which is enriched for circular DNA molecules including the 6.6 kbp and the higher

hybridising elements, amplification of sequences specific for the 6.6 kbp element were expected along with amplification of other sequences like those from intact minicircles and maxicircles that should theoretically also be enriched for. However, in the gel-purified 6.6 kbp DNA, sequences specific for the 6.6 kbp element were mainly expected to be amplified with a low level of amplification of contaminating genomic DNA. The AP-PCRs were performed simultaneously, as outlined in section 2.7, using 2 ng of each of the three target DNAs. The DNA fingerprints were analysed in parallel by size separating the products on agarose gels. By ethidium-bromide staining of the agarose gels, fragments that could contain the 'putative unique' sequences were identified. These fragments were those that amplified in the 6.6 kbp target DNA and simultaneously amplified as the same-sized products in the other two target DNAs. In order to eliminate both the *Nla* III repeat and other possible repetitive sequences, Southern blots of these gels were generated and hybridised with these sequences. Firstly, the Southern blots were probed with ^{32}P-labelled gel-purified 6.6 kbp DNA. A note was made of the same sized fragments which hybridised for all three target DNAs. These Southern blots were stripped and reprobed with the ^{32}P-labelled *Nla* III repeat. Fragments that hybridised to the *Nla* III repeat were not pursued further. The Southern blots were again stripped and reprobed with ^{32}P-labelled total genomic CP 547 DNA in order to identify and then eliminate other repetitive sequences that may have been amplified in the PCRs. Subsequently, through this screening strategy, putative unique and non-repetitive sequences specific for the 6.6 kbp element were identified and gel-purified for further analysis.

With this approach, 80 arbitrary primers were tested in the AP-PCR using the 3 target DNAs. However, it became apparent that the major set-back of using the AP-PCR technique was that the target DNA had to be purified away from any contaminating DNA. As was evident from previous experiences, when purifying the 6.6 kbp element from the agarose gels, some level of co-migrating contaminating DNA sequences was inevitable even when great care was exercised. Such sequences contributed to the many false positives in the AP-PCR amplifications. Another frequent readily amplified

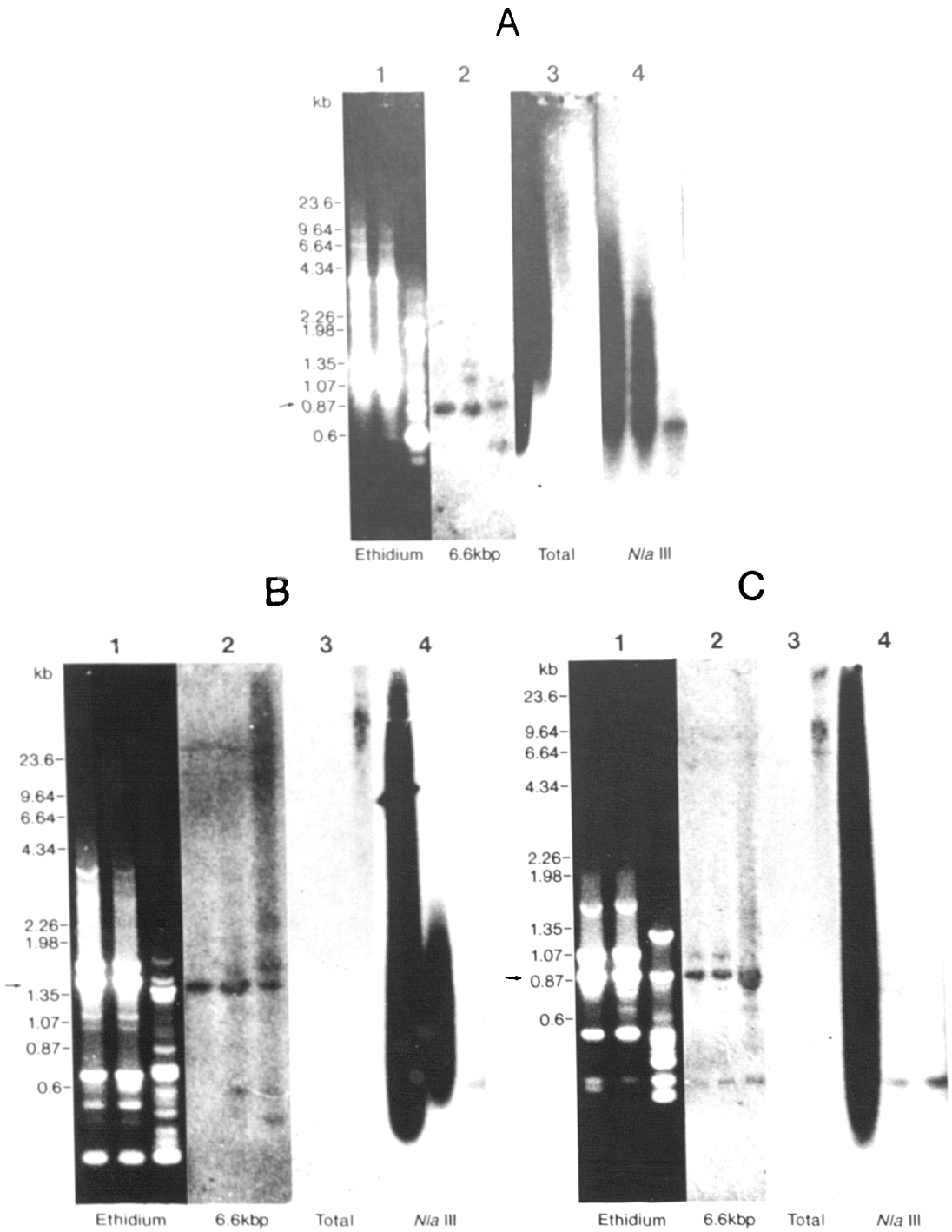
contaminant in the gel-purified 6.6 kbp target DNA was sequences that did not hybridise b ck to the 6 6 kbp element, but to the size markers. However, these concentrations were gre tly reduced on extensive washing of the commonly used electrophoretic tanks (the possible source of this contaminant) prior to electrophoresis.

Representative examples of two of the AP-PCR amplification experiments are shown in Figure 35 where different arbitrary primers were tested. The ethidium-bromide stained gels disclosed that not all arbitrary primers could prime and amplify sequences from the target DNAs. The Southern blots of such gels were then hybridised with gel-purified 6.6 kbp probe. From the resulting autoradiograms, sequences amplified in the gel-purified 6.6 kbp DNA were identified (Figure 35 a, b and c, blot 2). To eliminate sequences hybridising to the *Nla* III repeat, the blots were stripped and re-probed with this probe (Figure 35 a, b and c blot 4). It could not be discounted that the 6.6 kbp element has other repetitive sequences besides the *Nla* III repeat, and in order to avoid picking another repetitive sequence, the blots were stripped once again and probed with total genomic CP 547 DNA (Figure 35a, b and c blot 3) which would give strong hybridisation signals for repetitive sequences. Finally, in order to eliminate at least some of the false positives, the stripped blots were re-probed with ^{32}P-labelled DNA size markers. From this hybridisation screening, amplified sequences that hybridised to the gel-purified 6.6 kbp probe and not to the *Nla* III repeat, total genomic DNA or the size markers were selected for further analysis. These fragments were excised from agarose gels and used as ^{32}P-labelled probes on test Southern blots containing lanes of undigested CP 547 DNA. Of the 80 arbitrary primers tested, only nineteen primers gave amplification of sequences from the gel-purified 6.6 kbp target DNA. Of these, two PCR amplification products hybridised to the *Nla* III repeat and were thus not analysed further. Eleven samples were amplification products of contaminating genomic DNA, as these did not hybridise to the 6.6 kbp element but to other trypanosome sequences. Five samples were amplification products of spurious DNA that did not give any hybridisation to trypanosome DNA. Of the examples in Figure 35, only the 870 bp sequence amplification product of one primer, ILRAD oligonucleotide (ILO) 878 (Figure 35a blot

Figure 35

Search for non-repetitive sequences in the 6.6 kbp element using arbitrary primers (AP) in a PCR amplification (AP-PCR).

(A, B & C) These represent examples of DNA fingerprints generated using three different arbitrary primers (ILO 878, 868 and 1071, respectively) in each panel. In each panel (1) represents the ethidium-bromide stained gel together with the Southern blots of these gels (2, 3 & 4). As outlined in section 2.7, DNA fingerprints of three different target DNAs were generated in each panel. From left to right, these are represented by the alkaline lysis DNA preparation from CP 547 trypanosomes, total genomic CP 547 DNA and the gel purified 6.6 kbp DNA, respectively. Lanes 2 to 4 represent the hybridisation results with ^{32}P-labelled gel-purified 6.6 kbp DNA, (2) ^{32}P-labelled total genomic DNA isolated from CP 547 population, and (3) a ^{32}P-labelled *Nla* III repeat. The washing conditions for all the blots were of high stringency (0.1 X SSC, 65°C) and overnight exposure at -80°C with intensifying screens. The arrows in panels A, B and C indicate the fragments that hybridised to the 6.6 kbp element, but not to the total DNA and the *Nla* III repeat probe.


A
1
2
3
4
kb
23.6
9.64
6.64
4.34
2.26
1.98
1.35
1.07
0.87
0.6
Ethidium
6.6kbp
Total
Nla III
B
C

2), hybridised faintly to the 6.6 kbp and the higher hybridising elements, but not the *Nla* III repeat (Figure 36a). The presence of this unique sequence was verified by repeated amplifications with the ILO 878 primer using different 6.6 kbp isolations as the target DNA.

3.7.1 Analysis of the ILO 878-870 bp product generated by AP-PCR.

In most AP-PCR reactions using the ILO 878, the fragment specific to the 6.6 kbp element was approximately 870 bp. The 870 bp fragment could not be gel purified easily since it migrated closely with other amplified sequences (Figure 35a blot 1 , lane 3). Attempts at excising this fragment and reamplifying it to enrich and separate it from other fragments were unsuccessful as the same profiles were obtained. Therefore, the region encompassing the 870 bp fragment was carefully gel purified by geneclean® method, ^{32}P-labelled by random priming and used as a probe in Southern blot analysis. High post-stringency washes showed a hybridisation signal corresponding to the 6.6 kbp and the higher hybridising elements, and some restriction enzyme fragments (Figure 36). In order to rule out the presence of a possible *Nla* III repeat-containing fragment in the gel-purified DNA that may be responsible for the hybridisations, it was necessary to identify genomic clones specific for the ILO 878-870 bp PCR product. Therefore, about 15,000 recombinant clones from the CP 547 genomic library were plated at a density of 5,000 plaques per 150 cm^2. Of the duplicate library lifts, one set was hybridised with the gel-purified region encompassing ILO 878-870 bp and the second set was probed with a ^{32}P-labelled *Nla* III repeat. The screening of these identical lifts with the two different probes revealed that some of the genomic clones hybridising to the ILO 878-870 bp PCR probe also hybridised to the *Nla* III repeat (Figure 37, indicated by the asterisks), whereas some genomic clones hybridised only to the ILO 878-870 bp PCR product (Figure 37, marked by arrow head). To rule out the possibility that the gel-purified ILO 878-870 bp PCR sequence may be contaminated with some *Nla* III repeat repetitive sequences that had escaped detection, 11 clones hybridising to the ILO 878-870 bp PCR probe, but not the *Nla* III repeat, were picked and the DNAs prepared from them were used as

Figure 36

Southern blot analysis of DNA from the diminazene-resistant CP 547 clone 2 with ILO 878-870 bp as a hybridisation probe.

2 μg of total DNA from CP 547 diminazene-selected clone 2 was resolved on a 1.4% agarose gel as (U) undigested total genomic DNA, (1) *Nla* III digested, (2) *Alu* I, (3) *Sau*3A I, (4) *Taq* I, (5) *Bam*H I and (6) *Eco*R I digested DNA.

(A) The Southern blot of this gel was hybridised at high stringency conditions with the ^{32}P-labelled gel-purified ILO 878-870 bp fragment.

(B) A Southern blot similar to the one in panel A was hybridised with a ^{32}P-labelled *Nla* III repeat probe. The washing conditions were of high stringency (0.1 X SSC, 65°C) and exposure was carried out overnight at -80°C with intensifying screens. The brackets indicate the position of the 6.6 kbp element.

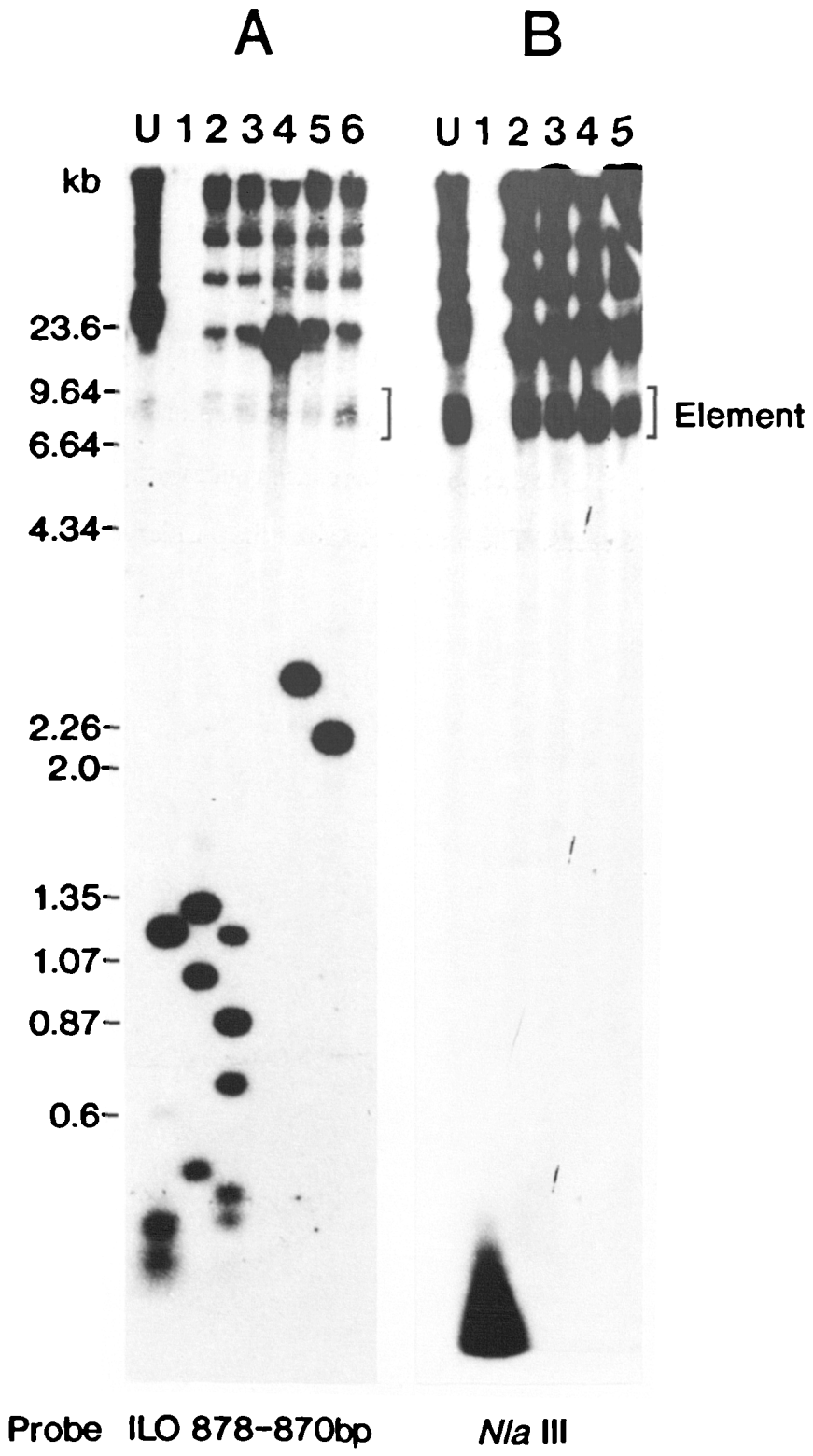
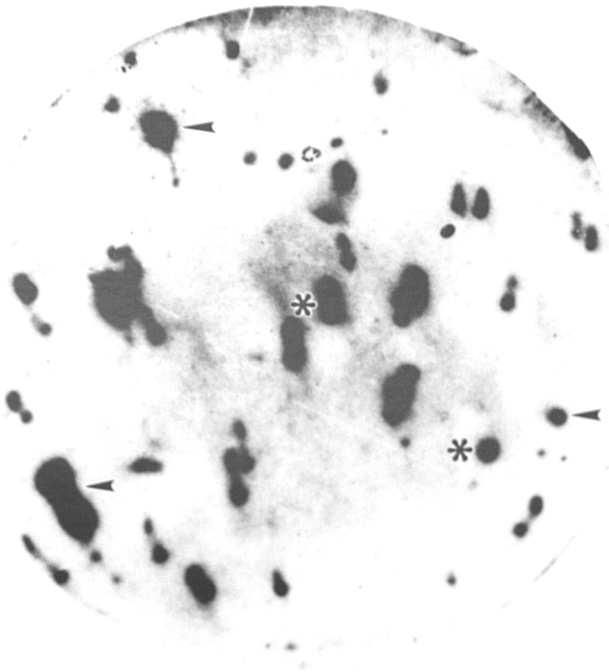

A
B
U 1 2 3 4 5 6
U 1 2 3 4 5
kb
23.6
9.64
6.64
4.34
2.26
2.0
1.35
1.07
0.87
0.6
Element
Probe ILO 878-870bp
Nla III

Figure 37

The ILO 878-870 bp sequence is linked to some copies of the *Nla* III repeat sequence in *T. b. brucei*.

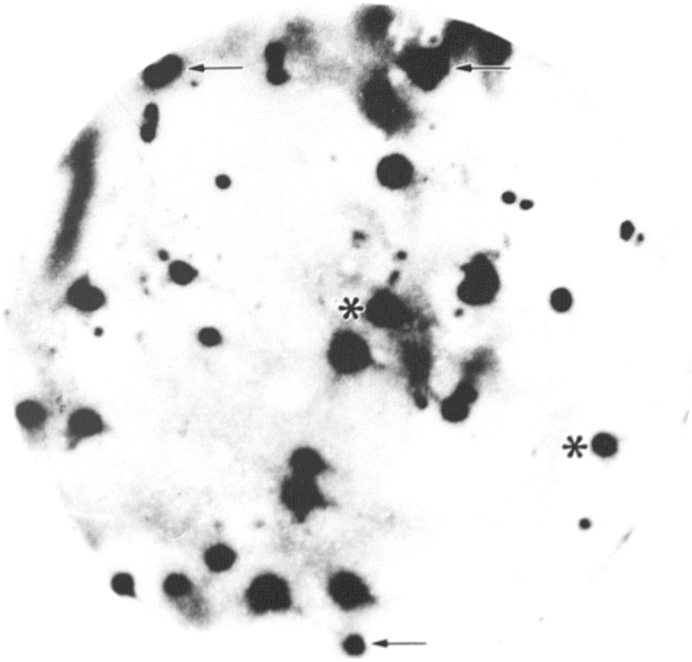
This figure shows the comparative screening of clones from a genomic library generated from *T. b. brucei* CP 547. About 15,000 recombinant clones from the genomic library were plated at a density of 5,000 plaques per 150 cm^2. Of the duplicate library lifts, one set was hybridised with ^{32}P-labelled gel purified ILO 878-870 bp fragment (panel A) and the second set was probed with a ^{32}P-labelled *Nla* III repeat (panel B). The arrow heads in panel A indicate some clones clearly specific for the ILO 878-870 bp probe; the asterisks in panels A and B indicate some clones clearly hybridising to both the ILO 878-870 bp probe and the *Nla* III repeat probe. The arrows in panel B point to some clones specific for the *Nla* III repeat probe only. The washing conditions were of high stringency (0.1 X SSC, 65°C) and exposure was carried out overnight at -80°C with intensifying screens.

A



ILO 878-870bp PCR probe

B



Nla III repeat probe

hybridisation probes on test Southern blots. Four clones hybridised to the 6.6 kbp element and the higher hybridising elements. One of these, pILO18, was selected for further analysis and was subcloned into plasmid Bluescript. A test Southern blot of undigested and restricted CP 547 DNA probed with ^{32}P-labelled pILO18 displayed a hybridisation profile identical to the one obtained with the gel-purified ILO 878-870 bp PCR probe (Figure 36), suggesting that the 870 bp sequence amplified by ILO 878 from gel-purified 6 6 kbp DNA is indeed present in the 6.6 kbp region and most likely on some species of the 6 6 kbp element However, this sequence is not unique to the 6.6 kbp element as hybridisation to the higher hybridizing elements was also evident. On rescreening the genomic library with ^{32}P-labelled DNA purified from pILO18 it became apparent that some of the positive clones also hybridised to the *Nla* III repeat, confirming the close proximity of the two sequences within the genome. Due to the limitations on time, the 870 bp is yet to be characterised.

It is thus hoped that future characterisation of these sequences will possibly unlock the purpose and function(s) of the 6.6 kbp element and the higher hybridising elements in the multidrug-resistant CP 547 clone and other isolates in which extrachromosomal elements have been identified.

CHAPTER 4

DISCUSSION

4.1 Background

African trypanosomes form a homogeneous group of unicellular flagellates that frequently cause severe diseases in tropical and subtropical Africa. Most of the salivarian trypanosomes are cyclically transmitted by several tsetse fly species in which they undergo a complex life cycle. The diseases caused by salivarian trypanosomes have proven to be particularly difficult to prevent or to effectively treat due to specific features of both the trypanosome and the insect vector, the tsetse fly. Attempts to develop a vaccine against African trypanosomes have also been thwarted by the parasites ability to change the composition of its exposed surface antigens. This process of antigenic variation allows the parasite to avoid the host's immune responses and presents the host with a seemingly endless antigenic repertoire. Chemotherapy has remained the most effective means of controlling trypanosome infections. However, the continued use over the last 30 years of a limited and chemically related set of chemotherapeutic agents has resulted in the appearance of drug resistance An increase in the incidence of drug-resistant infections in recent years and the occurrence of multidrug-resistant parasites particularly threaten the future of chemotherapeutic control in humans and livestock (Röttcher and Schillinger, 1985; Mbwambo *et al.*, 1988; Zweygarth and Röttcher, 1989; Codjia *et al*, 1993).

In view of the difficulties and expenses involved in the development and introduction of new trypanocides, it is important to maintain the efficacy of the currently used compounds. This can be carried out both in terms of modifying them and/or using them in improved combinations. However, in order to design better chemotherapeutic strategies or improve on those presently used, both the mode of action of the present

drugs and studies on the molecular mechanisms responsible for drug resistance require investigation.

At the onset of the work presented here no data addressing molecular mechanisms of drug resistance had been obtained for African trypanosomes. Hence, the initial objective of this study was to determine if gene amplification, one common mechanism by which cells become resistant to the action of selective agents (Cowell, 1982; Schimke, 1982), was employed as a mechanism of resistance in a multidrug-resistant *T. b. brucei* CP 547 field isolate Table 8 outlines the different tested drugs to which the CP 547 population was found to be resistant.

In experiments designed to detect amplified DNA, an extrachromosomal element of 6 6 kbp was identified in this population. This element represents the first extrachromosomal element identified in African trypanosomes, thus warranting its characterisation The identification of the element suggested a form of amplification in the drug-resistant isolate analogous to that found in drug-resistant *Leishmania* spp. (Beverley *et al.*, 1988) In *Leishmania* spp., DNA amplification was first shown to occur after selection with methotrexate (MTX) a dihydrofolate reductase inhibitor. It has also been observed to occur sometimes spontaneously in wild-type populations (Beverley *et al* , 1988) These amplified DNAs generally exist as extrachromosomal circles, most of which appear to have an involvement in drug resistance. The two best characterised amplified elements include the R and H circles Beverley, 1991). In many lines of *L. major*, R circles arise by circularisation and amplification of an approximately 30 kb region of chromosomal DNA in response to selection with either MTX, or CB3717, a thymidylate synthase inhibitor. The R circles contain the bifunctional thymidylate synthase-dihydrofolate reductase gene, and the production of these circles results in elevated levels of the enzyme, which confers resistance to antifolates. The H circles are approximately 70 kb in size. These elements are found in different lines of *Leishmania* spp. obtained by selection with MTX or drugs unrelated to antifolates (Karakura and Chang, 1989) and are believed to encode a novel mode of resistance. Finally, a homologue of the P-glycoprotein or multiple-drug resistance (MDR) gene has been

identified on H circles of *L. tarentolae* (Ouellette *et al.*, 1991). However, the role of the gene product does not appear comparable to MDR genes found in higher eukaryotes (Callahan and Beverley, 1991)

The aforementioned 6 6 kbp extrachromosomal element was initially identified in only the multidrug-resistant *T. b. brucei* CP 547. It was therefore tempting to conclude that the 6 6 kbp element played a role in drug resistance. Therefore, to ascertain the possible function of the element, its characterisation was necessary. A further reason for characterising the element was that its small size could make it a suitable candidate for the development of a shuttle vector for the genetic manipulation of *T. b. brucei*.

4.2 Summary of results

For the analysis of the 6 6 kbp extrachromosomal element, attempts to isolate pure and sufficient quantities of the element by standard techniques posed many difficulties Several different approaches were attempted before a sequence which hybridised to the 6 6 kbp element was finally isolated. This sequence is represented by monomers of 108 bp which form long arrays of tandem repeats. *Nla* III is the sole restriction enzyme that cuts within these repeats. In most of the sequenced clones, the tandem 108 bp repeat units are interspersed by a 5 bp spacer sequence. A unique 5 bp direct repeat flanks two complete copies and one partial copy of the 108 bp repeat. The possible significance of these 5 bp interspersed sequences will be discussed later with respect to transposition. In Southern blots generated from undigested total genomic DNA of CP 547, hybridisation with the *Nla* III repeat revealed extrachromosomal elements larger than the 6.6 kbp element. These elements were subsequently referred to as the 'higher' hybridising elements. The 6.6 kbp element and the 'higher' hybridising elements appear to be predominantly composed of long tandem arrays of *Nla* III repeats, since in the many restriction enzyme digests carried out, only *Nla* III appeared to cut these elements. Some results indicate that in addition to the *Nla* III tandem repeats, there are also other sequences on the 6.6 kbp element. One such sequence identified by the

PCR using arbitrary primers (AP-PCR) is the ILO 878-870 bp product, and, like the *Nla* III repeat, this appears to be present on the 6 6 kbp and the 'higher' hybridising elements.

The 6 6 kbp element and some of the 'higher' hybridising elements display features of circular DNA molecules The 6.6 kbp element also displays some level of size and sequence heterogeneity. Whether this heterogeneity is because of the presence of different species of the 6 6 kbp element will be discussed later. The copy number of the 6 6 kbp element is also not stable, and appears to be directly affected by the application of selective drug pressure. However, from the data presently available, the 6 6 kbp element can not be unequivocally correlated directly to drug resistance. This is discussed more extensively in later sections.

The *Nla* III repeat family represents a newly identified repetitive family specific to members of the *Trypanozoon* subgenus This repeat family of approximately 40,000 copies, represents about 5% of the diploid parasite genome. Pulsed field gel-electrophoresis analysis of the drug-resistant CP 547 population revealed that the *Nla* III repeat sequence is dispersed through all size classes of chromosomes, and is also present on extrachromosomal elements such as the 6.6 kbp element and the 'higher' hybridising elements identified by conventional agarose gel electrophoresis. There is also evidence to suggest that extrachromosomal elements containing *Nla* III repeats are also present in some drug-sensitive populations The *Nla* III repeat is arranged in tandem arrays, is dispersed throughout the genome, and is represented by a high copy number. These properties define the *Nla* III repeat as a dispersed highly repetitive sequence.

Transcriptional studies of the *Nla* III repeats have revealed that at least some of the copies are actively transcribed and their transcription is developmentally regulated. A heterogeneous array of transcripts ranging from greater than 10 kb to smaller than 300 bp are detected in the actively dividing long slender bloodstream and insect stage procyclic forms of the parasite but not intermediate or non-dividing stumpy bloodstream forms. The *Nla* III repeat lacks an open reading frame and transcripts do not appear to have a spliced leader sequence at the 5' end. Furthermore, there is almost an equal representation of polyadenylated and non-polyadenlyated transcripts.

4.3 Limitation of the parasite material

One of the problems in this project was the lack of an appropriate drug-sensitive control population. Ideally, the drug-resistant material should have been derived from a known, well characterised, drug-sensitive clone. However, such a control was unavailable as the CP 547 population was a field isolate which was resistant to the highest tolerated dose of diminazene aceturate in mice. Hence, since CP 547 was a drug-resistant population, the best available drug-sensitive control was the well characterised *T. b. brucei* clone, ILTat 1 4. During the course of this study, CP 547 clones showing reduced levels of resistance to diminazene were also produced, but in comparison to ILTat 1 4, these CP 547 clones still expressed high levels of resistance to diminazene aceturate and isometamidium chloride

4.4 Technical difficulties

The discovery of the 6 6 kbp extrachromosomal DNA suggested that an expeditious means for generating hybridisation probes was available. This probe was to be useful for identifying sequences specific to this element by screening recombinant libraries, and hybridising Southern blots. However, the major obstacle for such analysis and characterisation was that the 6.6 kbp element is present in very low quantities, estimated at approximately one copy per genome. Since these small quantities are not visible in ethidium-bromide stained gels, purification of the 6.6 kbp element from agarose gels presented many problems. Firstly, in order to visualise the element, a Southern blot was required for each experiment. Secondly, enrichment of the low amounts of the 6.6 kbp element from agarose gels to generate enough material for one labelling experiment required approximately 500 µg of total DNA; equivalent to approximately 6×10^9 trypanosomes. Both these steps were time consuming and relatively expensive to carry out.

In initial isolations of the element, the agarose gel slices corresponding to the 6.6 kbp region were localised with the use of linear size markers. In order to avoid missing the 6 6 kbp element, wide agarose slices were excised and DNA was then extracted either by geneclean® or electroelution. However, major losses of DNA were incurred with both techniques since the large volumes of the gel slice contained low quantities of DNA; the DNA was so dilute that its precipitation was inefficient. In addition, the presence of relatively large quantities of agarose resulted in co-precipitation of agarose components with the 6 6 kbp DNA. Consequently, the labelling of small quantities of agarose-contaminated 6 6 kbp DNA resulted in poor hybridisation probes. An additional problem encountered was that not all attempted gel purifications of the 6.6 kbp region contained DNA. This led to the realisation that the 6.6 kbp element had differential mobility in different percentage agarose gels, a property of circular DNA molecules As discussed in a later section, a further problem resulting in low 6.6 kbp yields was brought about by the continuous passage of the CP 547 population This resulted in a significant decrease in the copy number of the 6 6 kbp element and eventual loss of the element.

Since there was a suspicion that the 6 6 kbp element was a circular element, alternative techniques such as caesium chloride banding to enrich for circular elements were attempted. However, using this technique, enrichment of the 6.6 kbp element and the 'higher' hybridising elements was unsuccessful. The most likely reason for this was that the copy number of the extrachromosomal elements was probably too low to be visualised as a distinct band When Southern blots containing the fractions of the CsCl gradient were hybridised with a probe generated from the gel-purified 6.6 kbp DNA probe, hybridisation was evident to all the fractions, and no distinct bands were revealed. These results were explicable with further investigations.

When the different gel-purified 6.6 kbp DNA probes were used in Southern blot analyses, clear hybridisation occurred to a consistent set of restriction fragments, believed to be genomic copies of the 6.6 kbp element. However, there was very faint or no hybridisation to the 6.6 kbp element in lanes containing undigested and digested CP 547 DNA. Due to the weak hybridisation signal, it was virtually impossible to determine the

restriction enzymes that cut the 6 6 kbp element. Longer exposures of autoradiography only promoted higher background signals. Furthermore, depending on the 6.6 kbp probe used, there were inconsistencies in some of the hybridising fragments These variations in the Southern blot profiles were originally attributed to the differing concentrations of genomic contaminants co-migrating with the 6.6 kbp probe, and to variations in the transfer efficiency of DNA in the different blots. In conclusion, the problems with isolating even small quantities of the 6.6 kbp DNA, and the difficulties in interpreting the DNA hybridisation results, indicated that alternative approaches were required.

4.5 Overcoming the problems

One option to overcome the encountered technical difficulties was to directly clone sequences from the 6 6 kbp element by a 'shotgun' cloning approach. Gel-purified 6.6 kbp DNA was digested with selected restriction enzymes and attempts were made to clone sequences from it in a plasmid restricted with the same enzymes. The clones obtained using this approach did not hybridise back to the 6.6 kbp element. The explanations for this were that either the 6.6 kbp element was resistant to digestion with the restriction enzymes used, or that the gel-purified 6.6 kbp DNA occurred in too small a quantity to be cloned The cloned sequences were therefore believed to be contaminating genomic DNA that was co-purified from the 6.6 kbp region. Lack of success prompted attempts to clone the putative genomic copies of the 6.6 kbp element. However, this approach was also subsequently abandoned as sequences specific for the 6.6 kbp element could not be unequivocally identified.

The lack of apparent success with the different approaches prompted the following alternative strategies:

1) Purification of the 6.6 kbp element after localisation of the element by Southern blot analysis. This approach improved the level of recovery of the element to some degree and highlighted that the mobility of the 6.6 kbp element varied in different percentage agarose gels. This unusual mobility property, relative to that of linear DNA-size markers,

is characteristic of circular DNAs (Beverley, personal communication) and was the first indication that the 6 6 kbp element had properties of a circular DNA element. This observation also provided an explanation for the curious lack of radiolabelling of 6.6 kbp DNA from some gel isolations; cutting out a gel slice as determined by the linear size markers meant that the 6 6 kbp element was not localised properly, and in some cases none or only a small proportion of the DNA was recovered. With this purification method it also became apparent that with successive passage of the CP 547 population *in vivo*, in the absence of drug pressure, hybridisation signals to the 6.6 kbp element became increasingly less intense and eventually were not detected. Therefore, isolations were being made from the 6 6 kbp region, but with increasing passages, decreasing amounts of 6 6 kbp DNA were present in the purified material.

2) Generation of CP 547 clones Recognition of the instability of the 6.6 kbp element in the CP 547 population prompted the generation of clones of CP 547 that were derived with and without drug pressure Analyses of these clones established a correlation between the presence of the 6 6 kbp element and the application of drug pressure. Subsequently, regular and reliable isolations of the 6.6 kbp element were made from cloned populations of CP 547 that were maintained in mice under a selective pressure of 55 mg/kg body weight of diminazene aceturate.

3) Abundant quantities of probe were generated directly from the 6.6 kbp element using PCR. By exploiting the PCR, direct amplification of sequences from membrane-bound 6.6 kbp element was achieved using random hexanucleotides. This approach helped to generate abundant quantities of the random hexanucleotide-primed PCR probe with a sequence specificity essentially identical to the probe generated from the gel-purified 6.6 kbp DNA. However, as with the gel-purified 6.6 kbp DNA, this probe also hybridised weakly to the 6.6 kbp DNA. The specificity of the PCR probe endorsed the earlier results obtained with the probes generated from gel-purified 6.6 kbp DNA. The necessity to produce large quantities of parasites for generation of total genomic DNA from which

only low quantities of the 6 6 kbp element could be recovered was therefore overcome. Furthermore, with the availability of sufficient quantities of the PCR probe, a genomic library was screened to select clones which would hybridise strongly to the 6.6 kbp element.

Despite improving on the procedures to obtain purer and more abundant quantities of the 6.6 kbp DNA probe, none of the probes generated or purified from the 6 6 kbp element region ever hybridised strongly back to the element itself. This emerged as a major problem at the time, as no explanation was available for the lack of a strong hybridisation signal. In all the different approaches used, the main emphasis had been to identify sequences that hybridised strongly to the 6.6 kbp element. Since such a sequence could not be unequivocally identified, a comparative analysis of all the results was made. This involved a comparison of the hybridisation profiles obtained from Southern blots hybridised with the gel-purified 6 6 kbp DNA probe, ^{32}P-labelled putative genomic copies of the 6 6 kbp element, the random hexanucleotide-primed PCR probe, and clones selected with the PCR probe. A search for fragments that hybridised with the four different probes led to the eventual identification of a second, strongly hybridising element named as the > 40 kbp element. On using the gel-purified > 40 kbp element as a probe on Southern blots of CP 547 DNA, strong hybridisation was evident to the 6.6 kbp element, the 'higher' hybridising elements and the region of unresolved genomic DNA. This indicated that sequences similar to those on the > 40 kbp element are present on the 6 6 kbp and the 'higher' hybridising elements. Subsequently, the > 40 kbp element was used as a hybridisation probe to screen both genomic and cDNA libraries generated from the CP 547 population to obtain sequences that are contained on the 6.6 kbp element. In both the genomic and the cDNA libraries, since the average size of the fragments was less than 6 kbp, the full length element was not expected to be represented. Furthermore, since the sequences hybridising to the > 40 kbp element are repetitive, the chances of picking a clone derived directly from the 6.6 kbp element were unlikely. Nevertheless,

the availability of the > 40 kbp element offered an opportunity to identify and characterise a repetitive sequence contained on the 6.6 kbp element.

The clones selected for further analysis hybridised to both the 6.6 kbp and 'higher' hybridising extrachromosomal elements in the drug-resistant CP 547 population. However, in the drug-sensitive *T. b. brucei* populations, no extrachromosomal elements were apparent and hybridisation was only evident in the region of the gel comprising the unresolved genomic DNA. Characterisation of these clones has led to the identification and characterisation of a new family of repetitive DNA in *T. b. brucei*, named as the *Nla* III repeat family. The availability of this sequence as a probe for hybridisations has permitted further characterisation of the 6 6 kbp and the 'higher' extrachromosomal elements identified in the drug-resistant CP 547 population.

In this discussion, the features of the *Nla* III repeat family are outlined in addition to the features of the 6 6 kbp and the 'higher' hybridising elements. Secondly, the possible role of the 6 6 kbp element and the *Nla* III repeat family in the genomic organisation of trypanosomes is discussed.

4.6 The *Nla* III repeat family

Sequence analysis of selected clones hybridising to the 6.6 kbp element and the 'higher' extrachromosomal elements revealed the presence of a 108 bp tandem repeat sequence interspersed by a 5 bp sequence. Each sequenced 108 bp repeat contained overlapping copies of smaller repeats; these were represented by three copies of a 9 bp repeat, two copies of a 10 bp repeat and two copies of a 27 bp repeat. The interspersing 5 bp spacer sequence in most cases is a region of the 9 bp repeat. It would be interesting to know the events that might have given rise to such a curious repeated structure. It could be possible that the different blocks have originated from different parts of the genome to form a composite unit. This leads to the question as to what represents the active unit of this sequence. The 108 bp repeat sequence has been referred to as a 'unit' based on its tandem appearance and the presence of the *Nla* III sites. However, the active unit may be

the 6 6 kbp element or even still, a much larger sequence In one of the sequenced clones, two complete and a partial copy of the *Nla* III repeat were flanked by a unique 5 bp direct repeat Sequences flanked by direct repeats are generally indicative of transposable DNA elements (Singer and Berg, 1991) Whether the 108 bp sequences are transposable is discussed later

Southern blots of agarose gels containing undigested and restriction enzyme-digested CP 547 DNA when probed with the *Nla* III repeat revealed hybridisation to the 6 6 kbp, 'higher' hybridising elements and the 23 kbp main DNA region in these gels. In the track with undigested ILTat 1 4 DNA, hybridisation was only evident to the region corresponding to the 23 kbp main DNA region. Even frequently cutting enzymes such as *Sau*3A I or *Alu* I did not cut the 6 6 kbp element and the 'higher' hybridising elements. However, when total genomic DNA from either of the populations was digested with *Nla* III, the entire hybridising sequences were cut resulting in a ladder of monomers, dimers and trimers of approximately 113 bp repeat units, which represents the 108 bp repeat and its 5 bp end The ladder was not a result of partial digestions, as the reactions were carried out overnight with an excess of *Nla* III. These experiments suggest that the *Nla* III family of repeats is made up of very long tandem arrays of the 108 bp sequence, since digestion with the various enzymes other than *Nla* III did not result in any digestion products Therefore, a stretch of 23 kbp would have more than 200 copies in tandem. However, 200 copies in tandem represents the lower limit of tandem arrays in the genome since hybridisation to digested or undigested CP 547 DNA is mostly to the > 23 kbp region The multimers of the 113 bp unit generated after digestion with *Nla* III, most likely arise from repeats which have an altered or modified *Nla* III site, thus giving rise to sub-families.

Presently it is not known whether the *Nla* III repeat family is associated with sequences other than the interspersed 5 bp sequences, although one sequence named as the ILO 878-870 bp product, hybridising weakly to the 6.6 kbp element and the 'higher' hybridising elements, has been identified in close proximity to the *Nla* III repeat in some genomic clones. However, in comparison to hybridisation with the *Nla* III repeats, the

ILO 878-870 bp fragment hybridises only weakly to the extrachromosomal elements. This suggests that if this sequence is present on the same elements containing the *Nla* III repeats, it is not associated with every *Nla* III repeat present on these elements.

Sequence analysis has revealed that the *Nla* III repeat contains no open reading frame, yet the G/C content of this repetitive sequence is 47.8 mol%, equivalent to the G/C content of most protein coding sequences in the *Trypanozoon*. Structural analysis of the *Nla* III repeat revealed that it can not form any stable secondary structures as is also true for the Alu-satellite repeat sequence of *T. b. brucei* (Sloof *et al.*, 1983). A homology search of the (GenBank database release 77) has revealed that the *Nla* III repeat has the highest homology (87 5°₀ in a 40 bp overlap) with a subtelomeric sequence specific to a subset of minichromosomes of *T. b. brucei* (Weiden *et al.*, 1991). By using oligonucleotides generated to the different repeat blocks as hybridisation probes, it may be possible to establish whether the source of homology is specific for telomeres other than those of minichromosomes The possible significance of the homology between the *Nla* III repeats on extrachromosomal elements to the subtelomeric region is discussed later

PFGE and OFAGE revealed that on the basis of 12 *trypanozoon* populations examined, the *Nla* III family of repeats is dispersed in all chromosome size classes of *T. b. brucei* Although this family is highly repetitive, the lack of hybridisation to some chromosomes of *T. b. brucei* indicates that the *Nla* III repeat is not an integral feature of all the chromosomes. However, the repetitive nature and the dispersal of the *Nla* III repeats suggests that this family may be able to undergo transposition. Furthermore, hybridisation differences observed between the subclones of *T. b. brucei* GUTat 3.1 suggests that either the *Nla* III repeat is rapidly reorganising within the parasite genome or that transposition of the *Nla* III repeat may be occurring within short time durations. PFGE of different *T. b. brucei* DNA also disclosed the presence of some chromosome sized, *Nla* III repeat containing DNA molecules that have been referred to as curious as they are not visible on ethidium-bromide stained gels and do not hybridise to the telomere probe. In the GUTat 3.1 subclones, the curious molecules were found in the

mini-intermediate-chromosomes size range, whereas in the drug-resistant CP 547 population one curious molecule has an apparent size of approximately 750 kbp while others are smaller than 50 kbp minichromosomes. Whereas haploid chromosomes in the 750 kbp size range are easily visualised in ethidium-bromide stained gels, these curious molecules were only visualised by hybridisation with the *Nla* III repeat probe. There are several possible reasons for the inability to detect the curious molecules in ethidium-bromide stained gels One possibility is that these molecules exist in copy numbers of less than one per genome However, if this was the case, it would be difficult to explain the persistent presence of the 6 6 kbp element in different populations expanded from one CP 547 clone. A second possibility is that the molecules are actually much smaller than is apparent from their migration in the gels. If this was the case, the relative staining by ethidium-bromide of a smaller molecule would be much fainter in comparison to a larger DNA molecule found at the same place in a gel. In this context one of the physical properties of circular DNA molecules is aberrant slow migration through pulsed field gels, leading to a larger apparent size than is deduced either from sequence analysis or from migration of the same DNA when linearised (Beverley, 1988). Therefore, if the curious molecules are circular, then the inability to visualise them in pulsed field gels can be rationalised

A further feature of these curious molecules is that they did not hybridise to a consensus telomeric sequence that hybridises to all the chromosomes apparent on the ethidium-bromide stained gels This suggests that the curious molecules are either non-chromosomal elements with unconventional telomeric ends or that they are indeed circular molecules The possible circular nature of some of these molecules is further supported by the altered direction in migration of one curious molecule in the CP 547 population in both PFGE and OFAGE. PFGE of gamma irradiated CP 547 DNA also verified that the 750 kbp curious molecule was indeed circular; treatment with gamma irradiation resulted in nicking of this circular DNA to give an approximately 400 kbp linear molecule. Molecules displaying such anomalous mobilities, characteristic of circular molecules, were however not evident in the drug-sensitive *T. b. brucei*

populations that were examined From the evidence available, these large curious molecules are most likely oligomeric forms of the extrachromosomal elements evident in Southern blots of CP 547 DNA resolved on conventional agarose gels, analogous to the oligomeric forms of H circles in *Leishmania* spp. (White *et al.*, 1988).

Transcriptional studies of the *Nla* III repeats have revealed that their transcription is developmentally regulated, heterogeneous transcripts ranging from greater than 10 kb to smaller than 300 bp are present in actively-dividing long slender bloodstream forms and the procylic insect form of ILTat 1.4 and CP 547, but were absent in the short-stumpy and the intermediate forms of ILTat 1.4. In bloodstream and procyclic forms of *T. b. brucei*, transcription of telomeric repeats located at chromosomal ends also appears to result in generation of heterogeneously sized RNA (Rudenko *et al.*, 1989). It has been postulated that transcription of telomere repeats results from read-through downstream of telomeric genes The heterogeneous sized transcripts of the *Nla* III repeat may be a result of tandem repeats of varying sizes from different genomic loci. Alternatively, transcripts from the different loci could be hybrids with varying numbers of *Nla* III repeat units together with sequences unique to each locus. Since the smallest transcripts are approximately 300 bp, this indicates that even three units, or possibly less, of the *Nla* III repeats can be transcribed as single transcripts. An explanation for the smaller transcripts could be the possible dispersal of copies from the *Nla* III repeat clusters to new chromosomal locations, similar to dispersal by the ribosomal and histone genes away from their long tandem clusters (Childs *et al* 1980; Long and Dawid, 1980). However, by Southern blot analysis the smallest array of the *Nla* III repeats that is detected is the 6.6 kbp element. Three possible explanations for the smaller transcripts that are observed on Northern blots are as follows: firstly, transposition of the smallest possible unit of the *Nla* III repeat might result in modification of bases at the recipient locus, whereby a large area spanning the transposed unit is resistant to digestion by restriction enzymes. Although there is no precedent for such base modifications in trypanosomes, it would explain the lack of hybridisation of the *Nla* III repeat probe to fragments smaller in size than the 6.6 kbp element. Secondly, it is possible that there are simple sequences with

promoter activity interspersed among some of the *Nla* III repeat units that are responsible for the generation of the smaller transcripts Thirdly, it is possible that the tandem *Nla* III repeats encode their own internal promoter and that the differences in size of transcripts are due to initiation of transcription from different copies. The differences in size could also depend on the varying position of transcription termination signals among the *Nla* III tandem arrays, at different loci.

Among fundamental processes that are carried out differently by trypanosomes and mammalian cells, the method by which the parasites transcribe mRNA stands out (Borst, 1986) The 5' end of all mature transcripts of genes coding proteins in trypanosomes possess a 39 nucleotides capping sequence named the spliced leader (SL), often improperly the 'miniexon', which is added to the RNA by trans-splicing Borst (1986) In a PCR using CP 547 cDNA as target DNA, primers specific for the miniexon and those specific for the *Nla* III repeat failed to amplify sequences specific for the *Nla* III repeat This experiment indicated that the *Nla* III repeat transcripts lack the miniexon sequence at their 5' ends However, in the control PCR using CP 547 cDNA as target DNA, primers specific for oligo dT and the miniexon sequence resulted in amplification products

Determination of the copy number of the *Nla* III repeats within different *T. b. brucei* populations revealed that this family is present at up to approximately 40,000 copies in the diploid parasite genome, with no significant difference between the drug-resistant and drug-sensitive populations. This family of repeats can therefore be broadly classified as a 'highly' repetitive dispersed family (Singer and Berg, 1991), and represent 5% of the total parasite genome. However, this family of repeats is restricted to the subgenus *Trypanozoon*, since even at low stringency, no hybridisation was apparent to *T. congolense* or *T. vivax*. The absence of the *Nla* III repeat family from closely related kinetoplastida suggests its recent origin and amplification in the *Trypanozoon*. However, since the *Nla* III repeats are known to be involved in rapid genomic reorganisations, it is also possible that this family may have an early origin, but that its rapid evolution

resulted in significant divergence between *Trypanozoon* and other genus resulting in the lack of hybridisation.

Despite its relatively small size, (six times that of *Escherichia coli*) (Borst *et al.*, 1982), the genome of *T. b. brucei* contains an abundance of repeat sequences. In addition to the large collection of variant surface glycoproteins (VSG) genes (up to 1000 copies), which show local homologies (van der Ploeg *et al.*, 1982b), several copies of reiterated sequences have been described; the spliced leader (SL) sequence (Boothroyd and Cross, 1982), the 76 bp repeat sequences found 5' to the VSG genes (Campbell *et al.*, 1984b), and the telomeric repeat sequences (Blackburn and Challoner, 1984; van der Ploeg *et al.*, 1984d) In addition to these sequences, families of retrotransposon-like reiterated sequences have also been reported from a number of *Trypanosoma* species. For example, the ribosomal mobile element (RIME) in the subgenus *Trypanozoon* represents a 'middle' repetitive family of sequences (Hassan *et al.*, 1984). Two halves of a RIME element are also found flanking the trypanosome repeated sequence TRS1 (Murphy *et al.*, 1987) or 'ingi' element (Kimmel *et al*, 1987) which is closely related to mammalian retrotransposons and is widely distributed in many genomic sites in *T. brucei*. Both RIME and TRS1 ingi are present in about 400 copies in the *T. b. brucei* diploid genome, representing about 1 5% of the genomic DNA content (Kimmel *et al.*, 1987; Murphy *et al.*, 1987). Therefore, thus far, apart from the homologous sequences surrounding VSG genes (van der Ploeg *et al.*, 1982a), TRS1/ingi and RIME sequences have been considered to be the most abundant dispersed repetitive sequences in the *T. b. brucei* genome (Kimmel *et al.*, 1987). However, it now appears that the *Nla* III repeat family represents the most abundant class of repeats yet identified in trypanosomes. Finally, in addition to the presence of these reiterated families, the first examples of satellite DNAs in protozoa, the 177 bp Alu repeat were identified in *T. b. brucei* and *T. cruzi* (Sloof *et al.*, 1983).

Considering that the *Nla* III repeat family represents almost 5% of the *T. brucei* genome, it is surprising that this reiterated family has not been described previously. The probable reason for the lack of detection is that the *Nla* III repeat family exists in long

tandem arrays of *Nla* III repeats, generally greater than 20 kbp and lack sites for restriction enzymes other than *Nla* III, which is an infrequently used enzyme. Furthermore, in caesium chloride density gradients, unlike the *T. b. brucei* Alu-satellite repeat family, the *Nla* III repeat family does not form a satellite band since the G/C content of the family is equivalent to that of coding sequences which are known to equilibrate at the main band position in the density gradient (Newton and Burnett, 1972). Therefore, unlike satellite DNA, the *Nla* III repeat family most likely resolves with the main band, making this family quite inconspicuous.

In the work described here, a newly identified *Nla* III repeat family has been characterised The involvement of the *Nla* III repeats in genomic reorganisation, their transcriptional characteristics, and their presence on an extrachromosomal 6.6 kbp element and 'higher' hybridising elements, in a drug-resistant stock of *T. b. brucei* and clones derived from it, differentiates this repeat family from other families of *Trypanozoon* repeat sequences

4.7 The 6.6 kbp element and the 'higher' hybridising elements

Screening of the CP 547 genomic library with the > 40 kbp element led to the selection of genomic clones, all of which contained *Nla* III repeats in tandem arrays, interspersed with variable 5 bp sequences. The *Nla* III repeat sequence probe subsequently allowed partial characterisation of the 6.6 kbp and the 'higher' hybridising extrachromosomal elements in the CP 547 population.

High stringency hybridisations with the *Nla* III repeat revealed that the 6.6 kbp element and the 'higher' extrachromosomal elements contain sequences homologous to the *Nla* III repeats. Since *Nla* III is the only enzyme that cuts these elements, this suggests that the *Nla* III repeats exist as long tandem arrays on these elements. This was further established in PCRs in which primers designed from the *Nla* III repeat unit enabled amplification of sequences from the gel-purified 6.6 kbp when used as the target DNA. This PCR amplification product hybridised back to the 6.6 kbp and the 'higher' hybridising elements, and on agarose gels the PCR product resulted in a ladder

suggesting that the *Nla* III repeats are arranged in tandem on the 6.6 kbp element. The presence of heterogeneous sequences interspersed between the *Nla* III repeat would have resulted in gaps in the ladder of the PCR products. However, under the PCR conditions used, gaps in the ladder were not apparent, suggesting that the 6.6 kbp element is composed of the *Nla* III repeats Data from hybridisations with the *Nla* III repeat probe and the PCR analysis does not exclude the occurrence of randomly placed heterogeneous simple-sequence elements either between or at the ends of the *Nla* III repeats. However, in AP-PCR analysis, the ILO 878-870 bp sequence was amplified from the gel-purified 6 6 kbp DNA Hybridisation of the ILO 878-870 bp sequence to the 6.6 kbp element and the 'higher' extrachromosomal elements suggests that besides the presence of the *Nla* III repeats, these extrachromosomal elements may also contain other sequences. Further evidence for this is available from the identification of genomic clones which contain both the *Nla* III repeat and the ILO 878-870 bp sequence. Since the 6.6 kbp element and the 'higher' extrachromosomal elements are only digested by *Nla* III, it is likely that, as for the *Nla* III tandem repeats, the interspersed sequences present on these elements are also simple repeats, lacking sites for the restriction enzymes that were used. It is, however, worth noting that when a PCR was carried out that used primers designed from the *Nla* III repeat, there was no indication of the presence of any additional sequences on the 6 6 kbp element A likely explanation for this could be that the 6.6 kbp element is made up of many tandem repeats of the *Nla* III repeat followed by one ILO 878-870 bp sequence In such a situation, the majority of the products would represent the *Nla* III repeat and its multimers, the ILO 878-870 bp sequence would be under-represented. Since the ladder ran into a smear, the under-represented ILO 878-870 bp sequence may have been masked by the more abundant *Nla* III repeats. However, hybridisation with ILO878-870 bp would have resolved this. Another possible explanation is that the 6.6 kbp element may occur as a heterogeneous population in which the majority of 6.6 kbp species are made up entirely of the *Nla* III repeats. In this situation, the minority of the 6.6 kbp element species would contain other sequences like the ILO 878-870 bp product in addition to the *Nla* III repeat sequence. Finally, there may also be some species which

do not contain the *Nla* III repeat at all The presence of a heterogeneous 6.6 kbp population is supported by the evidence that is discussed in the next section.

As previously mentioned, the 6 6 kbp element and some of the 'higher' hybridising elements are most likely circular DNA molecules. Evidence to support this was obtained firstly, from their aberrant mobility in different percentage gels and in PFGE and OFAGE Secondly, evidence to support this was also obtained from results of *Bal* 31 exonuclease digestions of total CP 547 DNA. In these experiments the resistance of the > 40 kbp and some 'higher' hybridising elements in the 23 kbp region to exonuclease activity indicated their circular nature. From the *Bal* 31 experiments it was difficult to determine whether the 6 6 kbp element is circular in nature as it was continuously masked by the larger sequences that were sensitive to digestion by *Bal* 31. Thirdly, an oligonucleotide specifically designed from a consensus telomeric sequence which hybridised to all the *T. b. brucei* chromosomes resolved by PFGE did not hybridise to the 6 6 kbp element, the 'higher' hybridising elements and the curious molecules identified in PFGs These results indicate that the curious molecules are probably larger extrachromosomal circular elements and that, like the 6.6 kbp element and the 'higher' hybridising elements, these molecules either lack conventional telomeric ends or are circular in nature Finally, the 6 6 kbp element and the 'higher' hybridising elements are greatly enriched by the alkaline lysis protocol used in the isolation of circular plasmid DNA, suggesting their circular nature.

At present, it is not known whether the 6.6 kbp and 'higher' hybridising elements are specifically transcribed. From the transcriptional studies of the *Nla* III repeat, it is evident that it is highly transcribed. However, whether the *Nla* III repeats present on the extrachromosomal elements are also transcribed cannot be easily ascertained due to the repetitive nature and genomic distribution, of this sequence. For a meaningful transcriptional analysis of these extrachromosomal elements, either sufficient quantities of the cloned elements, or unique markers need to be identified on the elements to be used as hybridising probes. It may also be possible to specifically target exogenous

selectable marker genes such as the *neo* gene (Jiminez and Davies, 1980) to these elements by transfection

4.8 Size heterogeneity in the 6.6 kbp element

From various Southern blots of different preparations of undigested CP 547 DNA, it became apparent that the 6 6 kbp element also displays size heterogeneity. The reasons for this are undetermined However, to explain the size heterogeneity, the following possibilities are considered

1) If the 6 6 kbp element is made up entirely of *Nla* III repeats, some of these repeats may be deleted or added during repeated rounds of replication. The size variability could also be due to expansion and contraction of the region containing tandem copies of the *Nla* III repeat by intercistronic or intra-element recombinational events.

In *T. b. brucei*, fluctuations in sequence sizes have been observed in the 5' limit of transposition of VSG genes within the 76 bp tandem repeat units (van der Ploeg *et al.*, 1982b) These fluctuations are also believed to be due to expansion and contraction of the region containing the 76 bp repeats by intercistronic recombination events such as gene conversion (van der Ploeg *et al*, 1982b).

2) The size heterogeneity might be due to the presence of different species of the 6.6 kbp elements comprised of heterogeneous sequences. Evidence to support this is presented in the following section entitled 'sequence heterogeneity'.

4.9 Sequence heterogeneity in the 6.6 kbp element

As mentioned earlier, the different gel-purified 6.6 kbp probes hybridised consistently to a number of genomic restriction fragments but inconsistently to others. It was possible that hybridisation to the 'inconsistent' fragments was due to contaminating genomic sequences in the 6.6 kbp probe. However, this did not appear to be the case since the gel-purified 6.6 kbp probes did not hybridise to any of the abundant, highly

repetitive, trypanosome sequences, as would be expected from contamination with total genomic DNA Further, when random hexanucleotide primers were used to generate PCR probes from the gel-purified element, they were consistently specific for the restriction fragments that hybridised to the gel-purified 6.6 kbp DNA. It is curious that both the PCR probe and the *Nla* III repeat probe originate from the 6.6 kbp element, and yet the hybridisation profiles obtained using the PCR probe and the *Nla* III repeat probe are completely different. The differences in the two probes suggests, as previously mentioned, that there may be different populations of the 6.6 kbp element containing different sequences The random hexanucleotide-primed PCR product is possibly a result of selective amplification of sequences from one or more populations of elements which do not contain the *Nla* III repeat Although the random hexanucleotide-primed PCR probes gave hybridisation profiles essentially similar to the gel-purified 6.6 kbp DNAs, there was a problem in explaining why they did not hybridise strongly to the 6.6 kbp region In view of the possible occurrence of a heterogeneous population of 6.6 kbp elements, it is possible that the sequences contained within the PCR probe are derived from species of the 6 6 kbp elements that are present in copy numbers below the level of detection by hybridisation In order to validate these speculations, the different species of the 6 6 kbp element would have to be identified and characterised individually. However, it is difficult to envisage that in the random hexanucleotide-primed PCR there was selective amplification of sequences from some 6.6 kbp elements, whereas sequences specific to the *Nla* III repeat were not amplified. One possible explanation is that secondary structures may inhibit their amplification in PCR. However, from sequence analysis there is no evidence of stable secondary structures in the *Nla* III repeat which could rationalise its lack of amplification. In conclusion, the reasons for the lack of amplification of sequences specific for the *Nla* III repeat are as yet unclear.

The lack of hybridisation of the gel-purified putative genomic copies of the 6.6 kbp element back to the element can possibly be explained in a similar manner. The putative genomic copies possibly occur in only some members of the heterogeneous 6.6 kbp population, which are below the limits of detection by hybridisation. However, both

the putative genomic copies and the random hexanucleotide-primed PCR probe hybridised strongly to fragments in restriction enzyme digests of total genomic DNA. These fragments are most likely genomic copies of these sequences, with each hybridising band representing one or more copies of that sequence in the genome.

Further evidence for heterogeneity within the 6.6 kbp population was evident from the selection of a CP 547 population found to contain a unique species of the 6.6 kbp element This 6 6 kbp element contained sites for many restriction enzymes which are absent in all other 6 6 kbp elements. However, this unique species of the 6.6 kbp element changed to the form of the element that could not be digested with most restriction enzymes after the parasite population that contained it had been expanded in mice This suggested that sequences on the 6.6 kbp element vary in different populations of the same clone of *T. b. brucei*. Further evidence of heterogeneity was also obtained from hybridisations with the *Nla* III repeat probe to Southern blots containing digests of total CP 547 DNA with different restriction enzymes. These blots revealed that *Alu* I and *Apa*L I caused a reduction in hybridisation signal intensity to the 6.6 kbp element, but no alteration in its size No such differences were apparent between the hybridisation signal of the 6 6 kbp element in other restriction enzyme digests when compared to the signal for the undigested DNA samples. However, in some DNA samples isolated from other populations of CP 547, no such differences in the signal intensity of the 6.6 kbp element were apparent after *Alu* I or *Apa*L I digestion. These experiments therefore showed variations in the 6 6 kbp element in different CP 547 populations.

Some possibilities that could offer an explanation for the sequence heterogeneity observed in the 6.6 kbp element are as follows:

1) It is possible that the *Nla* III repeat probe hybridises to one population of elements whereas other populations in this size range are not detected by the probe. During expansion of the CP 547 parasite population in mice, selection for different 6.6 kbp species from a heterogeneous 6.6 kbp population may occur for undetermined reasons, resulting in variable 6.6 kbp DNA isolations. Some of the variable results may have occurred due to isolation of DNA for the probe from one population and hybridising the

probe to a second population The proposed heterogeneous probes could therefore have contributed to the inconsistencies observed in hybridisation to restriction fragments in Southern blots The presence of mixed species of 6.6 kbp elements also offers an explanation for the reduction in hybridisation signal intensity to the 6.6 kbp element that was observed when one isolation of total CP 547 DNA was digested with *Alu* I or *Apa*L I and not others. The simplest explanation for this latter observation is that only some *Nla* III containing 6 6 kbp species have the restriction site for these enzymes. As a result, digestion resulted in loss of only these species, accounting for the reduction in signal intensity. Similarly, one of the reasons for the earlier mentioned weaker hybridisation of the ILO 878-870 bp product in comparison to *Nla* III repeat probe to both the 6.6 kbp and the 'higher' hybridising elements, may be due to only some species containing the ILO 878-870 bp sequence

2) Some circular DNAs are thought to be derived from chromosomal tandem repeats as a result of homologous recombination (Schmookler Reis *et al*, 1983). It is clear that the *Nla* III repeated units, can also give rise to circular molecules. Dispersed *Nla* III repeats could carry 'passenger' sequences originally located between them. This would be analogous to, but distinct from, 'true' transposition according to bacterial models (Shapiro, 1979, Carlos and Muller, 1980). The variability could also be analogous to the unique variable arrangements of the *Crithidia* retrotransposable element 1 (CRE1) (Gabriel *et al*, 1990) identified in individual cells from the same population; the CRE1 element interrupts the tandemly arrayed miniexon genes of *C. fasciculata*. Although the specific genetic mechanisms underlying the frequent rearrangements of CRE1 are yet unknown, it has suggested that unequal crossing over between the flanking tandem arrays of miniexon genes may be responsible for the observed rearrangements (Gabriel *et al.*, 1990). If one assumes that there are unique sequences flanked by the *Nla* III repeat tandem arrays in the 6.6 kbp element, this organisation would then be analogous to the organisation of CRE1s within long tandem arrays of miniexon genes. It would also be similar to the situation in transgenic mice which contain a single copy of a Moloney murine leukemia virus proviral genome integrated into highly repetitive tandem

sequences on the sex chromosomes (Habers *et al.*, 1986). In both the CRE1 and Moloney murine leukemia virus proviral genome, the high frequency of rearrangements is thought to be due to unequal crossing over within the tandem arrays flanking the respective sequences.

In conclusion, it is possible that the observed sequence heterogeneity in the 6.6 kbp element may be a result of rearrangements within the 6.6 kbp element due to unequal crossing over within the tandem *Nla* III repeat units flanking unique sequences. Such a phenomenon could result in heterogeneous sequences among different 6.6 kbp element species Which species are detected will depend on: (i) The frequency with which these events occur, (ii) Whether there are 'hot spots' for these events; (iii) Whether these are produced by recombination or through duplication; (iv) The replication efficiency of these circular molecules whereby, some may replicate more efficiently than others becoming the dominant species

4.10 Is the 6.6 kbp element responsible for mediating drug resistance?

The aim of the present study was to try to determine if gene amplification is one of the mechanisms used by trypanosomes to overcome the lethal effects of trypanocidal drugs. The identification of an extrachromosomal 6.6 kbp element in the drug-resistant CP 547 population raised these expectations and encouraged its characterisation. As mentioned previously, the element suggested a form of amplification in the drug-resistant isolate analogous to that found in drug-resistant *Leishmania* spp. (Beverley *et al.*, 1988). The following section therefore discusses the possible biological significance of the 6.6 kbp and the 'higher' hybridising elements with respect to drug resistance.

Work in several laboratories has demonstrated that when the activity of an essential gene product becomes insufficient, either by mutation or because of the presence of a specific inhibitor, mutants that overcome the deficiency can often be found. Some mutants survive by at least one mechanism of resistance, that of gene amplification; an important step in the development of drug resistance *in vivo*. Gene

amplification may result in the over-production of the target gene product, a mechanism found in many drug resistant mammalian cells (Stark and Wahl, 1984) and *Leishmania* spp (Beverley, 1991, Segovia, 1994). Essentially the effects of the inhibitor are diluted by over-production of the target gene product through gene amplification. Multiple tandem repeats of the targeted gene are acquired, and the multiple gene copies produce a superabundance of mRNA and of the corresponding polypeptide. Sometimes, in response to drug pressure, amplification of genes from loci distant to the target gene can also occur (Stark *et al*, 1989). Mariani and Schimke (1984) have established that cytotoxic agents which enhance gene amplification are inhibitors of DNA synthesis. In *Leishmania* spp , the amplified DNAs exist primarily as extrachromosomal circular forms which can be easily visualised following electrophoresis under appropriate conditions (Beverley *et al*, 1988, Beverley, 1991). The 6.6 kbp element was identified in the multidrug-resistant CP 547 stock. When Southern blots containing undigested DNA were probed with the gel-purified 6 6 kbp element, in the lane with DNA from CP 547 stock, hybridisation was evident to both the 6.6 kbp extrachromosomal element and the total genomic DNA In the lane with DNA from the drug-sensitive ILTat 1.4 population, hybridisation was evident only to the total genomic DNA. It was therefore evident that sequences homologous to the 6 6 kbp element were present in the total genomic DNA of both CP 547 and ILTat 1.4. This suggested that the 6.6 kbp element arose from the genome and was most likely a product of gene amplification. Such an occurrence was not unlikely since trypanocides are also inhibitors of DNA synthesis, and may result in gene amplification as do mammalian cytotoxic agents inhibiting DNA synthesis. During the initial handling of CP 547 stock, the parasites were expanded from a CP 547 population that was maintained in mice by continuous passage. When Southern blots were generated from DNA purified from different passaged CP 547 populations, instability in the copy number of the 6.6 kbp element was revealed. With reference to Figure 21, if the drug pressure is withdrawn, the copy number of the 6.6 kbp element reduces and the element eventually becomes unnoticeable. The response of the 6.6 kbp element to selective drug pressure, its extrachromosomal and circular characteristics, and

its presence in the multidrug-resistant CP 547 stock and not the drug-sensitive reference clone, ILTat 1 4, raised the expectation that the 6.6 kbp element was analogous to the circular extrachromosomal elements mediating drug resistance in methotrexate-resistant *Leishmania* spp (Beverley, 1991) and methotrexate-resistant cultured mammalian cells (Schimke, 1984) In *Leishmania* spp and cultured mammalian cells the copy number of the extrachromosomal elements is also unstable in the absence of selective drug pressure.

With reference to Table 8, four clones referred to as the 'unselected' clones were generated from the CP 547 stock passaged in the absence of drug pressure. All the unselected clones displayed reduced levels of resistance to both diminazene and isometamidium in comparison to the parental CP 547 stock. Furthermore, the values for resistance differed between each of the four unselected clones. It therefore appears that the continuous passage of the parental CP 547 stock in the absence of drug pressure resulted in the selection of sub-populations that expressed lower levels of resistance to diminazene and isometamidium In three of the four unselected CP 547 clones, the reduction in resistance was also accompanied by the loss of the 6.6 kbp element. These observations suggested a correlation between the 6.6 kbp element and drug resistance. The variations in the drug-resistance profiles and the copy number of the 6.6 kbp element in the four 'unselected' clones emphasised the need for a uniform CP 547 population for the further characterisation of the 6 6 kbp element. Therefore, four additional clones were derived from the parental CP 547 stock after it had relapsed following treatment with the maximum tolerated dose of diminazene aceturate in mice (ie, 60 mg/kg body weight). These clones were referred to as the 'selected' clones. The four selected clones all displayed resistance to the maximum tolerated dose of 60 mg/kg of diminazene aceturate in mice and therefore their absolute level of resistance could not be established *in vivo*. All the four selected clones showed the presence of the 6.6 kbp element, suggesting a strong correlation between the presence of the 6.6 kbp element and drug resistance. However, this possibility became questionable since one of the unselected CP 547 clones (clone 3) contained the 6.6 kbp element, and yet was more sensitive to diminazene than the unselected clone 1 which did not have the element. This result alone

indicates that there is no direct correlation between the presence of the 6.6 kbp element and expression of resistance to diminazene. However the presence of the element in all the selected clones makes it difficult to discount a possible correlation between drug resistance and the presence of the element. Although the unselected clones were more sensitive to diaminazene than the selected clones, in comparison to the drug-sensitive control, ILTat 1 4, the unselected clones express higher levels of resistance to aceturate and isometamidium (Table 8)

From the limited data available on the different CP 547 clones, all evidence suggests that the 6 6 kbp element does not mediate drug resistance directly. This is apparent from the observation that the element is absent from the more resistant, unselected clone 1 but present in less resistant, unselected clone 3 In support of this, sequence analysis of the *Nla* III repeat, known to be present on the 6 6 kbp element and the 'higher' extrachromosomal elements, revealed no open reading frames which might encode a possible drug target Also, transcriptional analysis of the *Nla* III repeat did not reveal any differences in hybridisation between the drug-resistant CP 547 population and the drug-sensitive reference clone used, ILTat 1.4. Furthermore, no significant difference in copy numbers of the *Nla* III repeat was detected between CP 547, ILTat 1.4 and another drug sensitive-clone, GUTat 3 1 However, it should be noted that these copy number estimates are relatively inaccurate and an alteration of 10% , equivalent to about 4,000 copies, would not be detected Therefore, small differences in the copy number of the *Nla* III repeats between the drug-resistant and the drug-sensitive populations would probably be difficult to detect due to the limitations in the accuracy of the estimates. The combined results obtained from drug-sensitivity profiles, sequence analysis, transcriptional analysis, and determination of the copy number of *Nla* III repeats indicates that the *Nla* III repeat does not mediate drug resistance. However, in view of the suggestion that the 6.6 kbp element is composed of a heterogeneous population, it would be more appropriate to say that the 6.6 kbp elements containing only the *Nla* III repeats do not appear to mediate drug resistance. Since the 'higher' extrachromosomal

elements have not been analysed in detail, their possible involvement in mediating drug resistance cannot be ruled out

Although the 6 6 kbp element does not appear to mediate drug resistance, the maintenance of the element on application of 55 mg/kg diminazene aceturate indicates that it is generated or amplified as a direct consequence of drug pressure The response of the 6 6 kbp element to drug pressure may also be a general response of the CP 547 cell line, or even all *T. b. brucei* populations, to other external stresses The element may be an inherent or integral property which the population evolved to ensure its continued existence in a hostile environment As a follow up in this investigation, it would be useful to determine whether application of methotrexate-pressure generates such elements in trypanosomes It would also be useful to determine whether application of diminazene pressure would produce extrachromosomal elements in ILTat 1 4 or GUTat 3 1 Furthermore, it would useful to determine the drug resistance profiles of the other trypanosome isolates found containing similar extrachromosomal elements If all these isolates are resistant, then there may be a real correlation between the presence of the elements and drug resistance However, if even one of these isolates is drug sensitive, then there is no correlation and the elements must be produced in response to as yet unknown factors

To date, no data have been published on the molecular basis of resistance to diminazene in trypanosomes As shown in Table 8, the continuous passage of the parental CP 547 population in the absence of diminazene pressure led to the selection of clones displaying reduced levels of resistance in comparison to the parental CP 547 stock These results show that the resistance to diminazene is not stable and contradict previous results by Kaminsky and Zweygarth (1989b) and Gray and Roberts (1971a) who claimed that resistance to diminazene is stable The reason for the discrepancy is most likely due to fact that Gray and Roberts and Kaminsky and Zweygarth used parasite stocks However, in the light of the instability of resistance to diminazene aceturate that has been observed in the CP 547 clones, conclusions about stability of diminazene aceturate in

studies by Gray and Roberts and Kaminsky and Zweygarth require clarification. Quantitative rather than qualitative assays for drug resistance are required before definitive statements about the stability of resistance can be made (Peregrine, 1994).

4.11 Are the 'higher' hybridising elements multimers of the 6.6 kbp element?

Digestion of total CP 547 DNA with *Bal* 31 exonuclease, and the subsequent hybridisation of the resolved DNA with the *Nla* III repeat probe revealed that there are additional 'higher' hybridising elements containing *Nla* III repeats than is apparent from just resolving of undigested DNA on standard agarose gels and hybridising with the repeat Some of the 'higher' hybridising elements appeared to be resistant to *Bal* 31 exonuclease digestion, while others appeared sensitive. This indicated that the 'higher' hybridising elements contain a mixture of circular and linear molecules.

Both the 6 6 kbp and the 'higher' hybridising elements were resistant to digestion by a series of restriction enzymes; *Nla* III was the only enzyme identified that digested them to completion Therefore, it is likely that the 'higher' extrachromosomal elements also contain tandem arrays of the *Nla* III repeat similar to the 6.6 kbp element. *Apa*L I digestion of one of the preparations of total CP 547 DNA resulted in the concurrent loss of signal intensity of both the 6.6 kbp element and one of the 'higher' hybridising elements, the 13 kbp element. The likely interpretation of these results is that in that population of CP 547, only some species of the 6.6 kbp and the 13 kbp element have an *Apa*L I site. These results indicate that the 6.6 kbp element and the 13 kbp element probably have a similar structural organisation. Furthermore, the hybridisation signals of the 6.6 kbp, the 13 kbp, and the elements between 20 kbp and > 40 kbp display a level of periodicity in their sizes which may be expected for a multimeric series of extrachromosomal DNAs with size increments roughly corresponding to 6.6 kbp. These observations suggest that the sequence organisation of some of the 'higher' hybridising elements is likely to be similar to that of the 6.6 kbp element. Thus, at least some of the

Nla III repeat-containing elements may represent multimeric forms of the 6.6 kbp element

From experiments performed to verify the chemical nature of the 6.6 kbp element, it is apparent that not all the 'higher' hybridising elements are similar. Results of such an experiment showed that some of the 'higher' hybridising elements are resistant to digestion by RNase A and RNace 'It', but fully sensitive to digestion by RNase H, suggesting that these element may be DNA-RNA hybrid molecules. As is discussed in later sections, DNA-RNA hybrid molecules may represent intermediate products of reverse transcriptase.

Since the *Nla* III repeats are present on chromosomes of both the drug-resistant and the drug-sensitive populations, it is most likely that the extrachromosomal elements hybridising to the *Nla* III repeat are of chromosomal origin. The production of these extrachromosomal elements appears to be a directed phenomenon because if it had been random one would expect them to also contain some other highly repetitive *T. b. brucei* sequences in addition to the *Nla* III repeats. However, high stringency hybridisations using some of the well characterised highly repetitive *T. b. brucei* sequences such as the 177 bp Alu-satellite repeat, TRS1 ingi, minicircle DNA, the telomere consensus sequence, the miniexon sequence and RIME as hybridisation probes revealed that the 6.6 kbp element and its 'higher' forms do not contain any of these sequences. Irrespective of the true mechanism(s) of their production, there is a defined size range within which the 6.6 kbp element and the 'higher' hybridising elements appear. The 6.6 kbp element appears to be the lower size limit as no elements smaller than this size were detected. This indicates that these molecules are generated by a precise, non-random process. This observation is suggestive that the 6.6 kbp element and the 'higher' hybridising elements may play an important role in the genetic make-up of the parasites.

The instabilities displayed by the 6.6 kbp element are indicative of the occurrence of an active biological phenomenon involving genetic rearrangements. Thus, extrachromosomal elements displaying such instabilities could represent by-products or intermediates of events such as deletions, gene amplifications or transpositions.

4.12 Classification of the *Nla* III repeat family

Two general classes of repetitive elements occur in eukaryotic genomes. The first class contain clustered repeats, while the second is arranged as dispersed repetitive sequences.

The clustered repeat family may be composed of simple sequences with repeats no more than 6 bp long, repeated as many as 10^6-10^7 times per genome (Gall *et al.*, 1973), while others can be up to hundreds of base pairs long (Brutlag, 1980; Miklos, 1985). Because of their sequence content, many of these repeated sequence clusters have buoyant densities in caesium chloride density gradients that are different from the majority of an organism's DNA, and can be observed as 'satellites' to the main DNA band, and hence their name Satellite DNA is mainly found at the ends and centres of chromosomes However, more recently, the term 'satellite DNA' has encompassed any tandemly repeated sequence (Willard and Wayne, 1987). 'Minisatellites' for instance, are similar to satellites but are shorter while microsatellites are even shorter comprising only a few tandem repeats of a simple sequence that are dispersed through the genome of eukaryotes (Jeffreys *et al*, 1985). The number of tandem repeats at a given locus in the DNA from one individual may contain a short minisatellite while another may contain a long minisatellite. Polymorphisms in minisatellites can be used to make an individual specific DNA fingerprint that can be used to follow chromosomes in genetic analysis (Jeffreys *et al.*, 1985). Furthermore, defects in minisatellites and microsatellites are now associated with some cancers (Nowak, 1994)

Trypanosomes are the only known members of protozoans and lower eukaryotes to contain satellite DNA sequences (Sloof *et al.*, 1983). There are some features of the *Nla* III family which would indicate that it is similar to the 177 bp Alu-satellite repeat family present in the subgenus *trypanozoon*:

1) From the digestion profile of the 6.6 kbp and 'higher' hybridising elements it was apparent that these elements appear to be made up of very large numbers of tandem

arrays of the *Nla* III repeat These long tandem arrays of simple repeat units are typical of satellite DNA (Brutlag, 1980, Miklos, 1985). The previously identified *T. b. brucei* satellite DNA referred to as the 177 bp Alu-satellite repeat family, is made up of long tandem arrays of a 177 bp repeat (Sloof *et al.*, 1983). This repeat contains two copies a 19 bp sequence and several additional copies of part of this sequence. Similarly, the *Nla* III family exists as long tandem arrays of the 108 bp *Nla* III repeat which as previously mentioned is also made up of different sub-repeats. The satellites of higher eukaryotes are believed to have arisen by duplication of a simpler sequence (Brutlag, 1980; Miklos, 1985) Therefore, it is possible that the *Nla* III may also have arisen from multiple events bringing the different sub-repeats together to form one active unit. However, unlike the *Nla* III repeat family in which some of the 108 bp units are interspersed with a 5 bp sequence, there is no published data indicating the presence of interspersed sequences within the 177 bp Alu-satellite DNAs

2) Satellite DNA is highly species specific, whereby even closely related species appear to have completely distinct satellite DNAs (Brutlag, 1980; Dover, 1986). Like the 177 bp Alu-satellite repeat DNA, the *Nla* III repeat family is also specific for the subgenus *Trypanozoon* This is apparent from its lack of hybridisation, even at low stringency, to DNA generated from *T. congolense* and *T. vivax*.

3) Most satellite DNAs show a limited sequence difference between repeats within one organism Restriction enzyme digestion of most satellites shows that either they are completely resistant to specific enzymes or they are digested into very regular patterns of DNA fragment sizes (Brutlag, 1980; Miklos, 1985). After digestion of total *T. b. brucei* DNA with *Alu* I, 99% of the 177 bp Alu-satellite repeat can be isolated in a fraction containing oligomeric series of DNA fragments whose lengths are multiples of the monomer unit (Sloof *et al.*, 1983). Similarly, for the *Nla* III repeat family, when genomic *T. b. brucei* DNA is digested with *Nla* III, almost 100% of the family can be isolated in a fraction containing oligomeric series of DNA fragments whose lengths are multiples of the 113 bp monomer unit.

4) In higher eukaryotes, satellite DNAs tend to be restricted to specific loci such as centromeres, telomeres and nucleolar organising regions (Warburton and Willard, 1992). It is now recognised that satellite DNA in primates is organised in higher-order, chromosome-specific repeat units which may form arrays as large as several thousand kb (Willard and Wayne, 1987) Each chromosome in primates is characterised by a multimeric higher-order repeat unit whose organisation and nucleotide sequence is specific for the particular chromosome. The chromosome-specific subsets are in general greater than 95% identical (Warburton and Willard, 1992). Similarly, the *Nla* III repeat family is also dispersed in the genome of *T. b. brucei* in chromosomes of all size-classes, in addition to being present on extrachromosomal elements. However, whether these elements have a particular arrangement which identifies as being derived from specific chromosomes can only be established if the sequence organisation of tandem arrays between these elements, between chromosomes and within chromosomes is elucidated. Unlike the *Nla* III repeat, the 177 bp Alu-satellite repeat DNA sequence is localised specifically in the minichromosome size-class (van der Ploeg *et al.*, 1984b).

The aforementioned four characteristics displayed by the *Nla* III repeat family also apply to the highly repeated satellite DNAs of animals and plants (Miklos, 1985). However, some features of the *Nla* III family separate it from the *T. b. brucei* satellite DNA family and those of higher eukaryotes. Like most satellite DNAs, the 177 bp Alu-satellite repeat is easily visualised as a classical satellite peak well separated from the main band of nuclear DNA in buoyant density gradient, since its G+C content is 29 mol% (Sloof *et al.*, 1983). However, the *Nla* III repeat family does not separate after equilibrium centrifugation of total CP 547 DNA in a CsCl density gradient since its G+C content is 47.8 mol%. Furthermore, unlike the 177 bp Alu-satellite repeat DNA family and most other satellite DNAs, the *Nla* III repeat family is transcriptionally active, resulting in abundant heterogeneous transcripts. Nontranscribed, highly repetitive DNA, in a functional sense is 'dead' (Miklos, 1985). The transcription of satellite DNA in amphibian (Diaz *et al.*, 1981) and cultured mouse and rat cells (Sealy *et al.*, 1981) is considered as transcription by 'default' because it is yet to be proven that these transcripts

are not just adventitious products of a small cluster of repetitive DNA sequences that have been transposed to transcriptionally active regions of the genome, or due to transcription initiation sequences that have been transposed into a tandem satellite family.

The second family of repetitive sequences in eukaryotes represents the dispersed repetitive sequences Dispersed repetitive sequence are characteristic of most higher eukaryotic genomes. However, no single unifying description of their arrangement can be applied to them (Goldberg *et al*, 1975; Davidson *et al.*, 1985). Generally, dispersed repetitive DNA sequences are considered to be mobile DNA (Young, 1979) and are believed to have arisen either by DNA-mediated or by RNA-mediated transposition (Potter *et al*, 1979, Young, 1979). Because of lack of a structural similarity among the different dispersed repetitive sequences, it is clear that there is more than one mechanism for their generation Among these are DNA transposition events, gene duplications, chromosomal rearrangements and the integration of viral genomes. Although many of the above mechanisms may play a role in the formation and evolution of repetitive DNA sequences, the process of retroposition appears to be the dominant mechanism in forming the major mammalian repetitive DNA families (Weiner *et al.*, 1986). Each type of mobile element occurs many times in the genome. Thus, the elements constitute families of dispersed repeated sequences which foster further genomic rearrangements because they provide sites for nonallelic, homologous recombinations.

Several different mobile DNA elements are distinguishable in eukaryotes (Singer and Berg, 1991). Classifications primarily based on common structural features have led to the identification of three main families (Singer and Berg, 1991): the transposable elements, the retrotransposons and the retroposons. The former two elements transpose because they have the coding capacity for enzymes such as reverse transcriptase and integrase, required for their transposition. The retroposons include a very diverse group of sequences that, notably, do not necessarily themselves encode functions required for transposition. The evidence that retroposons are mobile is largely indirect. It includes: (1) the dispersal of family members to different genomic loci, (2) the presence of target site duplications, and (3) the existence of alleles that differ by the presence or absence of

a retroposon Although the mechanism of their transposition is unproven, they appear to transpose by what is believed to be a passive process, through an RNA intermediate (Singer and Berg, 1991)

Several features of the *Nla* III repeat family indicate that it may be able to undergo transposition Firstly, it is repetitive and dispersed throughout the *T. b. brucei* genome in all chromosome size-classes Secondly, members of the family are present on extrachromosomal elements, dispersed repetitive DNAs present on extrachromosomal elements are believed to represent intermediates in transposition (Krolewski *et al.*, 1984; Rogers, 1985). Therefore, the extrachromosomal elements comprised of *Nla* III repeats may also represent intermediates in transposition. Thirdly, the alteration in location of some of the *Nla* III repeats, a typical property of mobile DNAs (Wichman *et al.*, 1992). This was evident in the chromosome profiles of GUTat 3.1, with reference to Figure 29, whereby additional minichromosome-sized molecules that hybridised to the *Nla* III repeat sequence were revealed in only two out of four GUTat 3.1 subclones. This indicates that the *Nla* III repeat sequence may be rapidly translocating, although it is possible that such chromosomal rearrangements occur as a result of VSG-gene switching (Myler *et al* , 1988) Furthermore, it is also possible that the minichromosome-sized curious molecules in GUTat 3 1 represents extrachromosomal elements. Fourthly, one cDNA clone revealed two complete and one partial copy of the *Nla* III repeat flanked by direct repeats, which could represent a target-site duplication.

Since sequence analysis of the *Nla* III repeat revealed no open reading frames, the dispersal of the *Nla* III repeat family may be similar to the dispersal of retroposons, which, in a similar manner to the *Nla* III repeats, do not encode enzymes required for transposition (Weiner *et al.*, 1986; Singer and Berg, 1991).

Singer and Berg (1991) and Weiner *et al.* (1986) have further classified retroposons into two subfamilies containing firstly, the processed polypeptide pseudogenes, and secondly, the processed RNA pseudogenes. The general structural features of some of these retroposon families are: (1) the absence of terminally redundant

sequences, either direct or inverted, (2) the presence at the 3' end of one strand of a segment that is rich in A residues, and (3) variable sized target site duplications.

The best characterised sequences of retroposons and retrotransposons families are the short and long interspersed DNAs (SINEs and LINEs, respectively, in the terminology of Singer [1982]) Although these sequences represent the major mammalian repetitive DNA families (Singer and Berg, 1991), very little is known about their functions The *Alu* family of SINEs, found within the genomes of primates, is the best studied of all The *Alu* family represents one of the most successful classes of mobile elements, having amplified to copy numbers which approximate 5-6% of the primate genome by mass (Deininger and Daniels, 1986). *Alu* elements are distributed, on average, every 5 kbp within the human genome, but also exist as clusters within specific genomic loci (Lee *et al* , 1984, Flemington *et al.*, 1987). They are retropseudogenes that are ancestrally derived from 7SL RNA (Ullu and Tschudi, 1984), while other families of SINEs are derived from tRNAs (Daniel and Deininger, 1985). *Alu* family members contain an internal RNA polymerase III promoter, and their mobilisation is thought to occur via a polymerase III-derived transcript and retroposition (Schmid *et al.*, 1989). LINE family members appear to be directly diverged from a RNA polymerase II-transcribed protein coding gene of unknown function (Singer and Skowronski, 1985; Singer and Berg, 1991).

The existence of retroposition in trypanosomes was implied by the discovery of the ribosomal mobile element (RIME), which was found inserted into a rRNA gene in one *T. b. brucei* stock (Hasan *et al.*, 1984). The TRS1/ingi element (Kimmel *et al.*, 1987; Murphy *et al* , 1987) identified in *T. b. brucei* shares all the features described for mammalian LINEs, the most important, having the potential to encode reverse transcriptase. It was found that these trypanosome LINEs are flanked by separate different halves of RIME (Kimmel *et al.*, 1987). However, to date, no SINEs have been reported in trypanosomes.

Dispersal of genetic information from one locus to another is known to be either RNA-mediated or DNA-mediated. Therefore, the question arises as to how the *Nla* III

repeat sequence is dispersed in the *T. b. brucei* genome From restriction enzyme analysis it is evident that in the *T. b. brucei* genome, the *Nla* III repeats are found as long tandem arrays Therefore, dispersal would most likely involve mobilisation of clusters of these repeats The smallest array of such clusters appears to be the 6.6 kbp extrachromosomal element Therefore, the 6 6 kbp extrachromosomal element may be the progenitor or intermediate for transposition and dispersal of the repeat clusters. From the transcriptional studies, the smallest *Nla* III transcript is approximately 300 bp, which could represent the smallest *Nla* III repeat dispersal unit.

Tandem, highly repetitive DNA sequences appear only rarely to undergo translocations to other parts of the genome (Miklos, 1985). On the contrary, the *Nla* III repeat family may be undergoing active translocation, as evident by PFGE of GUTat 3.1 subclones and the presence of direct repeats flanking some *Nla* III repeat units. It has been postulated that in a dispersed family, clusters of repeats may represent an early stage in the growth of the dispersed repeat family (Anderson *et al.*, 1981). Anderson *et al.* (1981) also proposed that a DNA-mediated mechanism operates in which individual member units of the repeat family originate by the sequential excision from the tandem arrays of the repeat, the excised fragments are subsequently dispersed by a variety of mechanisms The DNA-mediated mechanisms involve transposition, chromosomal translocation between non-homologous chromosomes and unequal crossing over. It is therefore possible that as in 2034 repeat family in *Strongylocentrolus purpuratus* species of sea urchins (Anderson *et al*, 1981), the *Nla* III repeat family is also at an early stage in dispersal, whereby its repeats are transposed. The 2034 repeat family is believed to represent a sequence family that is still dispersing, because, in addition to dispersed clusters of the repeat in tandem, there are also a few singly dispersed members. Clustered arrangements of the *Alu* elements have also been found within specific genomic loci of the human genome Thus, the dispersed individual members of the family are believed by some investigators to have originated and dispersed from such clusters (Calabretta *et al.*, 1981).

Over the past few years, RNA-mediated transposition has emerged as a major evolutionary force contributing to continuous sequence duplication, dispersion, and rearrangements that maintain the remarkable fluidity of eukaryotic genomes (Rogers, 1985, Singer and Berg, 1991) In light of the dispersal of repetitive sequences such as the primate *Alu* repeats, the *Nla* III repeats may also be dispersed by retroposition. Since at least some of the *Nla* III repeats are transcribed, its retroposition is theoretically possible according to demonstrations that any mRNA can be inserted as cDNA into the genome if reverse transcription is active in a cell (Lania *et al*, 1987).

There are several features of the *Nla* III repeat family that place it amongst retroposon-like sequences

1) Evidence of possible target-site duplications. Insertion of nonviral retroposons usually generates a target site duplication of varying direct repeats (Soares *et al* , 1985). There is evidence showing that some *Nla* III repeat units are flanked by direct repeats In the cDNA pCA 15, two complete and one partial *Nla* III repeat are flanked by a 5 bp direct repeat, this suggests that these *Nla* III repeats may have transposed as one unit It has in fact been noted that retroposons have a tendency to integrate either adjacent to one another, or directly into each others' tails (Rogers, 1985), giving rise to clusters of members which can subsequently be mobilised as a single unit Such an explanation could explain the 5 bp direct repeat flanking two complete and one partial copy of the *Nla* III repeat among tandem arrays of the *Nla* III repeat units It is however worth noting that non-viral retroposons which do not generate a target site duplication have also been identified (Deninson and Weiner, 1982, Bernstein *et al* , 1983) Similarly, almost 20 to 3 °o of chromosomal mammalian *Alu* repeats are not flanked by direct repeats (Krolewski *et al* , 1982)

2) Retroposons usually have a 3' terminal poly (A) tract However, since the ends of the genomic copies of the *Nla* III repeat units were not characterised in the present study, it is not known whether this is also true for the *Nla* III repeat units There are, however, indications that the *Nla* III repeat units in the genome are in the vicinity of A rich sequences This is evident from the screening of the CP 547 genomic library, whereby

some genomic clones hybridising to the *Nla* III repeat also hybridised to oligo d(T). However, just as the presence of direct repeats is not obligatory for retroposition, most of the small nuclear (sn) RNA pseudogenes and all characterised artiodactyl SINEs that are retroposons do not have poly(A) tails (Weiner *et al*, 1986; Singer and Berg, 1991).

3) Non-viral retroposons such as the mammalian *Alu*-repeat are known to be co-transcribed along with adjacent chromosomal sequences, and thereby mobilise them along side (Roger, 1985) If the *Nla* III repeats are a family of non-viral retroposon-like molecules, then co-transcription of adjacent chromosomal sequences from different genomic locations would contribute to heterogeneous transposition intermediates. This could offer an explanation for the size and sequence heterogeneity observed in the 6.6 kbp element

4) A transpositionally active retroposon should be transcriptionally active. Thus, the extent of amplification of a retroposon correlates with its transcriptional competence (Daniels and Deininger, 1991) Both RNA polymerase II and III transcripts are known to give rise to retroposons and retrotransposons, named class II and class III, respectively (Weiner *et al*, 1986, Singer and Berg, 1991) In general, processed pseudogenes of snRNA genes are class II genes with external promoters whereas almost all small cytoplasmic RNA genes are class III with internal promoter elements (Singer and Berg, 1991) *Alu* family members are actively transcribed *in vitro* (Ullu and Weiner, 1985). However, studies on transposed 7SL pseudogenes, close sequence relatives of *Alu*, show that the internal promoter is not sufficient for *in vivo* transcription (Weiner *et al*., 1986). In the case of 7SL, the authentic gene requires upstream control sequences The *Nla* III repeat family is transcriptionally active, but presently it is not known which class of RNA polymerase is responsible for its transcription However, since the *Nla* III repeat sequence is a small sequence lacking an open reading frame, it could be transcribed by RNA polymerase III Most identified trypanosome mRNAs transcribed by RNA polymerase II have the SL sequence at their 5' ends, independent of their coding sequence (Parsons *et al*, 1984, Walder *et al*., 1986). The absence of the SL sequence from the 5'

region of the cDNAs of the *Nla* III repeats suggests that their transcription may be mediated by RNA polymerase III

If the *Nla* III repeat is a retropson-like sequence, then some of its transcriptional properties can be explained on the basis of transcription of other retroposons. The observed heterogeneous-sized transcripts may be a result of tandem repeats of varying sizes from different genomic loci Alternatively, transcripts from different loci could be hybrids with varying numbers of *Nla* III repeat units, together with sequences unique to each locus Heterogeneous transcripts have been observed as a transcriptional feature of some non-viral retroposons which are active transpositionally (Matera *et al.*, 1990). Unlike other RNA polymerase III-dependent genes, transposed *Alu* elements lack $d(T)_4$ termination signals and therefore rely on 3' flanking DNA for transcription termination. As a result, *Alu* sequences interspersed at different loci generate heterogeneous transcripts whose lengths and 3' sequences are variable and dependent on the position and efficiency of the termination signals encountered by RNA polymerase III (Jelinek and Schmid 1982, Maraia *et al*, 1992) Transcripts of B1, a murine homolog of the human *Alu*, are also heterogeneous in size This heterogeneity is mainly due to the variable 3' sequences as B1 also lacks transcription termination signals (Weiner *et al*, 1986, Maraia, 1991)

The *Nla* III repeat is actively transcribed in the long slender and procyclic forms of *T b. brucei* These forms represent the proliferating and actively-dividing life-stages of trypanosomes Retroposition is believed to be pronounced in such actively dividing cells (Carey *et al*, 1986, White *et al*, 1989) As for the *Nla* III repeats, the murine SINEs, B1 and B2, are known to be transcribed actively in early stages of development and states of proliferation and transformation (White *et al.*, 1989; 1990). This kind of transcriptional control appears to apply not only to particular families of SINEs, but also to many, and conceivably all genes transcribed by RNA polymerase III (White *et al.*, 1990) This gives rise to the question as to whether the *Nla* III repeat sequences are abundant because they are transcribed during the stage in development when retroposition is occurring more efficiently. Alternatively, the *Nla* III repeat and its

uncharacterised adjacent sequences contain sequences which make it a particularly efficient substrate for retroposition The transcription of both TRS1 and RIME is also developmentally regulated TRS1 is preferentially transcribed in bloodstream forms of the parasite (Murphy *et al*, 1987), but significantly less so in actively dividing procyclic forms

4.13 The 6.6 kbp extrachromosomal element and the 'higher' hybridising elements as intermediates of genomic rearrangements analogous to small polydisperse circular DNA

Many eukaryotic cells, derived from both tissue and culture, have been shown to contain small polydisperse circular DNAs (spc DNA) (Krolewski *et al.*, 1982, 1984; Stanfield and Hellinski, 1984, Schindler and Rush, 1985). These molecules range from a few hundred to a few thousand base-pairs per cell and are present at levels from less than 1 to more than 100 copies Such molecules are believed to be derived from, or related to, chromosomal DNA, whereby their buoyant density is virtually indistinguishable from that of total genomic DNA A variety of mechanisms have been proposed to explain the generation of spc DNAs since they are known to contain heterogeneous sequences ranging from unique to repetitive chromosomal sequences (Riabowol *et al.*, 1985). Among the repetitive chromosomal elements identified are the short interspersed *Alu* retroposons (Krolewski *et al*, 1982), the long interspersed nucleotide sequence known as *Kpn*-I (Schindler and Rush, 1985) and the tandemly repeated 172 bp sequence known as α-satellite (Bertelsen *et al*, 1982) Presently, the biological function(s) and significance of the spc DNAs remains mostly speculative. However, the most optimistic speculation is that they are intermediates and/or by products of gene rearrangements (including transpositions) Such rearrangements are thought to occur commonly in such apparently disparate phenomena as development (Lewis *et al.*, 1982), ageing (Shmookler Reis *et al.*, 1983) and cancer (Calabretta *et al.*, 1982). Certain species of spc DNAs are characterised by a relatively reproducible size and structure. These characteristics indicate that precise

mechanisms are responsible for their synthesis, and contrast markedly with the structures of other spc DNAs that probably arise from more or less random and/or non-reproducible events It is therefore possible that the 6 6 kbp extrachromosomal element, and some of the 'higher' hybridising elements, are analogous to the *Alu*-containing spc DNAs. Since there is as yet no unifying characteristic feature of the family of spc DNAs, the hypothesis that the *Nla* III repeat containing-extrachromosomal elements are analogues of the spc DNAs of higher eukaryotes is based on similarities between the features displayed by the spc DNAs and the 6 6 kbp element. These include: 1) The 6.6 kbp element and some of the 'higher' hybridising elements are circular DNA molecules containing the highly repetitive *Nla* III sequences, which are also present in chromosomal DNA sequences, suggesting a chromosomal origin of these elements. Furthermore, since the 6 6 kbp element and the 'higher' hybridising elements cannot be distinguished as a distinct band on CsCl gradients, this suggests that these elements, in a similar manner to spc DNAs, may have a buoyant density which is indistinguishable from that of chromosomal DNA

2) The 6 6 kbp element displays size and sequence heterogeneity, as discussed earlier, in which the *Nla* III repeat-containing elements may represent one particular species with a distinct size range Similarly, the spc DNA exist as heterogeneous populations However, within these populations, there are distinct-sized species of the spc DNAs, such as the *Alu* 1-containing and the *Kpn* 1-containing subspecies of discrete size classes (Krolewski *et al*, 1982, Schindler and Rush, 1985).

What, if any, is the significance of the 6 6 kbp element and the 'higher' hybridising elements? The instability of copy number, sequence and size heterogeneity of the 6 6 kbp and the larger elements suggests that these elements, like the spc DNAs of higher eukaryotes, are possibly intermediates or by-products (or both) of genetic events such as transposition and extrachromosomal amplification.

The presence of potentially mobile dispersed genetic elements such as *Alu* retroposons (Jelinek and Schmid, 1982) on some discrete-sized spc DNAs has led to speculations that, like some other eukaryotic elements, *Alu* repeats may have the ability to

move from one chromosomal site to another by means of an intermediate molecule such as the spc DNAs (Krolewski and Rush, 1984). Although more than one model can explain the generation of circular molecules, there has been some speculation that genetic elements such as *Alu*-containing spc DNAs are generated by reverse transcription of *Alu* specific RNA (Jagdeeswaran *et al.*, 1981; Sharp, 1983). It is also believed that such *Alu*-containing circles disperse further by recombining with other *Alu* sequences elsewhere in the genome. Although only limited, there is some evidence to suggest that at least some of the *Nla* III repeat-containing extrachromosomal elements may also be products of reverse transcription-mediated transposition, when CP 547 total genomic DNA was subjected to digestion with RNase H, it became apparent on Southern blots that two regions among the 'higher' hybridising elements were sensitive to RNAse H digestion. Although these molecules were not characterised further, their sensitivity to RNAse H indicates that they may represent DNA-RNA hybrids. In *Drosophila melanogaster* DNA-RNA complexes have been detected in cells grown *in vitro* (Flavell and Ish-Horowicz, 1981, Arkhipova *et al*, 1984). The properties of such complexes are consistent with those expected from the model of a reverse transcription pathway of transposition and amplification of dispersed genetic elements.

On theoretical grounds, several authors have proposed a relationship between the processes of gene amplification and the generation of spc DNAs (Lambert *et al.*, 1983; Fujimoto *et al*, 1985). In *Leishmania* spp (Garvey and Santi, 1986) and human cells (Maurer *et al*, 1987) extrachromosomal elements containing sequences encoding dihydrofolate reductase have been interpreted as polydisperse circles representing intermediates in gene amplification. In a given cell type, the amount of spc DNA varies in response to certain influences. Thus, protein synthesis inhibitors such as puromycin or cycloheximide greatly increase spc DNA levels in all cells studied (Sunnerhagen *et al.*, 1986, 1989). This response to inhibitors is very similar to the changes in the 6.6 kbp element that are observed when trypanosomes are exposed to diminazene aceturate. In view of these similarities, the 6.6 kbp and the 'higher' hybridising extrachromosomal elements are probably analogous to spc DNA and therefore represent intermediates of

gene amplification generated by reverse transcription. Selective drug pressure might act by enhancing this process; as mentioned earlier, such a phenomenon could be tested by applying drug pressure on drug sensitive ILTat 1.4 and GUTat 3.1 clones.

The newly identified *Nla* III repeat family appears to be the most abundant set of dispersed repetitive sequences in the *T. b. brucei* genome, contributing up to approximately 5% of the nuclear DNA. The copy number and dispersion of members of this family make it unlikely that they are without influence on the organisation of the genome. The function, if any, of highly repetitive DNAs in eukaryotic genomes has been difficult to define. It is generally believed that most of it represents the so-called 'selfish DNA', contributing nothing to the organisms whose genomes they occupy (Doolittle and Sapienza, 1980; Orgel and Crick, 1980). Their transposition does, however, have mutagenic effects, disrupting genes into which they are transposed, or altering the expression of genes in their proximity. They may therefore be of some advantage in the evolution of the host organism (Rogers, 1985). One obvious influence of the dispersed sequences would be to provide many dispersed sites of homology, where pairing and recombination can occur. One may therefore expect translocations or chromosome fusions to occur as a result of recombinations between elements located on different chromosomes or between circular elements and chromosomal loci. Such a situation can be found in *Drosophila* in recombinations between the long interspersed 'Doc' elements (Schneuwly *et al.*, 1987). In tumour cells, amplification of specific genes, such as DHFR, have been implicated in a variety of adaptive responses to environmental stress (Schimke, 1984). However, concomitant with the amplification, DNA sequences acquire an increased mobility in the genome with extrachromosomal intermediates transposing and translocating to other chromosomal segments (Stark and Wahl, 1984). Events of these kinds have been shown to occur in the smaller chromosomes of *T. b. brucei* (van der Ploeg and Cornelissen, 1984; Bernards *et al.*, 1986) and may play significant roles in the processes of antigenic variation and evolution of VSG-gene repertoires (Bishop, 1983; van der Ploeg and Cornelissen, 1984; Pays, 1988a, b). In these instances, DNA rearrangements are instrumental in producing changes in gene expression. However, in

trypanosomes these events differ from the transposition mentioned above in that they are non-random.

Estimates of the frequency of putative *Nla* III transpositions under different conditions would be informative in establishing the degree of plasticity as a result of this repeat sequence. Differences found in chromosome distribution of the *Nla* III hybridising sequences between the GUTat 3 1 subclones could help identify newly transposed *Nla* III-containing elements, confirm the occurrence of transposition, and give estimates on the frequency of transposition

Retroposon sequences have been considered rare outside mammals and only there have they become predominant dispersed repetitive sequences. Therefore, if the *Nla* III repeats represent a retroposon-like family, it would be unusual that trypanosomes contain them A possible explanation for their presence is that such sequences were acquired prior to divergence early in evolution, but were mostly lost from the genomes of most lower eukaryotes except trypanosomes. It is also possible that these sequences were acquired after their divergence, for example, through gene transfer of viral genomes. The identification of RIME, TRS1 and SLACS, and now the *Nla* III repeat family, suggests that transposable elements may be more widely distributed among trypanosome genomes than has previously been apparent The presence of these elements suggests that a considerable proportion of the trypanosome genome is represented by "selfish DNA" sequences This is somewhat surprising when one considers that the genome size of trypanosomes is only six times that of *E. coli* It is also surprising since trypanosomes undergo a complex life cycle, involving both an insect and mammalian hosts. A considerable proportion of the coding capacity is probably employed for survival in the hostile environment of the mammalian bloodstream. However, it should be remembered that the genome of trypanosomes is continuously undergoing rearrangements, particularly with regards to the VSG genes It is therefore probable that rather than selfishly self-propagating, the *Nla* III repeats and the extrachromosomal elements containing *Nla* III are indeed functional in the trypanosome genome. In this respect, it is possible that there is an enhancement of retroposition by selective drug pressure, with the

various elements constituting intermediates. The *Nla* III repeats and probably sequences associated with it thus, via extrachromosomal intermediates, may be actively dispersing in the *T. b brucei* genome, generating mutagenic effects such as switching on new genes, destroying of control elements and promoting plasticity by insertions of the *Nla* III repeats The insertion or excision of such elements has probably confirmed a selective advantage and drug pressure probably acts to preserve such an insertion or excision. Therefore, such events could have resulted in generation of populations with novel properties, including the multidrug-resistant phenotype of the CP 547 population.

As discussed earlier, the *Nla* III repeat family has some features typical of satellite DNAs It is therefore important that these concluding remarks consider this characteristic in relation to *T. b. brucei*. In complex eukaryotes, as mentioned earlier, satellite DNA is localised mainly in the heterochromatin in apparently genetically inert and compact parts of the chromosomes, at both centromeric and telomeric regions (Warburton and Willard, 1992) Simple protists like trypanosomes have a primitive mitotic apparatus; the chromosomes do not condense in any phase of the mitotic cycle (Vickerman and Preston, 1970), therefore making it difficult to use microscopy to obtain reliable information on the chromosomal location of specific DNA sequences (van der Ploeg *et al*, 1984b). Homology searches have revealed the highest homology of the *Nla* III repeat to part of a subtelomeric sequence that is specific for some minichromosomes of *T. b. brucei*. Although the significance of this sequence is unclear, Weiden *et al.* (1991) have shown that the organisation of A + T and G + C rich blocks in subtelomeres of some *T. b. brucei* minichromosomes is similar to some subtelomeric domains of the distantly related *P. berghei* (Dore *et al*, 1990) and the X and Y elements in *Saccharomyces cerevisiae* (Horowitz and Haber, 1985) The X and Y elements in yeast cells can function as autonomous replicating sequences and have also been identified as mobile genetic elements, with a circular episomal intermediate form (Horowitz and Haber, 1985). The *P. berghei* subtelomeric elements are also mobile and can be transferred to subtelomeres of other chromosomes by an as yet unknown mechanism (Dore *et al.*, 1990). It is therefore possible that the *Nla* III repeat family represents subtelomeres of some

trypanosome chromosomes, and that the circular *Nla* III repeat-containing elements represent the episomal subtelomeres found in yeast cells. In *T. b. brucei* minichromosomes the 177 bp Alu repeat was the only satellite sequence to be identified (Weiden *et al*, 1991), leading to the suggestion that it may be a candidate for a trypanosome centromere. However, the *Nla* III repeat family, represented as tandem repeats, is a more reasonable candidate for the centromere since, unlike the 177 bp repeats, it is present on chromosomes of all size-classes, although not on all chromosomes. Its presence on circular elements may represent a mobile form of a centromere, providing 'services' to chromosomes where these tandem arrays are absent The increase in copy number of the 6 6 kbp element in response to selective drug pressure may therefore be a mechanism used by the parasite to increase its number of mobile centromeres This may allow for more effective parasite replication when under stress e g , drug pressure

Reviewing the two major classes of repetitive DNA has revealed that the *Nla* III repeat family cannot be assigned completely to either one of the two above-mentioned general families, the *Nla* III repeat family displays some features which are characteristic of dispersed repetitive families and some features which are characteristic of satellite DNA families

The biological significance of the *Nla* III repeat and the 6.6 kbp and the higher hybridising elements is unclear at present Nevertheless, in view of the possible transposition of *Nla* III repeats, and in view of the instability of the 6 6 kbp extrachromosomal element containing the *Nla* III repeats, the *Nla* III repeat family reported here may provide a unique system in which one can study the mechanism of chromosomal instability If a unique marker could be inserted into the extrachromosomal DNA, the element may be used as a convenient marker in analysing the dynamic state of chromosomal and extrachromosomal elements Such analyses could contribute to the better understanding of the plasticity of the parasite genome. Taking into consideration that VSG expression occurs, in part, through genomic rearrangements (Roth *et al.*, 1991), such analysis may consequently give a better insight into regulation of gene expression.

The repetitive DNA sequence may also be used as a diagnostic probe specific for *Trypanozoon*. In conclusion however, from the identification and the partial characterisation of the 6 6 kbp and the higher hybridising elements many questions have arisen (i) What is the structure of the active portion of this repeat that may be transposed, and what is the complete structure of the extrachromosomal elements? (ii) How may these structures have been constituted? (iii) Is this repeat sequence still proliferating in the *Trypanozoon* genome? If yes, how may it be doing so? (iv) What RNA polymerase is responsible for it transcription? (v) Can this repeat shed light on the organisation and functions of 'Selfish' DNA in higher eukaryotes? The answers to these may shed light on the function(s) of this repeat sequence and the extrachromosomal elements containing them

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