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Antigenic Variation and Telomere Structure in Trypanosoma brucei

Jorge Luis Munoz Jordan

Antigenic Variation and Telomere Structure in *Trypanosoma brucei*

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by

Jorge Luis Muñoz Jordán

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To Pati

Todo es hermoso, todo es constante,

Todo es música y color.

Y todo, como el diamante,

antes que luz es carbón.

All is beautiful, all is constant,

all is music and color.

And all, like in a diamond,

before splendor is coal.

José Martí

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List of Abbreviations:

AMT	4' aminomethyltrioxalen
BF	Bloodstream form (of *T. brucei*)
cDNA	Complementary DNA (synthesized from RNA)
DC	Doxycycline
DSB	Double-strand break
dsDNA	Double-stranded DNA
EDTA	Ethylenediaminetetraacetate
EGTA	Ethylene glycol-bis (ß-aminoethylether)-N,N,N',N'-tetraacetate
EM	Electron microscopy
EMSA	Electrophoresis mobility shift assay
ES	Expression site
ESAG	Expression site associated gene
FISH	Fluorescence *in situ* hybridization
FITC	Fluorescein isothiocyanate
5FOA	5-fluoro-orotic acid
GAD	GAL4 activation domain
GBD	GAL4 binding domain
GPI	Glycosylphosphatidylinositol
GPIPLC	GPI-specific phospholipase C
IF	Immunofluorescence
J	ß-D-glucosyl-hydroxymethyluracil
mRNA	Messenger RNA
NAP1	Nucleosome assembly protein 1
Nap1p	Yeast nucleosome assembly protein 1
PARP	Procyclic acidic repetitive protein (procyclin)
PF	Procyclic form (insect midgut form of *T. brucei*)
P_{CYC1}	CYC1 promoter
P_{GAL1}	GAL1 promoter
P_{T7}	T7 promoter

RAGE	Rotating agarose gel electrophoresis
rRNA	Ribosomal RNA
SDS	Sodium dodecylsulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SSB	*E. coli* single-stranded-binding protein
ssDNA	Single-stranded DNA
TDB	Trypanosome dilution buffer
TET	Tetracycline
TET_R	Repressor of the *E. coli* TET operator
T7RNAP	T7 RNA polymerase
TPE	Telomere position effect
TRF	Telomeric-repeat-binding factor
VSG	Variant surface glycoprotein
VSG	VSG gene
X-Gal	5-bromo-4-chloro-3-indoyl-ß-D-galactoside

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ABSTRACT

Trypanosoma brucei survives immune attack in its mammalian hosts by periodically changing the variant surface glycoprotein (VSG). From the ~1000 VSG genes (*VSGs*) scattered throughout the genome, a few are located at telomere loci called expression sites (ESs) and only one is expressed at any time, suggesting a stringent regulatory mechanism. Here, it is demonstrated that trypanosomes engineered to express two VSGs simultaneously from the same ES can multiply in cultures and infect animals normally. Mechanisms involved in the control of ES-associated gene expression are thought to involve some kind of telomere position effect. Telomeres typically contain simple DNA repeats (TTAGGG in animals and trypanosomatids) that are the binding sites for telomeric proteins, and end in 3' protrusions known as overhangs. In mammals, telomeres fold back in structures known as t-loops, in which the overhang is intercalated in the duplex DNA. The length of the TTAGGG tracts was measured (~ 15 kb) and the presence of 3' overhangs was established in trypanosome telomeric DNA. EM studies revealed the presence of t-loops in trypanosome telomeric DNA and at both ends of minichromosomes. Therefore, trypanosome telomeres have conserved features and probably contain proteins with conserved functions. Binding of the human protein TRF1 to telomeres in procyclic trypanosomes caused transient cell cycle arrest and abrupt telomere shortening, indicating that TRF1 is interfering with a presumed trypanosome telomere complex. Trypanosome protein extracts did not exhibit a detectable TRF1-like DNA-binding activity. A yeast one-hybrid screen was developed to detect trypanosome proteins with the ability to bind TTAGGG. However, this screen was interfered by the presence of an endogenous TTAGGG-binding activity and by the trypanosome nucleosome assembly protein NAP1.

INTRODUCTION

The order Kinetoplastida embraces unicellular organisms with one flagellum and a compact mitochondrial genome: the kinetoplast. All of these protozoa are parasitic; but their lineage probably originated more than 500 million years ago, before the arrival of their metazoan hosts (Stevens and Gibson, 1999). Within the family *Trypanosomatidae*, various species of *Leishmania* and *Trypanosoma* are transmitted to mammals by insect vectors, and have been studied for their medical importance. The genus *Trypanosoma*, subgenus *Trypanozoon*, is of particular interest. The subspecies *T. (T.) brucei brucei* (Plimmer & Bradford, 1899), one of the causative agents of the disease Ngana in African cattle, is used widely in laboratories as a model for the study of *T. brucei gambiense* (Duttun, 1902) and *T. brucei rhodesiense* (Stephens & Fantham, 1910), the causative agents of human Sleeping Sickness. Animal and human trypanosomiasis are endemic to central and sub-Saharan Africa, the area of distribution of *Glossina* species of flies commonly known as the tsetse, which transmit the parasite to men and animals. But 'African' trypanosomiasis also occurs in South America and Asia, through subspecies of trypanosomes that escaped the restraints of tsetse-specific transmission (Davila et al., 1999; Pholpark et al., 1999; Tuntasuvan et al., 2000).

The biology of Trypanosoma brucei

T. brucei is an extracellular parasite that populates the vascular system and interstitial tissue spaces. The bloodstream form (BF) trypomastigote (Fig. 1) has an elongated shape and is approximately 20 μm long. A cytoskeleton, composed of microtubules and

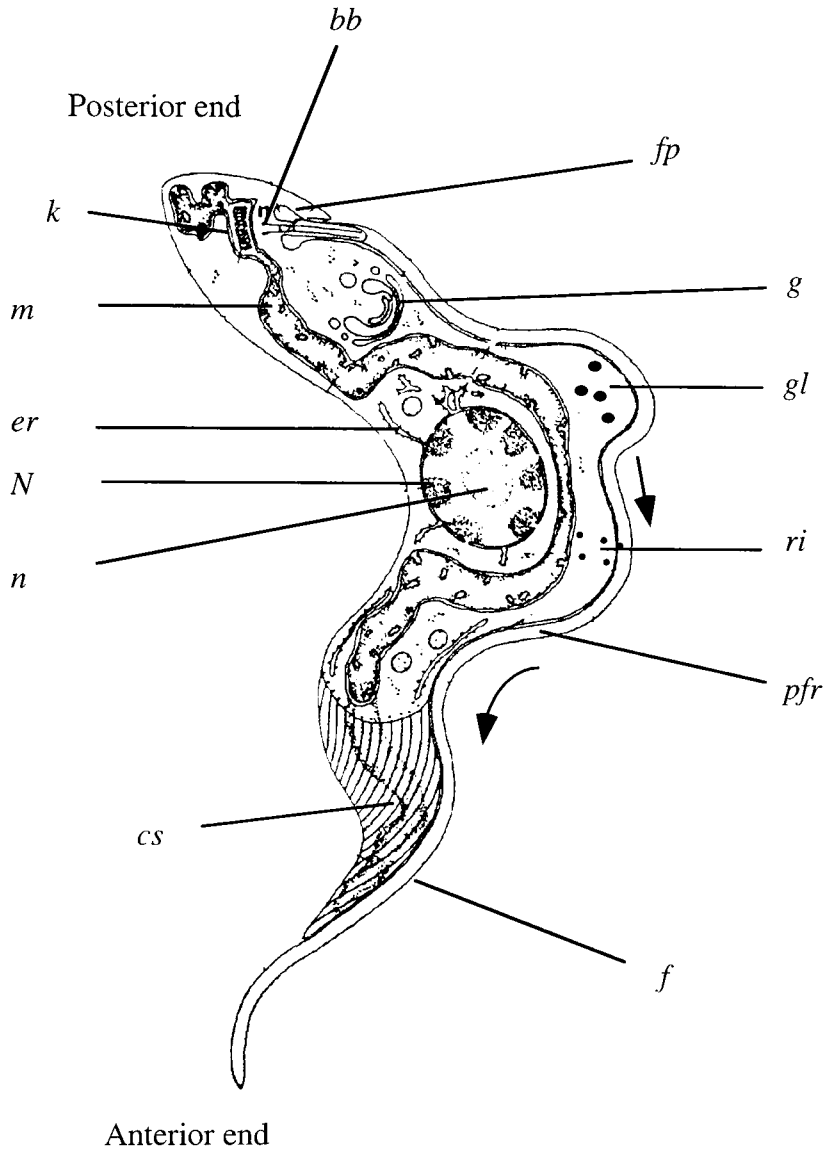


Fig. 1. Diagram of the ultrastructure of *Trypanosoma brucei* bloodstream trypomastigote. Lettering: *bb*, basal body; *cs*, cytoscheleton; *er*, endoplasmic reticulum; *f*, flagellum; *fp*, flagellar pocket; *g*, Golgi apparatus; *gl*, glycosome; *k*, kinetoplast; *N*, nucleus; *n*, nucleolus; *m*, mitochondrium; *pfr*, paraflagellar rod. Anterior and posterior ends are defined by the direction of motion (indicated by the arrows). Based on Vickerman, 1969.

connected to the inner plasma membrane, maintains cell shape during differentiation and cell division (Gull, 1999). The flagellum is composed of nine pairs of microtubules and a structure named the paraflagellar rod, composed of two major proteins (69 and 72 kDa) (Bastin et al., 1996). It emerges from the basal body and through the flagellar pocket. The flagellar pocket is an invagination of the plasma membrane at the flagellar attachment zone. All endocytosis and exocytosis in trypanosomes appear to occur in the flagellar pocket (Overath et al., 1997).

Nuclear and mitochondrial division are coordinated (Fig. 2). The G1 cell has a single nucleus, kinetoplast and flagellum. Kinetoplast division precedes nuclear division, and the basal body appears to mediate segregation of the kinetoplast (Robinson and Gull, 1991). The first indication of cell division is the division of the basal bodies, concomitant with initiation of nuclear S phase. The daughter flagellum elongates from the newly matured basal body during nuclear S phase and at the beginning of kinetoplast G2. The basal bodies separate before the new flagellum reaches its complete length. This is followed by the initiation of mitosis and formation of the spindle within the nucleus. Finally, cleavage occurs between the two basal bodies and cytokinesis is completed (Woodward and Gull, 1990).

The parasite undergoes a major developmental program during its life cycle (Fig. 3). When BF parasites are taken up by the feeding tsetse, parasites pass to the insect midgut and differentiate to procyclic form (PF) trypanosomes. The morphologies of PF and BF trypanosomes do not differ much, except for the position of the kinetoplast relative to the nucleus, which is closer in the PF than in the BF. In their metabolism, however, BF and PF differ substantially.

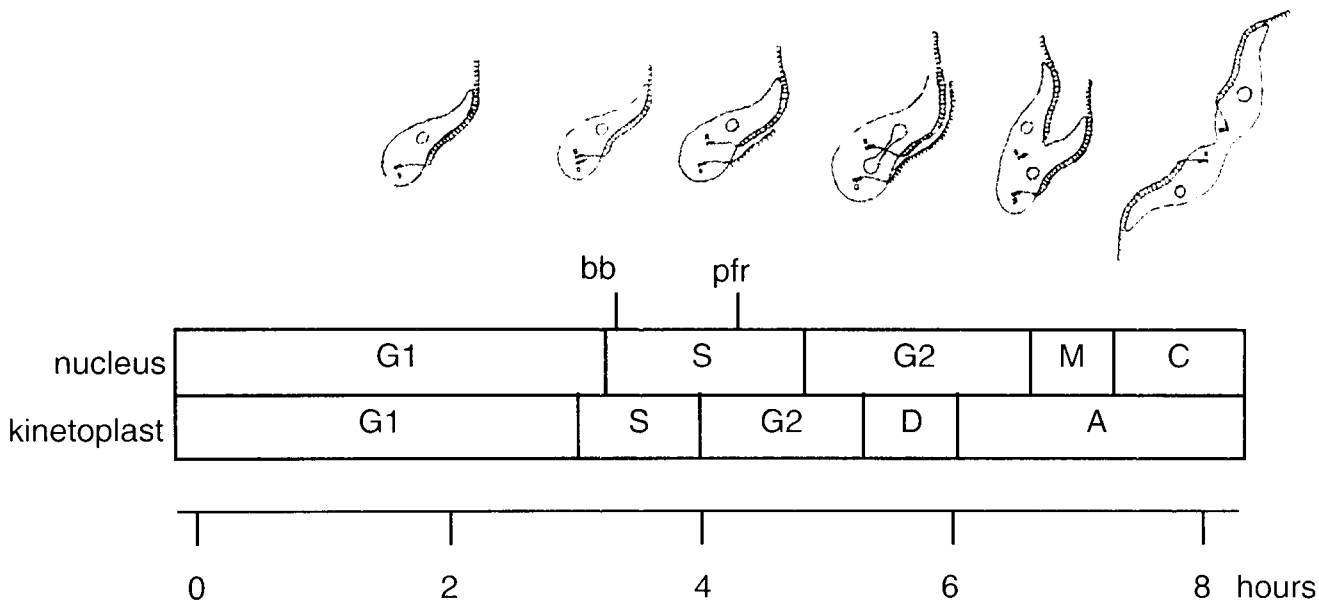


Fig. 2. Schematic representation of *T. brucei* cell cycle. The calculated duration and sequence of the periods G1, S, G2, M or D and C or A are indicated for nuclear and kinetoplast divisions. D indicates division of the kinetoplast, and A is the period during which the dividing cell contains two distinct kinetoplasts. The division of flagellar basal body (bb) and the paraflagellar rod (pfr) is indicated (Woodward and Gull, 1990).

The PF mitochondrion is capable of oxidative phosphorylation, and the parasite expresses a cell-surface protein of unknown function named procyclic acidic repetitive protein (PARP) or procyclin (Roditi et al., 1989). In addition, PF trypanosomes possess a membrane-bound trans-sialidase that transfers sialic acid residues to procyclin (Engstler et al., 1993). The PF differentiates into epimastigote and later into the non-dividing VSG-bearing metacyclic form in the salivary glands of the tsetse. Metacyclic trypanosomes are infectious and transmitted to the mammalian bloodstream during a blood meal and express the variant surface glycoprotein (VSG). Metacyclics quickly differentiate into BF trypanosomes, which undergo an elaborate process of antigenic variation by expressing different VSGs (discussed below). The BF lacks most of the Krebs cycle and oxidative phosphorylation enzymes in the mitochondrion, whose genome is dispensable. Instead, BF trypanosomes utilize glucose from the blood at a high rate, and most of the enzymes necessary for glycolysis are contained in specialized organelles called glycosomes (Opperdoes and Borst, 1977); for review see (Michels et al., 2000). The major cell surface proteins, VSGs and procyclin, are linked to the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor, whose composition varies according to the antigenic variant and life cycle stages (Ferguson, 1999).

Antigenic variation in T. brucei

As an extracellular blood parasite, *T. brucei* faces continuous immune attack. An intricate process of antigenic variation keeps the parasite population one step ahead of the hosts' immune defenses. Ten million identical VSG molecules crowd the entire surface of the cell membrane, making up 7-10 % of the total cellular protein (Vickerman, 1969; Cross, 1975),

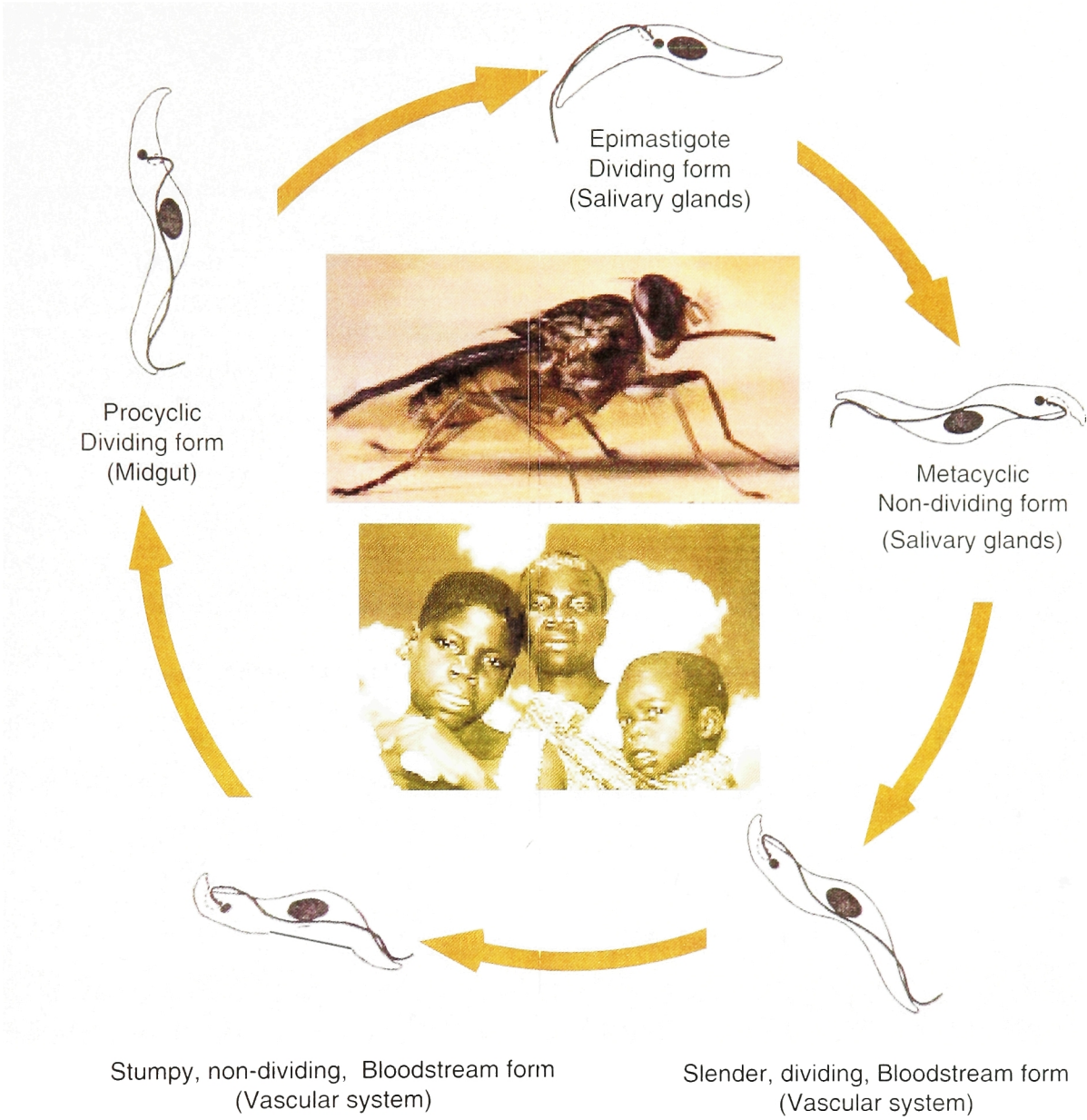


Fig. 3. The life cycle of *T. brucei*. The bloodstream form trypomastigote is found mainly in the vascular system of the mammalian host. In the bloodstream, the dividing slender-form trypanosomes progressively become stumpy and cease division. During the blood meal, bloodstream trypanosomes pass through the proboscis of the tsetse. Procyclic trypanosomes migrate to the midgut, where they multiply and attach to endothelial cells. Parasites migrate periodically to the proboscis and differentiate into epimastigotes, with the flagellum emerging anterior to the nucleus. Epimastigotes divide and attach to the walls of the salivary gland of the tsetse by their flagellum. Epimastigotes develop into the non-dividing, infectious Metacyclic trypanosomes, with the flagellum emerging from the posterior end. The cycle is completed when metacyclics are transmitted to the mammalian host and quickly differentiate into bloodstream-form trypomastigotes.

and apparently mask other underlying surface proteins (Ziegelbauer and Overath, 1992). VSGs are highly immunogenic, and as a result most of the parasites are cleared from the bloodstream. However, replacement of the VSG coat occurs at a variable rate (about 1 in 10^2-10^7 trypanosomes) (Myler et al., 1985; Lamont et al., 1986; Turner and Barry, 1989), resulting in a few parasites unrecognized by the immune system that re-populate the blood. This process results in the continuous, intermittent waves of parasitemia, characteristic of African trypanosomiasis.

VSGs are GPI-anchored on the cell surface, where they form homodimers. Each monomer consists of two domains separated by a hinge region (Johnson and Cross, 1979; Carrington et al., 1991). The crystal structure of two amino-terminal domains (about two thirds of the molecule) reveals that, in spite of considerable variation in amino acid sequence, the three-dimensional structure of each VSG is very similar (Carrington et al., 1991). The carboxyl-terminal domains are more conserved in sequence, consisting of one or two tightly folded subdomains, and can be grouped into three major sequence classes. The most conserved sequence tract in the VSG amino acid sequence is the carboxyl-terminal precursor peptide for GPI anchoring (cleaved prior to GPI addition) (Cross, 1990), which at the same time is substantially different from the one present in procyclin (Mowatt and Clayton, 1989).

Although only one VSG gene (*VSG*) encodes the entire antigenic coat, *VSG*s comprise a family of approximately 1000 genes (van der Ploeg et al., 1982), providing a large repertoire of *VSG*s to be transcribed one at a time. The expressed *VSG* is always located at the end of a polycistronically transcribed operon or expression site (ES) composed of

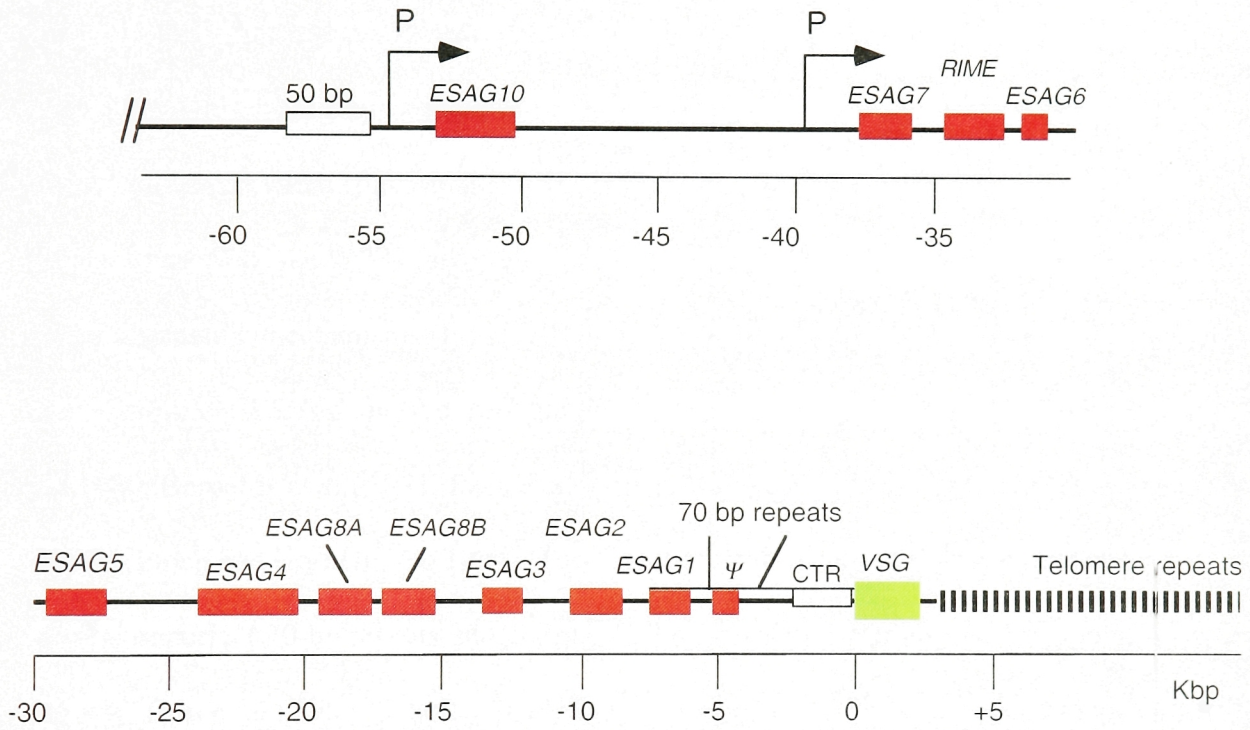


Fig. 4. Anatomy of an expression site (ES). A number of expression site associated genes (*ESAGs*) and a *VSG* gene are distributed through a region of 40-50 kb. *ESAG1* is a 36- kDa transmembrane protein; *ESAG2* codes for a 50-kDa GPI-anchored cell-surface glycoprotein; *ESAG3* is a 43-kDa secreted or membrane glycoprotein; *ESAG4* is a 139 kDa flagellum-associated surface receptor with cytoplasmic adenylate cyclase domain; *ESAG5* is a 46 kDa soluble (hydrophobic) protein; *ESAG6&7* form a 100 kDa transferrin receptor complex; *ESAG8* is a 70 kDa nucleolar protein with zinc ring and leucine-rich repeat domains; *ESAG10* is a transmembrane folate/biopterin transporter, and *ESAG11* is a 43-kDa GPI-anchored cell-surface glycoprotein. The expression site is located proximal to the telomeric TTAGGG repeats. Two promoters (P), as occurs in about 50% of ESs, are depicted (arrows) in this combined scheme with information from different ESs. The 70 bp repeats are located upstream of the *VSG*. The co-transposed region (CTR) is indicated. Based on a composite of different ESs, by G. Cross.

several ES-associated genes (*ESAGs*) and one *VSG* (Fig. 4) (Cully et al., 1985; Johnson et al., 1987; Kooter et al., 1987; Pays et al., 1989; Crozatier et al., 1990). The transcribed *VSG* is the most distal of the genes in the ESs and always located proximal to the end of the chromosome (de Lange and Borst, 1982). There are about 20 ESs, each consisting of a 40 to 60-kb polycistronic transcription unit. Only one ES-linked *VSG* is transcribed while all the others remain silent (Pays et al., 1981; Michels et al., 1984). In PF trypanosomes, ESs are not transcribed, and *VSGs* are not expressed. Antigenic variation is achieved by two major alternative mechanisms (Fig. 5). The transcribed *VSG* can be substituted by a copy of one of the silent *VSGs* dispersed throughout the genome (gene conversion) (Hoeijmakers et al., 1980; Bernards et al., 1981; Pays et al., 1981; Majiwa et al., 1982; Michels et al., 1983; van der Ploeg and Cornelissen, 1984). The 5' end of the transposed segment contains several imperfect 70-bp repeats and a sequence known as the co-transposed region next to the *VSG* (Liu et al., 1983; Scholler et al., 1988; Aline et al., 1989). The second mode of antigenic variation involves coordinated activation/inactivation of different ESs (transcriptional switch) (Michels et al., 1984) (Fig. 5). Therefore, the changing of the VSG coat in *T. brucei* is mediated by a combination of transcriptional control and genetic rearrangements occurring at telomeric loci.

Telomere position effect (TPE) and ES-transcription regulation

The ends of chromosomes, telomeres, are the site for the expression of *ESAGs* and *VSGs* in *T. brucei*. Considerable effort has been dedicated to understand how *T. brucei* alternates transcription of *VSGs* in the BF, but represses transcription of all *VSGs* in the PF. The

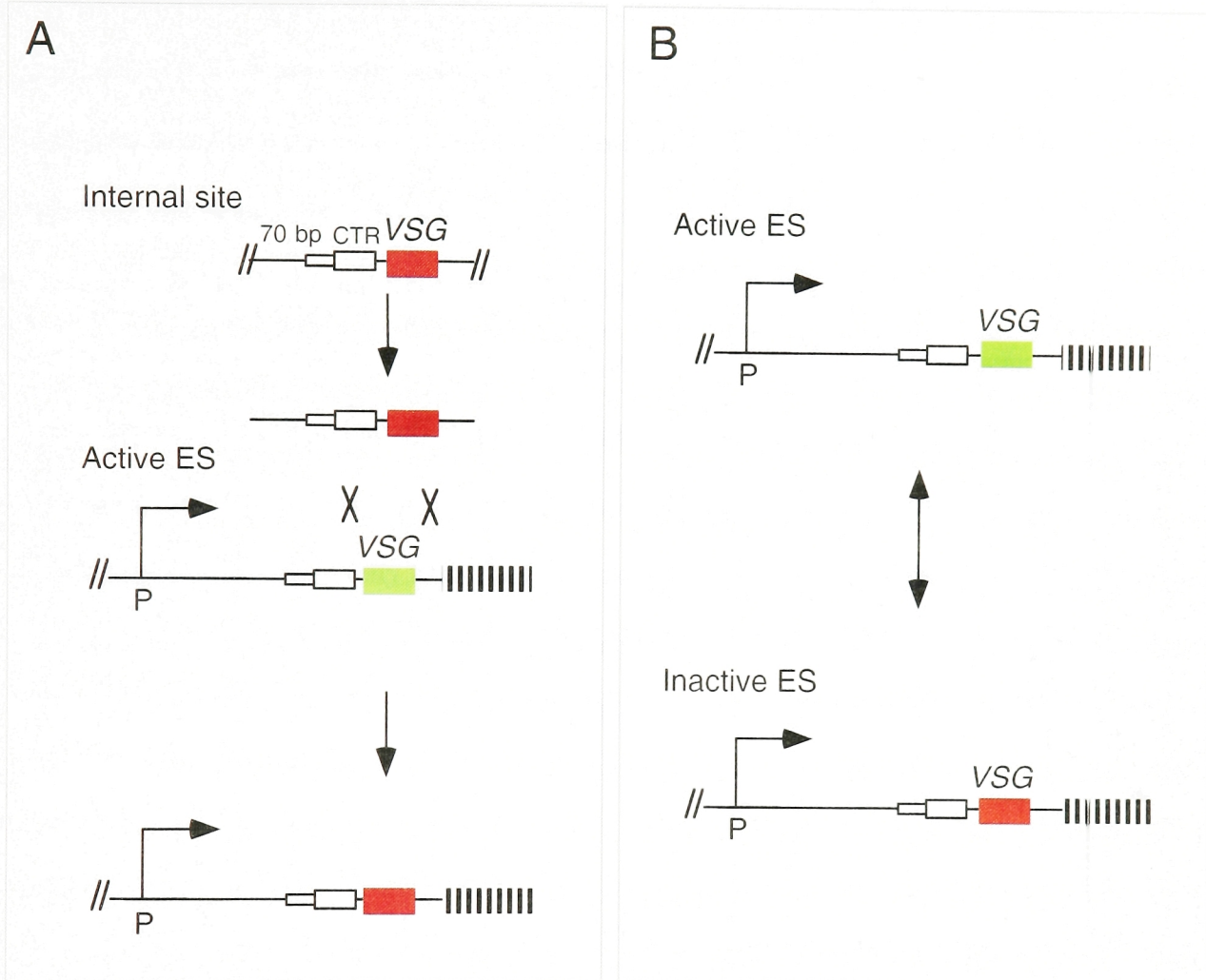


Fig. 5. Schematic representation of the two main mechanisms of antigenic switch in *T. brucei*. (A) Gene conversion switch: a *VSG* gene is duplicated and transposed by a process of recombination into an active expression site, replacing the previously transcribed *VSG*. (B) In-situ transcriptional switch: an actively transcribed ES becomes inactive while a previous inactive ES is turned into an active expression site. Either process results in antigenic variation.

evidence indicates the occurrence of some kind of TPE in trypanosomes (Gottschling et al., 1990), and points to telomeres as possible regulators of antigenic variation. The actively transcribed VSG is also more susceptible to DNAse I cleavage than the inactive VSG, an initial evidence of differences in chromatin structure between active and inactive ESs (Greaves and Borst, 1987). By various genetic manipulations, the ES-promoter can be replaced with the ribosomal RNA (rRNA) promoter, which is normally constitutively active but is regulated when it replaces the ES promoter in the BF. This promoter replacement in BF caused no effect in transcription of the active ES, and no change of the switching rates (Rudenko et al., 1995). In contrast, in PF all ESs are normally silenced and transcription is repressed only from the ES promoter, and not from the rRNA promoter. Because transcription initiation is promoter-independent in the BF but not in the PF, a developmentally regulated position effect has been proposed (Horn and Cross, 1995; Horn and Cross, 1997).

Transcription from the T7 promoter replacing the ES promoter in PF trypanosomes expressing T7 polymerase was, however, strongly repressed. Moreover, a form of transcriptional silencing operates through the entire ES in PF and transcription is more strongly repressed than in BF inactive ESs. This indicates a developmentally regulated process of chromatin remodeling (Navarro et al., 1999). In addition, repression of promoters inserted in both directions into the inactive ES in BF is more pronounced closer to the telomere (Horn and Cross, 1997). In agreement with this finding, basal transcription of *ESAG6* and *ESAG7* occurs in the inactive ES at levels that even compensate the loss of function of the allele in an active ES (Ansorge et al., 1999; Vanhamme et al., 2000).

However, *VSGs* located further downstream, very proximal to telomeres, are only transcribed from the active ES and never from inactive ESs (Vanhamme et al., 2000).

Further evidence implicates an epigenetic mechanism in transcriptional switching. Replacement of the active ES promoter by the T7 promoter prompts a rapid transcriptional switch in BF, presumably because the T7 promoter does not sustain transcription of the most distal genes of the ES such as the *VSG* (Navarro et al., 1999). Therefore, compromising an active ES leads to immediate activation of another ES. This agrees with a previous finding showing that insertions into an active ES can increase the incidence of ES switches (Davies et al., 1997). Furthermore, insertion of drug resistance genes next to two separate ES promoters caused rapid switches between the two ESs (Chaves et al., 1999). These two alternating ESs localize together towards the center of the nucleus, and separate from the rest of the telomeres that normally cluster around the periphery (Chung et al., 1990; Chaves et al., 1999).

Thus, it appears that in the BF, all inactive ESs seem to be at a state in which they can be easily activated and destabilization or incomplete transcription of the active ES can trigger a rapid switch. Basal transcription is attenuated at some early point in inactive ESs before the *VSG*, such that some ESAGs are transcribed at basal levels from various ESs, but *VSG* transcripts are synthesized only from the active ES.

The structure and function of telomeres

The first indication that telomeres are essential for accurate segregation of chromosomes dates to several decades ago, when it was found that chromosome breaks induce cell division arrest and that cells that escaped this arrest had repaired their chromosomes without losing their telomeres, though other parts of the chromosomes could be missing (Muller, 1938). Broken chromosome ends were found to fuse to one another, generating concatenated chromosomes with two centromeres (dicentric chromosomes) and consequential chromosome breakage and fusion during meiosis, indicating that the ends of chromosomes had to be protected (McClintock, 1941). The requirement of RNA primers to complete DNA synthesis, yielding incomplete replication at the very end of chromosomes, could potentially result in loss of DNA at every cell division (Watson, 1972). Telomeres therefore overcome the potential problems of generating a DNA-damage response in the cell, fusion of chromosomes, and shortening of the DNA ends by lack of replication. The molecular characterization of telomeres has propelled our understanding of these functions in recent years.

Telomeres were first characterized in the ciliate protozoan *Tetrahymena thermophila* and found to contain tracts of 5'-TTGGGG-3' repeats (Blackburn and Gall, 1978). Similar telomeric sequences have been found in other eukaryotes. The sequence 5'-TTAGGG-3' is perhaps the most widely distributed of telomeric repeats, present in trypanosomes as well as in several fungal species and in all vertebrates (Blackburn and Challoner, 1984; van der Ploeg et al., 1984; Moyzis et al., 1988; Meyne et al., 1989; Coleman et al., 1993; Javerzat

et al., 1993). Some telomeric repeats are considerably more heterogeneous, such as the ones in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. The length of these telomeric tracts can range from a few hundred bp in yeast and *Tetrahymena* to 10-100 kb in mice (Shampay et al., 1984; Larson et al., 1987; Shampay and Blackburn, 1988; Kipling and Cooke, 1990; Zijlmans et al., 1997; Hiraoka et al., 1998). Human and trypanosome telomeres are 4 to 30 kb (van der Ploeg et al., 1984; Moyzis et al., 1988; Cross et al., 1989). In the macronuclear DNA of ciliates, the G-rich strand protrudes 12 to 16 bp (Klobutcher et al., 1981). This characteristic of ciliate telomeres, known as the 3' overhangs or G tails, extends to other eukaryotes. In yeast, telomere 3' overhangs are transient and only occur for short periods at the end of S phase when telomeres are replicated (Wellinger et al., 1993; Wellinger et al., 1996). In mammals, G tails are approximately 45-200 bp long (Makarov et al., 1997; McElligott and Wellinger, 1997; Wright et al., 1997). Telomeres must circumvent the possible activation of DNA damage processing normally signaled by exposed single-stranded DNA (Garvik et al., 1995; Lydall and Weinert, 1995).

The fruit fly *Drosophila melanogaster*, rather than having simple telomeric repeats, has telomeres composed of transposable elements (Biessmann et al., 1990; Levis et al., 1993). Some organisms have sequences immediately internal to the telomere repeats with some similarity to *Drosophila* telomeres. In yeast the subtelomeric sequences X and Y' are found in most telomeres and, interestingly, if telomere repeats are lost, Y' elements can take over telomere function (Lundblad and Blackburn, 1993). In various species of trypanosomes, subtelomeric repeats have also been recognized (van der Ploeg et al., 1984; Fu and Barker,

1998; Chiurillo et al., 1999; Freitas-Junior et al., 1999; Chiurillo et al., 2000). In most organisms, the function of sub-telomeric repeats is not known.

The 5' terminus of the newly synthesized DNA strand is not completely extended when the last RNA primer is removed after lagging-strand synthesis (Fig. 6). In the absence of a mechanism to elongate telomeric DNA, this lack of telomere end replication would result in the continuous trimming of the chromosome ends over subsequent rounds of DNA replication (Watson, 1972). The DNA polymerase activity of telomerase (Greider and Blackburn, 1985) is the primary mechanism of telomere replication. Telomerase is composed of an RNA molecule that provides the template for the synthesis of telomere repeats (Greider and Blackburn, 1987; Greider and Blackburn, 1989; Singer and Gottschling, 1994; Blasco et al., 1995; Feng et al., 1995) and a catalytic protein component, EST2 in yeast and TERT in humans, with homology to reverse transcriptases (Counter et al., 1997; Harrington et al., 1997; Lingner et al., 1997). Elimination of telomerase activity in *S. cerevisiae* and *Kluyveromyces lactis*, by disruption of the telomerase RNA gene, causes progressive telomere shortening, decreased cell viability, and activation of alternative mechanisms of telomere maintenance in the surviving population (Singer and Gottschling, 1994; Cohn and Blackburn, 1995; McEachern and Blackburn, 1995). Consistent with the important role of telomerase, progressive telomere shortening has been observed in telomerase-negative human primary fibroblasts (Harley et al., 1990), and the number of cell divisions that these cells undergo correlates with telomere length (Allsopp et al., 1992). Telomerase is also active in the germ line, where telomeres are relatively long, and inactive in most somatic

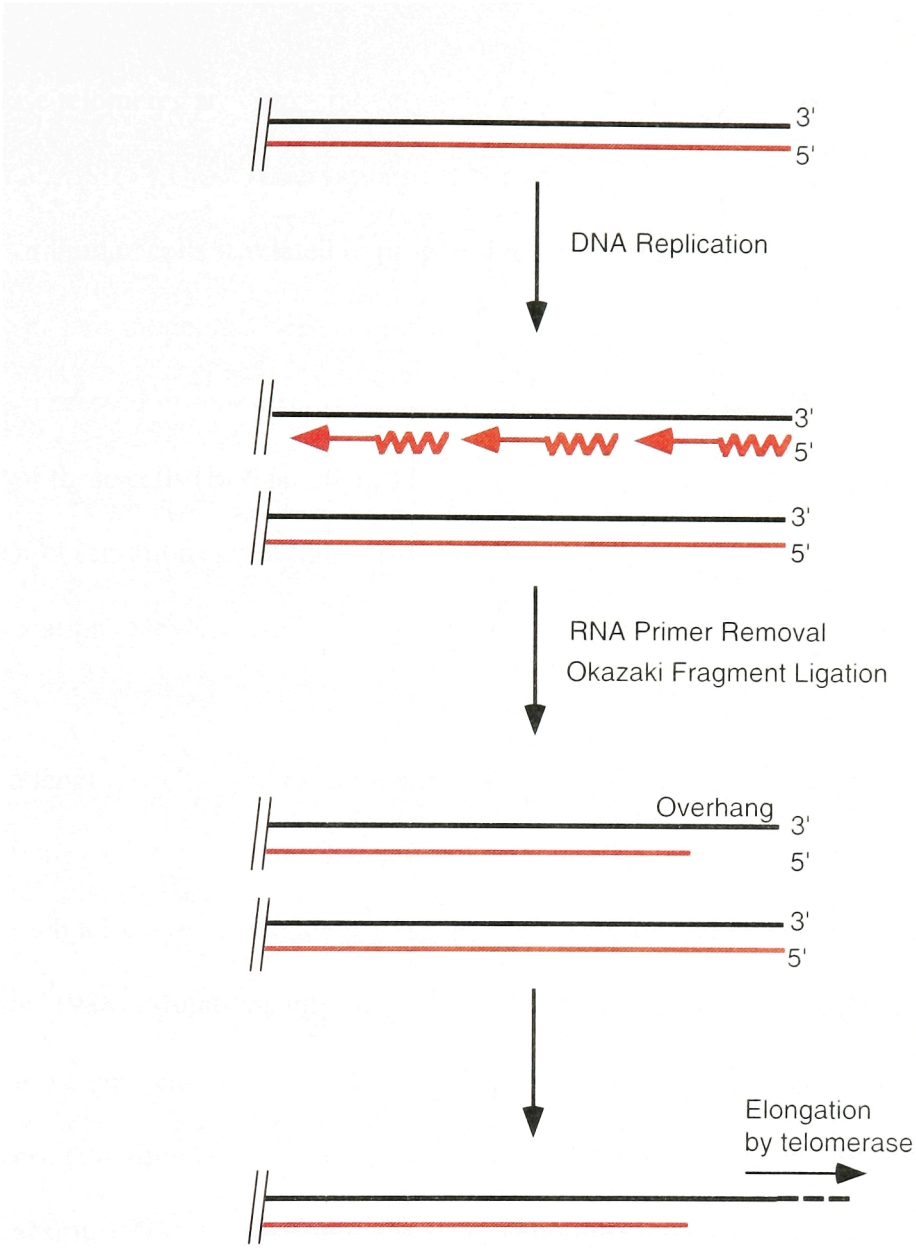


Fig. 6. The dogma of telomeric DNA replication and the end-replication problem. The DNA strands at one end follow two different processes of replication. One strand follows discontinuous replication (lagging-strand synthesis). Upon removal of Okazaki fragments, the 5' end of the newly synthesized strand cannot be completely replicated. The strand that followed continuous replication (leading-strand synthesis) is expected to be completely elongated. Telomerase adds telomeric repeats to the 3' end of the overhang, compensating for the loss of sequence.

cells whose telomeres are comparatively shorter (de Lange et al., 1990; Harley et al., 1990; Hastie et al., 1990). These observations are consistent with the view that the finite doubling capacity of human cells is related to progressive telomere shortening due to lack of telomerase. This model has been confirmed by the finding that stable expression of hTERT, normally repressed in senescent cells, results in the unlimited extension of the dividing capacity of these cells (Bodnar et al., 1998; Vaziri and Benchimol, 1998). In addition, a number of observations point to a correlation between active telomerase and cellular immortalization (Meyerson et al., 1976; Counter et al., 1994; Kim et al., 1994).

Telomere length is regulated by competing factors in equilibrium. Early work in yeast suggested the existence of a mechanism that establishes a telomere length equilibrium, keeping each telomere independently regulated (Walmsley and Petes, 1985; Shampay and Blackburn, 1988). Mutations introduced in the telomerase RNA template are incorporated initially at the very end of the telomere, and subsequently more internally after several generations, revealing concomitant occurrence of both shortening and elongation (McEachern and Blackburn, 1995). Furthermore, the latter experiment, and a similar one in *Tetrahymena* (Yu et al., 1990), showed various defects in cell division and growth. Alterations of the telomeric-DNA sequence therefore results in loss of telomere protection, an initial indication of a telomeric protein complex. Telomere length regulation, then, is a complex mechanism involving not only telomerase, but also proteins that bind specifically to the telomere repeats.

Telomeric-DNA-binding proteins

Several telomere-binding proteins have been described and can be subdivided in two classes. First, some proteins bind to telomere termini by association with the single stranded telomeric DNA repeats in the 3' overhang. A second set of proteins bind to the duplex part of the telomeric DNA. The identification of single-stranded DNA-binding proteins has been considerably hindered by their presumed low abundance in most organisms. One exception to this was *Oxytricha nova*, whose macronuclear genome is fragmented and amplified to generate thousands of copies of gene-size minichromosomes, which are subsequently protected with telomeres. These telomeres are composed of just 20 bp of duplex telomeric repeats and 14 bp of 3' overhang (Klobutcher et al., 1981). Abundance of telomeres in the macronucleus facilitated the identification of a telomeric protein (TP) composed of two subunits (∝-TP and ß-TP) (Gottschling and Zakian, 1986; Hicke et al., 1990; Gray et al., 1991; Price et al., 1992). This protein binds to the single stranded TTTTGGGG 3' overhang present at the telomere ends, forming a high-affinity and very stable complex (Fang et al., 1993). Clearly, *Oxytricha*'s TP has a capping function reflected by its extreme stability and tenacious binding. It remains unclear if analogous activities are present in other organisms apart from ciliates, or if this type of protein arose in ciliates as part of their unique genome.

In yeast, screens for mutants with telomere shortening phenotypes similar to telomerase defects have yielded the identification of *EST1* (ever shorter telomeres 1) as well as the telomerase catalytic subunit *EST2* (mentioned before), *EST3*, and *EST4* (Lendvay et al., 1996). The mutation that allowed the identification of *EST4*, which codes for Cdc13p, also causes telomere shortening and senescence. Cdc13p establishes a direct interaction with the 3' overhang and recruits Est1p, which ultimately results in assembly of telomerase to telomeres (Nugent et al., 1996; Evans and Lundblad, 1999). Cdc13p also protects the

opposite strand (the 5' end) from degradation by recruiting Stn1p, and mutations that disrupt this interaction result in elongated overhangs due to degradation of the C-strand 5' end as well as concomitant activation of DNA damage response (Garvik et al., 1995; Grandin et al., 1996). In addition to the single-strand end binding proteins, telomeric DNA is bound by specific proteins along the length of the double-stranded telomeric repeats. The *S. cerevisiae* multifunctional protein Rap1p (originally described as a Repressor activator protein 1) is both a transcription factor needed for expression of a variety of genes and a transcriptional silencer (Shore and Nasmyth, 1987). Rap1p is also the predominant telomeric-repeat-binding protein in vivo (Conrad et al., 1990). A central DNA-binding domain in Rap1p is composed of two Myb-type repeats (Myb domains) (Henry et al., 1990; Konig et al., 1996). This protein exemplifies the complex mechanisms that regulate telomere length. While mutations in the Myb domain of Rap1p cause telomere shortening (Lustig et al., 1990), suggesting that Rap1p binding to telomeres is needed to stop telomere shortening, truncation or overexpression of the carboxyl-terminal domain, not including the DNA-binding domain, results in telomere elongation, presumably by keeping negative regulators of telomere length away from the telomeres (Conrad et al., 1990; Kyrion et al., 1992; Buck and Shore, 1995). The proteins Rif1p and Rif2p (Rap1p interacting factors 1 and 2) interact with the C terminus of Rap1p and help in maintaining the length of the telomeres (Hardy et al., 1992; Wotton and Shore, 1997). The number of telomere repeats is proportional to the number of Rap1p molecules bound at any time. By substituting telomeric repeats with Gal4 binding sites and expressing Gal4 binding domain (GBD)-Rap1p-fusions, it has been shown that as long as a threshold level of Rap1p molecules is present at telomeres, a mechanism of repression of elongation operates. If the number of Rap1p molecules decreases, this mechanism inclines towards elongation. Presumably Rap1p, through its interaction with other proteins, inhibits telomerase binding or activity (Marcand et al., 1997). The mechanism by which telomerase and Rap1 might be interconnected is unknown. The unusually long telomeres in certain yeast strains are

unstable and undergo frequent large deletions, probably by recombination events. The truncated telomeres can be re-elongated. This instability of telomeres is also seen in *K. lactis* and in trypanosomes, and suggests that the cell can only maintain a certain number of extra telomeric repeats (Bernards et al., 1983; van der Ploeg et al., 1984; McEachern and Blackburn, 1995).

End-binding proteins have not been identified in mammals. Instead, the homodimeric proteins TRF1 and TRF2 (Telomeric Repeat Factors 1 and 2) bind to the duplex part of the telomeres (Chong et al., 1995; Bianchi et al., 1997; Bilaud et al., 1997; Broccoli et al., 1997). TRF1 and TRF2 possess similar architectures, both carrying very similar Myb-related DNA binding motifs at the carboxyl-terminus and a dimerization domain near the amino-terminus. The two proteins differ from each other in that TRF1 amino-terminus is acidic whereas TRF2 has a basic amino-terminus. They form homodimers, never heterodimers (Broccoli et al., 1997). Overexpression of TRF1 and TRF2 in telomerase active human cells leads to shortening of telomeres, and deletion of the Myb domain of TRF1 results in progressive telomere growth (van Steensel and de Lange, 1997; Smogorzewska et al., 2000). These findings indicate their roles as negative regulators of telomere length. In addition, lack of binding of TRF2 to telomeres generates the loss of terminal G-strand overhang and covalent fusion of the chromosome ends as evidenced by formation of chromosome bridges during mitosis and resistance of chromosome ends to *Bal*31 digestion (van Steensel et al., 1998). The exposed telomeres in TRF2 dominant negative mutants activate cell cycle arrest responses and p53/ATM-mediated apoptosis (Karlseder et al., 1999). TRF1 and TRF2 therefore protect the ends of the telomeres by binding to the duplex part of the telomeric repeats.

Electron microscopy (EM) has provided an explanation for how these mechanisms of telomere protection operate. Mammalian telomeric DNA was found in a loop conformation

(t-loop, for telomere loop) at chromosome ends in vivo (Griffith et al., 1999). Binding of single-stranded-binding protein from *E. coli* to the tail-loop junction indicated that there is a segment of single stranded DNA at this site, most likely formed by strand displacement upon invasion of the 3'overhang in the duplex telomeric repeat tract. Therefore, in the t-loop structure, the overhang is inserted into the duplex part of the telomeres (Fig. 7). In accordance with its role in protecting the end of telomeres, TRF2 was found to bind specifically to the junction *in vitro* (Griffith et al., 1999). The binding mode of TRF1 to telomeres is also consistent with the t-loop model as TRF1 can pair duplex telomeric tracts and bend telomeric DNA (Bianchi et al., 1999). Loops have also been identified at the ends of amplified micronuclear chromosomes in the ciliate *Oxytricha fallax* (Murti and Prescott, 1999). These loops were not found in DNA from macronuclei, in which telomeres are presumably too small to form loops, and the single strand binding TP protein complex is bound to the overhang (Murti and Prescott, 1999). In Chapter 2, the presence of t-loops at the ends of *T. brucei* chromosomes is documented, indicating an active mechanism of telomere protection in this organism. Taz1p (Telomere-associated protein in *Schizosaccharomyces pombe*), originally found by a one hybrid screen, binds to yeast telomeric repeats (Cooper et al., 1997) and emerges as a possible TRF ortholog due to their similar Myb domains. In addition to a role in telomere length regulation, Taz1p has a function in meiosis. Loss of Taz1p function results in lack of telomere clustering around the spindle pole body in the premiotic stage (Cooper et al., 1998; Nimmo et al., 1998). A second line of evidence underlining the role of telomeres in *S. pombe* meiosis is that lack of telomeres results in a transient reduced viability; however, survivors emerged exhibiting circular chromosomes and defective meiosis. (Naito et al., 1998). The structural similarities between TRF proteins and Taz1p, the reported clustering of telomeres around the centrosome in mammalian cells during meiosis, and the specific localization of TRF1 and TRF2 to meiotic telomeres, point to a role of mammalian telomeric proteins in meiosis (de Lange, 1998; Scherthan et al., 2000).

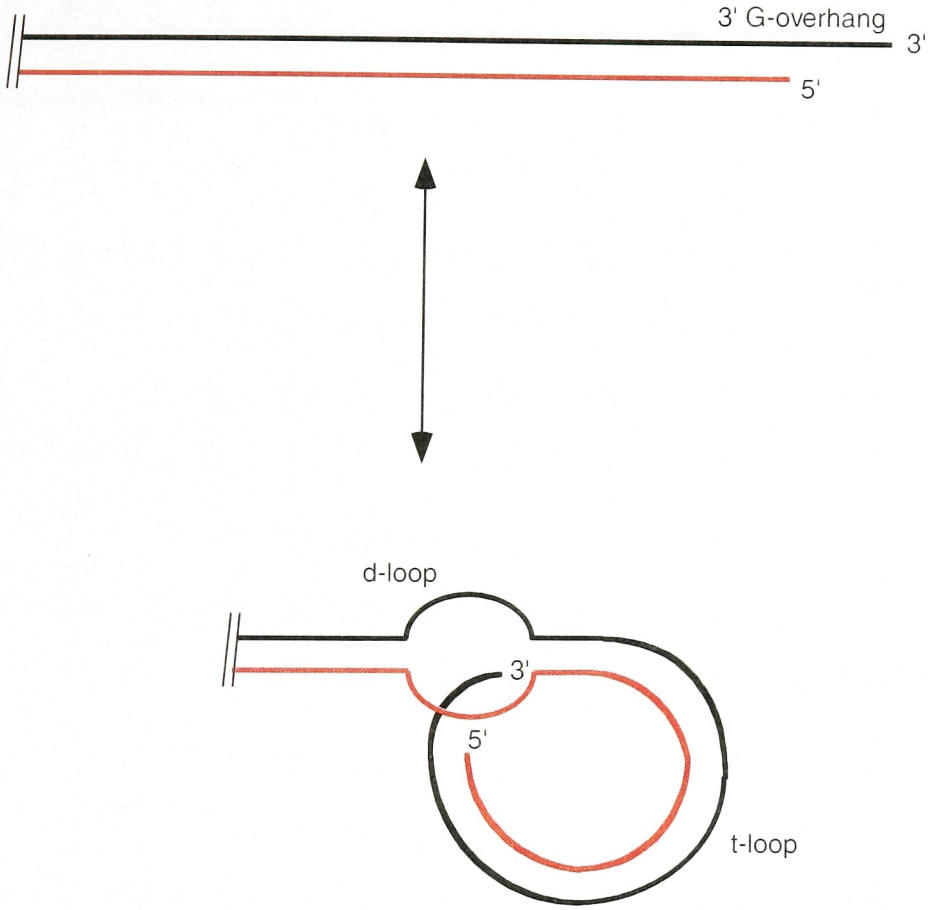


Fig. 7. Schematic representation of the t-loop structure. The telomere end folds back in itself and the 3' G-rich overhang is inserted into a displacement loop (d-loop) of the duplex part of the telomere repeats, forming a telomere loop, or t-loop. The 3' end is therefore hidden in this DNA structure. The folding mechanism is proposed to be protein-mediated. (Griffith et al., 1999).

Proteins with affinity for the duplex tracts of telomere repeats seem to be a common theme of telomeres. Although yeast Rap1p and human RAP1 have diverged in their modes of interaction with telomeres, it still stands that in both budding and fission yeast and in mammals, dsDNA binding proteins are important regulators of telomere length. A characteristic common to these proteins is the presence of Myb-like DNA-binding motifs. Because trypanosome telomeres maintain the length of their telomeres tightly, and their telomeres contain the conserved TTAGGG repeats, it is tempting to hypothesize that they maintain their telomeres by mechanisms involving dsDNA binding proteins.

The Telomere protein complex

Telomeric DNA-binding proteins bring other proteins to telomeres. In yeast, a large protein complex including Sir2p, Sir3p, and Sir4p (Silent Information Regulators) is recruited to telomeres by Rap1p and Sir3p and Sir4p also interact with histones H3 and H4 (Hecht et al., 1995; Lustig et al., 1996; Marcand et al., 1996). In addition, mutations in Sir3p and Sir4p cause telomere shortening, possibly by competing with Rif1p and Rif2p for binding to Rap1p (Palladino et al., 1993). The DSB-repair complex MRE11-RAD50-XRS2/NBS is also involved in homeostatic regulation of telomere length in yeast, as demonstrated by epistasis analysis with Tel1p (Ritchie and Petes, 2000).

Several proteins bind to mammalian telomeres by their interactions with TRF1 and TRF2. TIN2 interacts with TRF1 and deletions of its TRF1-binding domain results in telomere elongation (Kim et al., 1999). Tankyrase, a Poly(ADP-Ribose) polymerase, has 24 ankyrin

repeats in a domain responsible for its interaction with TRF1. Poly ADP-ribosylation of TRF1 by Tankyrase results in decreased binding of TRF1 to telomeres (Smith et al., 1998). A human homolog of Rap1p (hRap1) has been found to bind TRF2 at telomeres (Li et al., 2000) and co-precipitates with TRF2 in stoichiometric amounts (Zhu et al., 2000). In addition, Rad50/hMre11/Nbs1 associate with TRF2 and localize to telomeres in vivo (Zhu et al., 2000).

The human heterodimeric protein Ku70/Ku80 is involved in V(D)J recombination (Nussenzweig et al., 1996) and has affinity for dsDNA ends and for nicks and gaps in the DNA (Mimori and Hardin, 1986; Blier et al., 1993; Falzon et al., 1993). Ku also binds to circular DNA at the proximity of replication forks and is able to slide internally into duplex DNA after initial binding to the end (de Vries et al., 1989). In yeast, the Ku heterodimer also participates in nonhomologous end joining (Boulton and Jackson, 1996; Milne et al., 1996; Siede et al., 1996; Tsukamoto et al., 1996; Tsukamoto et al., 1997). Loss of yeast Ku function results in telomere shortening, impaired TPE, and alteration of subnuclear localization of telomeres and degradation of the 5' end of telomere to generate longer 3' overhangs (Boulton and Jackson, 1996; Porter et al., 1996; Boulton and Jackson, 1998; Driller et al., 2000) and Ku80 deficiency in mice results in accumulation of telomere fusion (Hsu et al., 2000). In *S. pombe*, Ku also protects telomeres from fusing and prevents chromosome circularization (Baumann and Cech, 2000). Direct binding of yeast and human Ku to telomeric DNA has been demonstrated in vitro (Gravel et al., 1998; Bianchi and de Lange, 1999). However, Ku proteins probably access telomeres through their interactions with other proteins in vivo. In human cells, Ku70 appears to bind telomeres

through a high affinity interaction with TRF1 and Ku80 (Hsu et al., 2000), and yeast Ku participates in Sir/Rap1 protein complex and counteracts telomeric silencing (Haber, 1999; Mishra and Shore, 1999).

Trypanosome telomeres

In addition to providing the genomic environment for antigenic variation to occur, trypanosome telomeres exhibit a combination of unique features. Two initial findings brought together trypanosomes' antigenic variation and telomeres. First, the transcribed *VSGs* are invariably located at telomeres (de Lange and Borst, 1982). Second, different telomere growth rates correlate to the transcriptional status of the ESs. Transcriptionally inactive telomeres grow at a rate of ~7 bp per cell division and the telomere of the active ES grows at ~10 bp per cell division (Bernards et al., 1983; Pays et al., 1983; van der Ploeg et al., 1984). The gradual growth of trypanosome telomeres can extend up to 20 kb (Bernards et al., 1983; Myler et al., 1988). Transcriptionally active telomeres undergo frequent deletions whereas inactive one rarely break (Bernards et al., 1983; Pays et al., 1983; Horn and Cross, 1997). In addition, a rare type of glycosylation is present at trypanosome telomeres, the glycosylated base J (ß-D-glucosyl-hydroxymethyluracil) (Gommers-Ampt et al., 1991). J results from glycosylation of thymidine residues (dT) in the DNA and is abundant at telomeres in all the *Kinetoplastidae*. J is also abundant within the ESs of BF, less abundant in the active ES, and not synthesized in the PF parasites (Gommers-Ampt et al., 1993; van Leeuwen et al., 1997; van Leeuwen et al., 1998). The function of J in the *Kinetoplastidae* has not been elucidated, nor is its possible involvement

in antigenic variation in *T. brucei* clear. Despite considerable interest in these aspects of trypanosome telomere biology and some efforts in trying to identify telomere binding factors (Eid and Sollner-Webb, 1995; Field and Field, 1996; Eid and Sollner-Webb, 1997), no specific telomeric proteins have yet been reported for trypanosomatids, and little is known about the function of telomeres in protecting the ends of trypanosome chromosomes.

Because trypanosome telomeres grow at a steady rate, an active mechanism of telomere replication had long been suspected. Only recently was telomerase activity discovered in kinetoplastids (Cano et al., 1999); however, the telomerase genes for the RNA template or the catalytic subunit of telomerase have not been identified. Introduction of a critically short telomere in *T. brucei* triggers rapid healing by addition of telomere repeats, suggesting the involvement of a mechanism that senses the length of the telomere repeats. Although the mechanisms involved are unknown, the healing is progressive, suggesting the involvement of telomerase (Horn et al., 2000). As presented earlier, telomeres are distributed around the nuclear periphery during G1 and S phase and cluster towards the center during division, which may indicate association with components of the nuclear matrix. In various species of trypanosomatids the 3' overhang ends in a specific nucleotide: the third G residue of the TTAGGG repeats in *T. cruzi* and *T. brucei* and the first T residue in *L. donovani* (Chiurillo et al., 1999; Chiurillo et al., 2000). This finding is in accordance with a precise mechanism of telomere elongation by telomerase (adding nucleotides in multiples of 6 bp), but is in discrepancy with the reported growth rate of 7 or 10 bp/cell division in *T. brucei* mentioned earlier.

Trypanosome telomeres contain TTAGGG repeats, allowing direct comparisons with vertebrate telomeres. Trypanosomes are amenable for genetic manipulations in the laboratory, therefore trypanosome telomeric proteins could be studied in this organism and mammalian telomeric proteins could be studied in dissociation from their natural environment if expressed in trypanosomes. This could allow evolutionary comparisons. Because trypanosomes possess 100 minichromosomes of 25-100 Kb, 11 chromosomes of 1-6 Mb and several intermediate-size chromosomes (Melville et al., 1998; Melville et al., 2000), these organisms have abundant telomere ends and their telomere repeats account for about 10% of the genomic DNA, making this organism a fruitful system in which to study telomeres. The study of trypanosome telomere structure and composition may yield insights into both universal and specialized biological principles.

Chapter 1

Stable Expression of Mosaic VSG Coats in *T. brucei*

A central question in antigenic variation is what regulates the expression of only one *VSG* from such an extensive gene repertoire. Metacyclic and BF trypanosomes express 10^7 copies of a GPI-anchored VSG. Only one VSG is expressed at a time, and during the time in which one VSG coat is replaced by another, mixed coats are expected to occur. This intermediate state would presumably be stable for the parasite to succeed in the infection. This mode of switching could potentially result in disadvantages for the parasite. For example, antibodies for the first VSG could recognize the protein in the context of the mixed coat or the synthesis of two VSGs could be unstable. The switching population would be then at risk of disappearing, which could explain the absence of these double-expressing trypanosomes in natural infections. Simultaneous multiple expression of VSGs would also represent an inefficient use of the VSG repertoire. A feedback mechanism to sense the concomitant occurrence of VSGs on the surface may therefore exist.

VSG has a half-life of 30-40 hours and *VSG* mRNA 4.5 hours (Ehlers et al., 1987; Bulow et al., 1989); hence, switching intermediates simultaneously express two VSGs could remain in the bloodstream for significant periods of time. Co-expression of VSGs had been previously reported for metacyclic trypanosomes. In the process of adapting to the bloodstream, metacyclic VSGs are replaced at a high frequency, resulting in the occurrence of 2.7 % of the trypanosome population expressing two VSGs simultaneously (Esser and Schoenbechler, 1985). However, these parasites did not multiply for long in the bloodstream, probably because metacyclic *VSGs* are transcribed independently, without *ESAGs* that are essential for survival (Graham et al.,

1990; Graham and Barry, 1991; Alarcon et al., 1994; Ligtenberg et al., 1994; Salmon et al., 1994; Steverding et al., 1994). Therefore, the expression of more than one VSG in metacyclic trypanosomes, despite its high probability, is not stable for prolonged times. Naturally occurring BF parasites of *T. equiperdum* with double coats of VSGs were stable for 30-50 generations *in vitro*, but unstable when inoculated into mice. One possible interpretation was that the parasites were isolated during switching, and that the cells expressing both *VSGs* were somehow stabilized *in vitro* but continued the process of switching *in vivo* due to an intrinsic incapacity to maintain the simultaneous transcription of both ESs or the mixed VSG coat in the animal (Baltz et al., 1986). In the absence of evidence for mechanisms that enforced the expression of singular *VSGs*, models based on immunological selection against certain trypanosomes had been offered under the assumption that cells expressing more than one VSG would be in disadvantage over cells expressing only one. Because trypanosomes expressing more than one VSG are eliminated, and immune selection act against the trypanosomes over time, the model predicted relapsing parasitemia patterns of homogeneous populations expressing only one VSG, similarly to what is found in natural infections (Agur et al., 1989; Barry and Turner, 1992). The questions of whether trypanosomes are intrinsically incapable of co-expressing different VSGs, or if constraints in the synthesis or assembly of VSGs enforce the expression of only one, had never been experimentally tested.

Trypanosomes permanently expressing two VSGs have been genetically engineered. A second *VSG* was inserted into an active ES. Because transcription is polycistronic, both the inserted and the resident *VSGs* will be transcribed at similar rates, providing an ideal situation in which to

study the stability of the protein products and the consequences for the growth and virulence of these trypanosomes.

Results

Two different VSGs can be co-expressed homogeneously on the trypanosome surface

To generate trypanosomes expressing two VSGs, the construct pKD4, designed to insert a neomycin phosphotransferase gene (*neo*), conferring resistance to G418, between the pseudo *VSG (Ψ VSG)* and the 5' end of *VSG 221* in the active 221 ES (Fig. 8). By this mean, the *VSGs* MITat 1.5 clone 117, MITat 1.6 clone 121, or ILTat 1.24 (Cross, 1975; Carrington et al., 1991) were introduced into the active *VSG 221* ES of *T. brucei* 427 clone 221a. Because this construct does not contain a promoter, expression of the inserted genes and G418-selection of the positive clones can only be obtained by transcription from the 221 ES promoter. In each case, 6 to 10 G418-resistant clones from 2 independent transfections were obtained. Initial immunoblots revealed that all clones expressed VSG 221 as well as the second VSG. One clone of each double-expressor was selected for further immunological analysis. Indirect immunofluorescence (IF) analysis of fixed, non-permeabilized cells demonstrated that the two VSGs are expressed on the cell surface (Fig. 9). In all cases, the VSG 221 was stained in green with a specific antibody coupled to fluorescein isothiocyanate (FITC). Specific antibodies conjugated with rhodamine stained the second VSG with red fluorescence. All of the approximately 200 double-expressing cells observed under the microscope exhibited the green and the red fluorescence

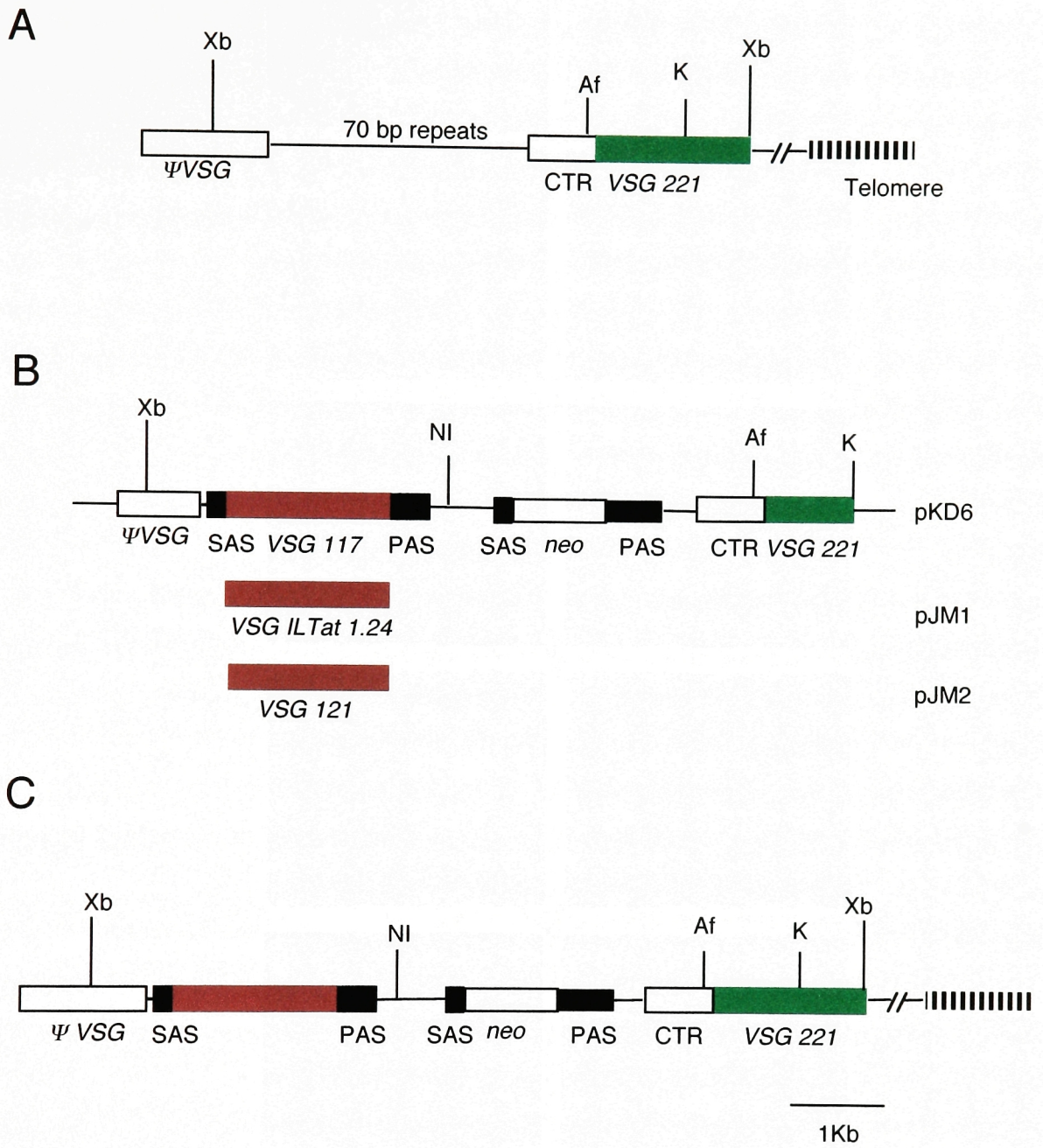


Fig 8. Integration of *VSG-NEO* cassettes into the active *VSG 221* expression site. Maps of (A) the telomere proximal region of the wild-type 221 ES, (B) the three plasmids constructed to insert each *VSG-NEO* cassette, and (C) the ES after cassette insertion by homologous recombination. CTR is the *VSG* co-transposed region. The *NEO* gene was flanked by a splice acceptor site (SAS) and a polyadenylation site (PAS) from *T. brucei* aldolase gene, and *VSGs* were flanked by SAS and PAS from the *T. brucei* actin gene. Only relevant restriction sites are indicated: Af, *Afl* II; NI, *Eco*NI; K; *Kpn*I; *Xba*I, Xb.

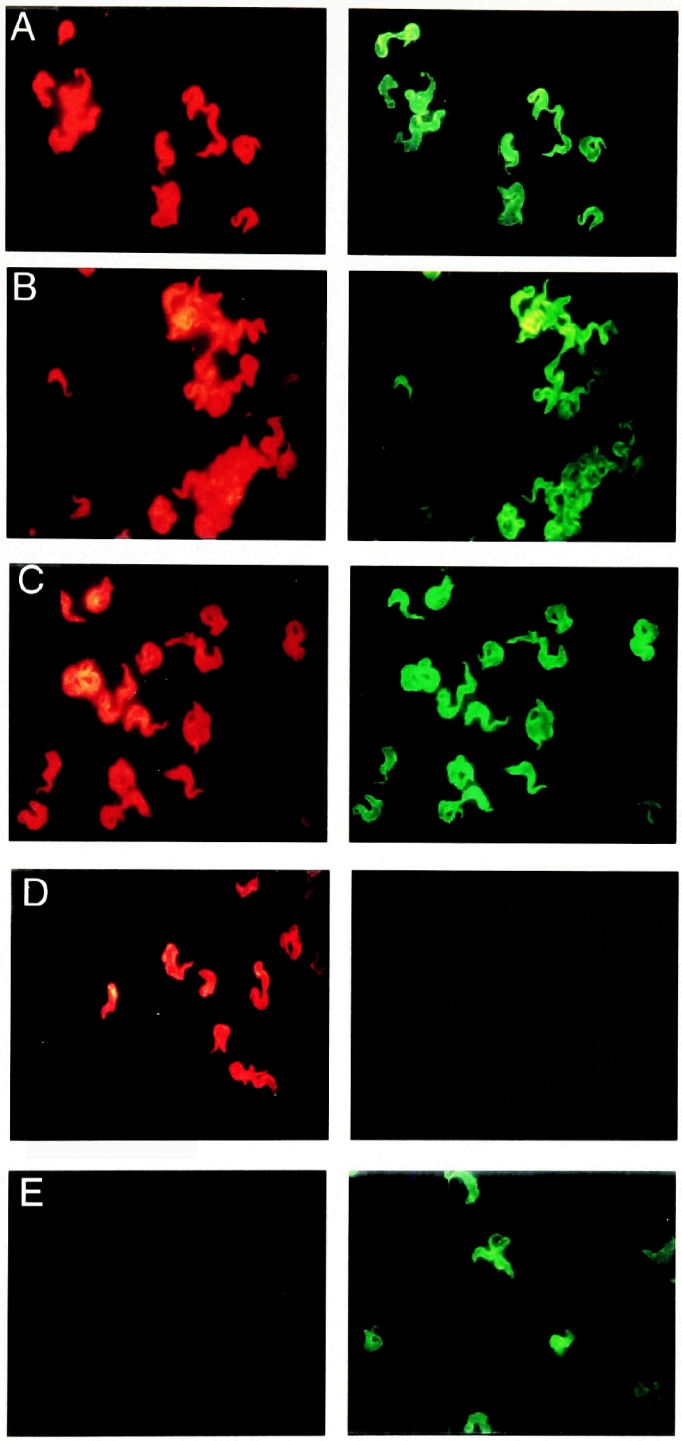


Fig. 9. Immunofluorescence of double-expressing and wild-type trypanosomes. BF trypanosomes were grown in vitro to a density of 10^6 parasites/ml, fixed, and dried onto microscope slides. Rabbit antibodies against VSG 117, 121, and ILTat 1.24 were individually combined with chicken anti VSG 221. Goat anti-chicken IgG coupled to FITC, and anti-rabbit coupled to rhodamine were used as second antibodies. The red fluorescence in the left panel of each pair represents the second VSG being expressed on the double-expressing cells (A) 221-117, (B) 221-ILTat 1.24, and (C) 221-121. (D) Wild type 121. The green fluorescence in the right panels identifies the constitutively expressed VSG 221 in the three double-expressing cell lines and in the wild-type 221 cells (E). No reaction was detected on the wild-type 221 cells when incubated with antibodies for 121 (E, left) or on the wild type 121 cells incubated with antibody for 221 (D, right).

simultaneously and uniformly distributed on the cell surface, confirming the homogeneity of cell populations expressing both VSGs. Parental lines expressing VSG 221, 121, or 117 showed no cross-reaction among the antibodies used.

The mosaic coats are composed of equal amounts of two VSGs

Brief osmotic shock results in cleavage of GPI anchors by endogenous GPI-specific phospholipase C (GPI-PLC), allowing rapid purification of soluble VSG (Cross, 1984). BF trypanosomes were briefly resuspended in water at 0°C and then in phosphate buffer at 37 °C. The released VSG was analyzed by immunoblotting. (Fig. 10A), which showed that each of the recombinant cell lines expressed both VSGs in substantial amounts. Mobility differences between VSGs facilitated their identification after SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and staining with Coomassie brilliant blue. This showed that both VSGs in the double-expressing cells appeared to be present in similar amounts (Fig. 10B, upper panel). This result was confirmed by metabolic labeling. BF trypanosomes were transferred to methionine-free medium supplemented with ^{35}S-labeled methionine-cysteine mixture. VSGs were extracted by osmotic shock as above and separated by SDS-PAGE. When this pairs of VSGs were treated with VSG 221-specific antibodies, there was no coprecipitation of the second VSG (Fig. 10B, lower panel). Therefore, these VSGs do not form detectable heterodimers.

Trypanosomes expressing two VSGs grow and infect normally

Trypanosomes expressing two VSGs grew at the same rate (population doubling time 6 h) as wild-type parental cells *in vitro*, independent of the presence of G418 in the medium. To evaluate the replicative capacity and survival of the double-expressing trypanosomes *in vivo* and while facing immune recognition, rats were infected with 5 trypanosomes in the absence of G418 and parasitemia was followed daily by tail puncture and hemocytometer counting. The double-expressing cells were as infective as the wild-type parental 221 line (Fig. 11). In each instance, two successive peaks of parasitemia were produced. The first peak was seen at 8 to 9 days after infection, when all of the parasites were shown by IF to be expressing both VSGs on their surfaces. The second peak of parasitemia was observed 13 to 14 days after infection, which the rats were unable to control and parasitemia escalated to more than $2x10^8$ trypanosomes ml^{-1} of blood. This second peak represents variants that are negative for both initial VSGs, and was fatal in all cases. No significant differences were observed between the two growth kinetics of double-expressing and wild-type 221 cells in different rats and in different experiments. Therefore, parasites expressing two different VSGs have no intrinsic growth disadvantage *in vivo* or *in vitro*.

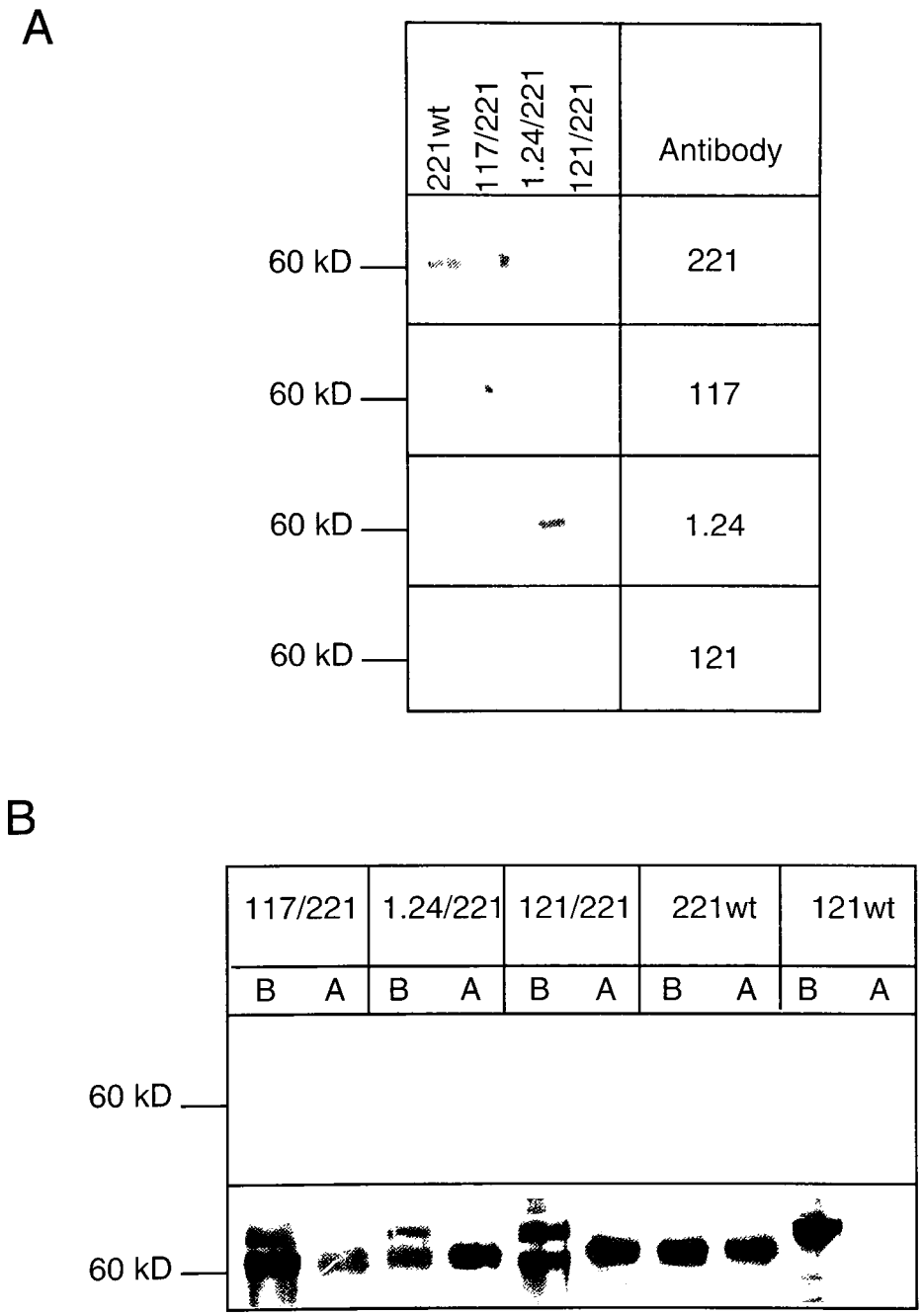


Fig. 10. Two VSGs are present in equal amounts in the mosaic coats. (A) Protein immunoblots of VSG preparations separated by SDS-PAGE. (B) Immunoprecipitation of partially purified 35S-labeled VSGs with Sepharose-coupled anti-VSG 221 (B and A denote before and after immunoprecipitation). The gels were stained with Comassie brilliant blue (upper panel) and then dried and exposed to autoradiographs (lower panel).

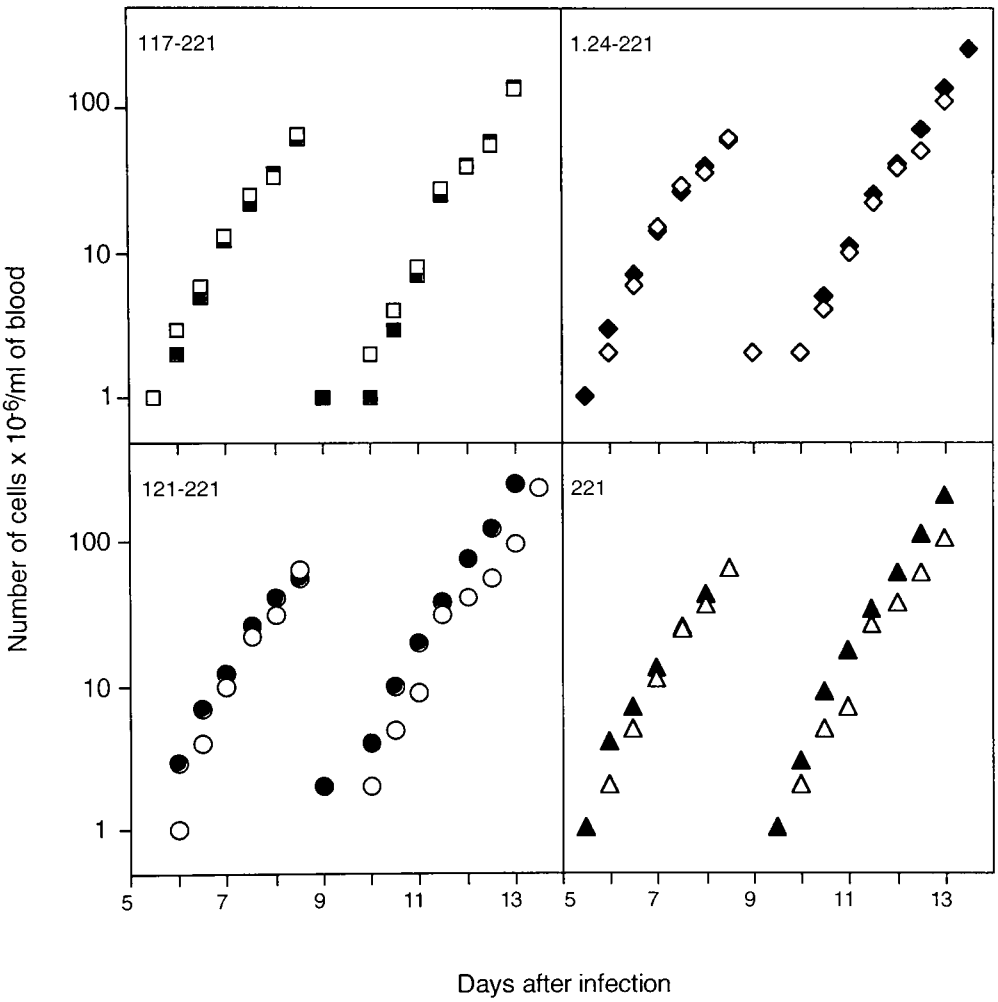


Fig. 11. Infection of rats with *T. brucei* 221 wild-type and double-expressing trypanosomes. Pairs of rats (300 g) were each infected with about 5 wild-type 221 or double-expressing trypanosomes. From the fifth day after infection, the parasitemia in each rat was checked every 12 hours by tail puncture and hemocytometer counting. In each panel, closed and open symbols represent the course of parasitemia in individual rats. The lower limit of counting was 106 cells/ml.

Discussion

Three different lines of trypanosomes have been generated which express VSG double coats. Expressing more than one VSG had no consequence for the survival and virulence of *T. brucei*. The VSG-switching rate in the animal host is too infrequent to be observed (Lamont et al., 1986), except during the initial metacyclic to BF transition (Esser and Schoenbechler, 1985; Turner and Barry, 1989). The engineering of trypanosomes to express two VSGs, thereby prolonging an intermediate in antigenic variation that could not hitherto be studied, provides a tool to investigate several aspects of antigenic variation, including the interplay between the parasite and the immune system.

The sequences of VSGs 221, 117, ILTat 1.24 and 121 are known (Allen et al., 1982; Boothroyd et al., 1982; Allen and Gurnett, 1983; Carrington et al., 1991) and the X-ray crystalographic studies of the amino-terminal domains of VSGs 221 and ILTat 1.24 have revealed that, despite substantial sequence difference between them, the three-dimensional structures are practically identical (Freymann et al., 1990; Blum et al., 1993). Two major VSG classes have been distinguished on the basis of sequence conservation of their carboxy-termini (Ferguson et al., 1988; Ferguson and Williams, 1988; Cross, 1990; Carrington et al., 1991). The mature sequences of these two VSG classes terminate in either aspartate (for example VSG 117, 121, and ILTat 1.24) or serine (for example VSG 221), forming the GPI attachment site, after removal of a 23- or 17- amino acid signal sequence, respectively. Therefore, the three trypanosome lines generated (221/117, 221/ILTat 1.24, and 221/121) express VSGs of different classes. The two VSGs were expressed in similar

quantities on the cell surface of the three double-expressing trypanosome line, as evidenced by the GPI-PLC-mediated release of both VSGs. The two VSGs were expressed by all the cells in the cultures and appeared to be equally distributed on their cell surfaces. Immunoprecipitation of VSG 221 does not bring down the second VSG, indicating that, despite the similarity in their three-dimensional structures, these VSGs do not form heterodimers. This observation is consistent with the situation observed during one case of naturally occurring expression of two VSGs in *T. equiperdum* (Baltz et al., 1986), and with observations of purified VSGs attached to nitrocellulose membranes, where only homodimer interactions were detected (Cardoso de Almeida et al., 1984). Examination of the dimer-interface region of the amino-terminal domains of VSG 221 and VSG ILTat 1.24 (Blum et al., 1993) shows a high degree of homodimer interaction, suggesting that VSGs would be unlikely to form heterodimers of similar stability. On the basis of these reports, heterodimer formation between two VSGs was not expected to occur. These findings suggest that the singularity of VSG expression and the reported semi-ordered appearance of VSGs on the cell surface (Kosinski, 1980; Agur et al., 1989) is not determined at the cell surface or by interactions between the VSGs. The semi-ordered appearance of VSGs is possibly a consequence of epigenetic mechanisms involved in VSG switching but not yet defined.

It was previously reported that *T. equiperdum* expressing two VSGs from different ESs were outgrown by cells expressing only one of the VSGs (Baltz et al., 1986). This suggested the possible existence of some intrinsic obstacle to the simultaneous expression of two VSGs, or the simultaneous transcription of two ESs. In the present study the

expression of two VSGs simultaneously resulted in no alteration in the capacity of *T. brucei* to replicate or to infect animals. Switching intermediates expressing two VSGs probably remain in the bloodstream for significant periods of time, as suggested by the half-life of VSG mRNA and protein. The rate of destruction of mosaic VSG trypanosomes compared to single VSG-expressing cells, could therefore be affected by any selective advantage or disadvantage that this situation confers. T-independent B-cell responses are important for immune clearance in experimental trypanosomiasis (Mansfield, 1994). This type of response is generated by a repetitive array of a single epitope in the dense VSG array on the surfaces of trypanosomes.

Perhaps expressing one VSG at a time has evolved as the most economical strategy of immune evasion, but an unknown mechanism of allelic exclusion ensures that only the active ES is thoroughly transcribed from the ES promoter.

Simultaneous activation of two different ESs can be detected, or enhanced, by inserting drug selection markers next to two different ES-promoters such that both ESs could be switched back and forward, or be maintained active at the same time. Rather rarely, however, could expression of the two VSGs be sustained, even when the two ESs were forced to be active by double drug pressure (Horn and Cross, 1997; Rudenko et al., 1998; Chaves et al., 1999; Navarro et al., 1999). Recent work shows that low levels of transcription occur within the inactive ESs in BF trypanosomes; however the VSG gene is not transcribed from the inactive ESs (Vanhamme et al., 2000). How an ES switch is precipitated is not known. Because the telomere of the active ES grows faster than

telomeres of inactive ESs, it is possible that some interconnection exists between the transcription of telomeric genes and the telomere structure in trypanosomes. For example, a telomeric complex may become increasingly repressive in the active ES as this telomere grows, resulting in inactivation of the VSG and concomitant activation of another. Also, the active telomere undergoes frequent shortening, sometimes associated with a transcriptional switch (Bernards et al., 1983; Pays et al., 1983). Perhaps these telomere deletions could result in destabilization of the active ES and immediate switch. These tentative models await the characterization of telomere structure and composition in trypanosomes.

There are no readily detectable differences in chromatin between active and inactive ESs (Navarro et al., 1999). One palpable difference between active and inactive ESs is the glycosylated base J, less abundant in the active ES (van Leeuwen et al., 1997). Other than this, and despite the identification of a J-specific binding protein with an unknown function (Cross et al., 1999), the mechanisms involved in determining the singularity of VSG expression remain obscure. The coordinated activation/inactivation of ESs (Horn and Cross, 1997; Navarro and Cross, 1998; Chaves et al., 1999) suggests an autoregulatory element involved in the switching, such as a gene product from the ES. A potential role for ESAG8 in RNA-Polymerase I transcription has been recently reported; however, ESAG8 localizes to the nucleolus and the two ESs involved in the switch don't (Chaves et al., 1999; Hoek et al., 2000).

In sum, the present evidence points to a model in which constraints at the level of VSG synthesis or assembly are not involved in keeping only one ES active at a time. Rather, epigenetic mechanisms involving some kind of telomere effect, possibly involving the product of an *ESAG*, seem to modulate the expression of only one *VSG* from only one ES at any time.

Chapter 2

The Structure of *T. brucei* Telomeres

Unprotected ends of linear dsDNA molecules generate DNA-damage responses. A failure of bacteriphage T4 in protecting its linear DNA molecule during injection into *E. coli* results in its degradation by a bacterial exonuclease and in a halt of bacterial replication (Silverstein and Goldberg, 1976; Appasani et al., 1999). The *E. coli* prophage N15 and the spirochete *Borrelia* protect their genomes by covalently linking the 3' and 5' ends of the opposite DNA strands (Hinnebusch and Tilly, 1993). *Streptomyces rochei* large linear plasmids have telomeres that end in palindromic repeats and have 3' single-stranded protrusions of exactly 280 bp. These 3' overhangs fold back on themselves and a protein is covalently bound to the 5' end of the DNA(Chang and Cohen, 1994). Most linear dsDNA viruses and phages, however, integrate their genomes into the host chromosomes and adopt circular modes of replication, and the vast majority of bacterial genomes are circular. Eukaryotic cells escaped from this norm and emerged to face the danger of having their linear chromosome ends exposed, a problem circumvented by the advent of telomeres, and their associated proteins.

Electron microscopy (EM) of mammalian telomeric DNA has revealed the presence of t-loops at chromosome ends in vivo. T-loops were isolated by cross-linking the DNA with psoralen AMT (4' aminomethyltrioxalen) and purifying protein free telomeric DNA by differential gel-filtration after digestion with frequent-cutting restriction enzymes. T-loops are composed of duplex TTAGGG repeats and their size varies with the length of the telomeric repeat array, from 2 to 25 kb. Binding of single-stranded-binding protein from *E. coli* to the tail-loop junction indicated that there is a segment of single-stranded DNA at this

site, most likely formed by strand displacement upon invasion of the 3' overhang in the duplex telomeric repeat tract. T-loops were found to be relatively abundant (15-40%) in a variety of mammalian DNA sources, including HeLa cells, mouse liver, HT1080 cells, and primary peripheral blood leukocytes (Griffith et al., 1999). Although the mechanism by which t-loops are formed in vivo has not been established, both TRF1 and TRF2 have biochemical features suggestive of a role in loop formation or maintenance. TRF1 and TRF2 bind duplex telomeric TTAGGG repeats (Chong et al., 1995; Broccoli et al., 1997). Whereas TRF1 can loop and pair telomeric DNA (Bianchi et al., 1997), TRF2 has been observed to promote the formation of t-loops from a model telomere substrate (Griffith et al., 1999). Both TRF1 and TRF2 control telomere length (van Steensel and de Lange, 1997; Smogorzewska et al., 2000), and interference with TRF2 function results in unprotected telomeres, as evidenced by loss of telomere 3' overhang, activation of the ATM/p53-dependent DNA-damage-checkpoint pathway, and apoptosis (Karlseder et al., 1999). T-loops have been proposed to provide an architectural solution to the problems posed by telomeres (Greider, 1999; Griffith et al., 1999). By sequestering the telomere terminus in the duplex part of the telomeric repeat array, t-loops could provide the means of hiding the chromosome end from DNA damage checkpoint machinery and DNA repair enzymes. In addition, insertion of the 3' terminus in the duplex DNA would presumably block telomerase from adding repeats to the chromosome end, providing a mechanism for regulation of telomere length maintenance.

To address the possible conservation of the t-loop structure, the advantages of the trypanosome genome were employed. The 11 chromosomes of 1-6 Mb contain all essential genes of *T. brucei* including telomeric copies of *VSGs* that can be used to recognize specific

telomere restriction fragments in genomic blots (de Lange and Borst, 1982; Melville et al., 1998; Melville et al., 2000). In addition, *T. brucei* contains 100 mainly transcriptionally inactive minichromosomes of 25-100 kb, which can be separated from the large chromosomes on sucrose gradients (Weiden et al., 1991). As in mammalian telomeres, the lack of recognition sites for endonucleases in the TTAGGG repeats make it possible to digest the rest of the DNA using frequent-cutting endonucleases, and purify telomeres by size fractionation (de Lange et al., 1990), and the TTAGGG repeats are substrates for psoralen cross-linking at TA steps. The abundance of TTAGGG-containing telomeres and the presence of telomeric *VSGs* have been used in this chapter to study the structure of trypanosome telomeres.

3'-overhangs are present in TTAGGG-containing telomeric restriction fragments

It was previously shown that *T. brucei* telomeres are composed of TTAGGG repeats and these repeats are exclusively found at chromosome termini, based on their sensitivity to exonuclease treatment of intact genomic DNA (Blackburn and Challoner, 1984; van der Ploeg et al., 1984). In the present study, digestion of DNA from BF and PF of *T. brucei* with frequently-cutting enzymes yielded telomeric fragments in the 10-20 kb range (Fig. 12A), suggesting that the telomeres contain long arrays of TTAGGG repeats. It was next determined whether *T. brucei* chromosome ends have overhangs of the TTAGGG repeat strand. Mammalian chromosomes have up to 200 nt of single-stranded TTAGGG repeats at their 3´ termini (Makarov et al., 1997; McElligott and Wellinger, 1997; van Steensel et al., 1998; Huffman et al., 2000) and these overhangs are presumed to be important for the formation of t-loops. The presence of single-stranded TTAGGG repeats can be assessed by annealing labeled C-strand specific oligonucleotides to genomic DNA. Using this approach, the native DNA from *T. brucei* was found to contain single-stranded TTAGGG repeats (Fig. 12B). The signal was not detected in DNA digested with *E. coli* exonuclease I, which is specific for 3´ single-stranded tails, or in a control hybridization with an oligonucleotide representing the G-rich telomeric strand, consistent with the signal being derived from 3´ single-stranded TTAGGG tails. Furthermore, the signals were present on large fragments (>10 kb) in DNA that was digested with *Alu*I/*Hinf*I/*Rsa*I, as would be expected if the G-tails are present at the ends of the trypanosome telomeres. The presence of single-stranded telomeric tails was corroborated by EM analysis of

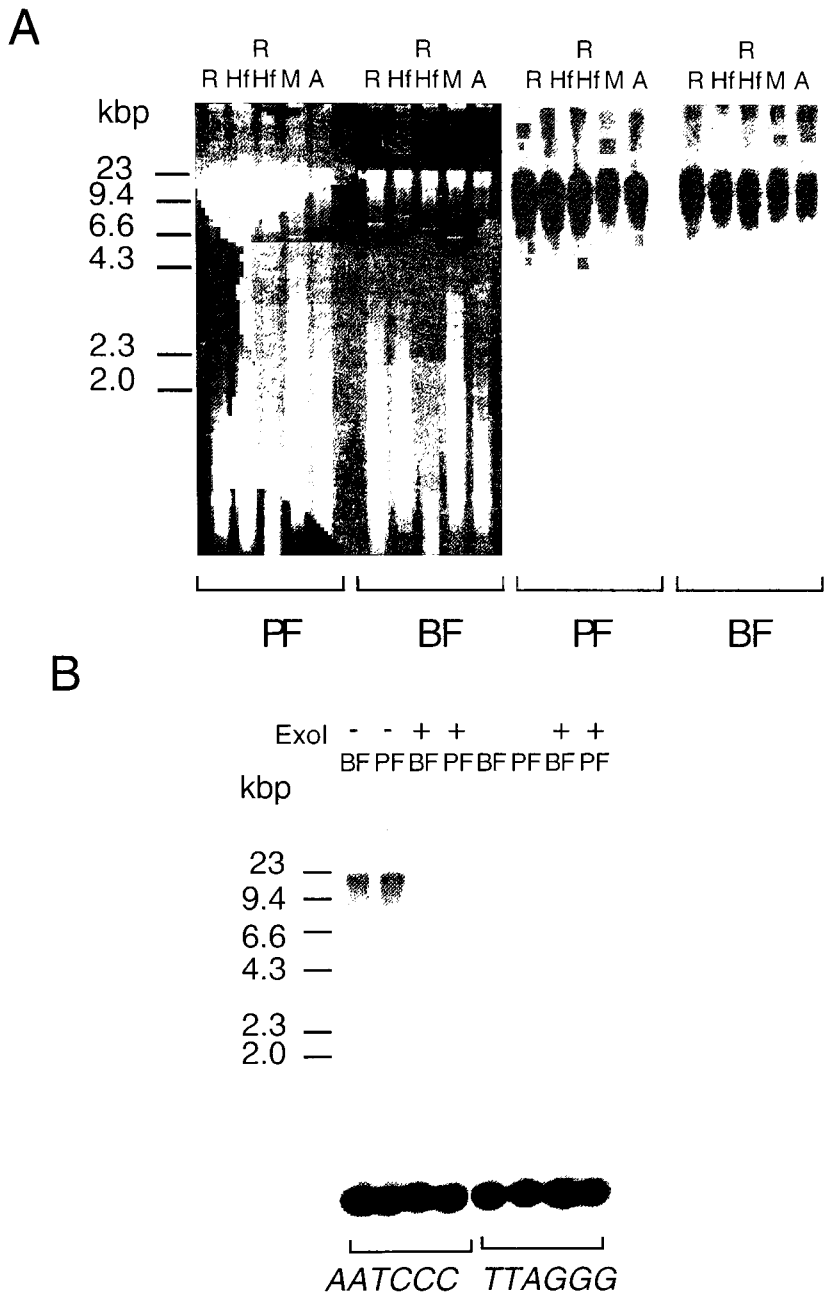


Fig. 12. Analysis of *T. brucei* telomeric DNA (A) Southern blotting analysis of telomeric restriction fragments in DNA from procyclic (PF) and bloodstream (BF) form trypanosomes. The DNAs were digested with *Rsa*I (R), *Hinf*I (Hf), *Rsa*I/*Hinf*I (R/Hf), *Mbo*I (M) or *Alu*I (A). The left panel shows the ethidium bromide; the right panel shows a Southern blot probed with a (TTAGGG)27 probe. (B) Overhang assay. DNA from BF and PF was digested with *Alu*I/*Hinf*I/*Rsa*I after treatment with (+) or without (-) *E. coli* exonuclease I. DNA was incubated with radiolabeled ssDNA probes TTAGGG or AATCCC as indicated and separated by agarose gel electrophoresis. The gel was dried and exposed on a PhosphorImager. The signal at the front represents free probe.

minichromosomes coated with *E. coli* SSB (see below). These data indicate that *T. brucei* telomeres resemble human telomeres in both the length of the TTAGGG repeat arrays and the presence of a 3´ (TTAGGG)n overhangs.

T. brucei TTAGGG repeat arrays are 15 kb average length

In order to determine the median length of the TTAGGG repeat arrays in the *T. brucei* line used in this study, a technique previously used to measure the length of human telomeres was applied (Saltman et al., 1993). The rate at which the exonuclease *Bal*31 removes TTAGGG hybridization signal is compared to the rate at which the enzyme shortens terminal DNA fragments. In *T. brucei*, this approach is facilitated by the availability of probes for subtelomeric *VSGs* and the detailed knowledge of the restriction maps of these loci, allowing precise measurements of *Bal*31 digestion rates on well-defined terminal restriction fragments. To measure the length of the telomeric repeat arrays directly, intact PF-DNA was treated with *Bal*31 exonuclease for increasing times, digested with *Hinf*I/*Alu*I/*Rsa*I and hybridized to a TTAGGG repeat probe (left panel of Fig. 13A). Quantification of the TTAGGG repeat signal at each time point indicated that the exonuclease removed approximately 2.6% of the TTAGGG repeat signal per min. The rate at which *Bal*31 shortened the telomeric fragment that carries *VSG* 221 was determined in parallel (center panel of Fig. 10A; for restriction map see Fig. 13B). The same shortening rate was found for the telomeric restriction fragment carrying *VSG* 121 (left panel Fig. 13A; for restriction map see Fig. 13B). As expected, *Bal*31 did not affect chromosome-internal restriction fragments,

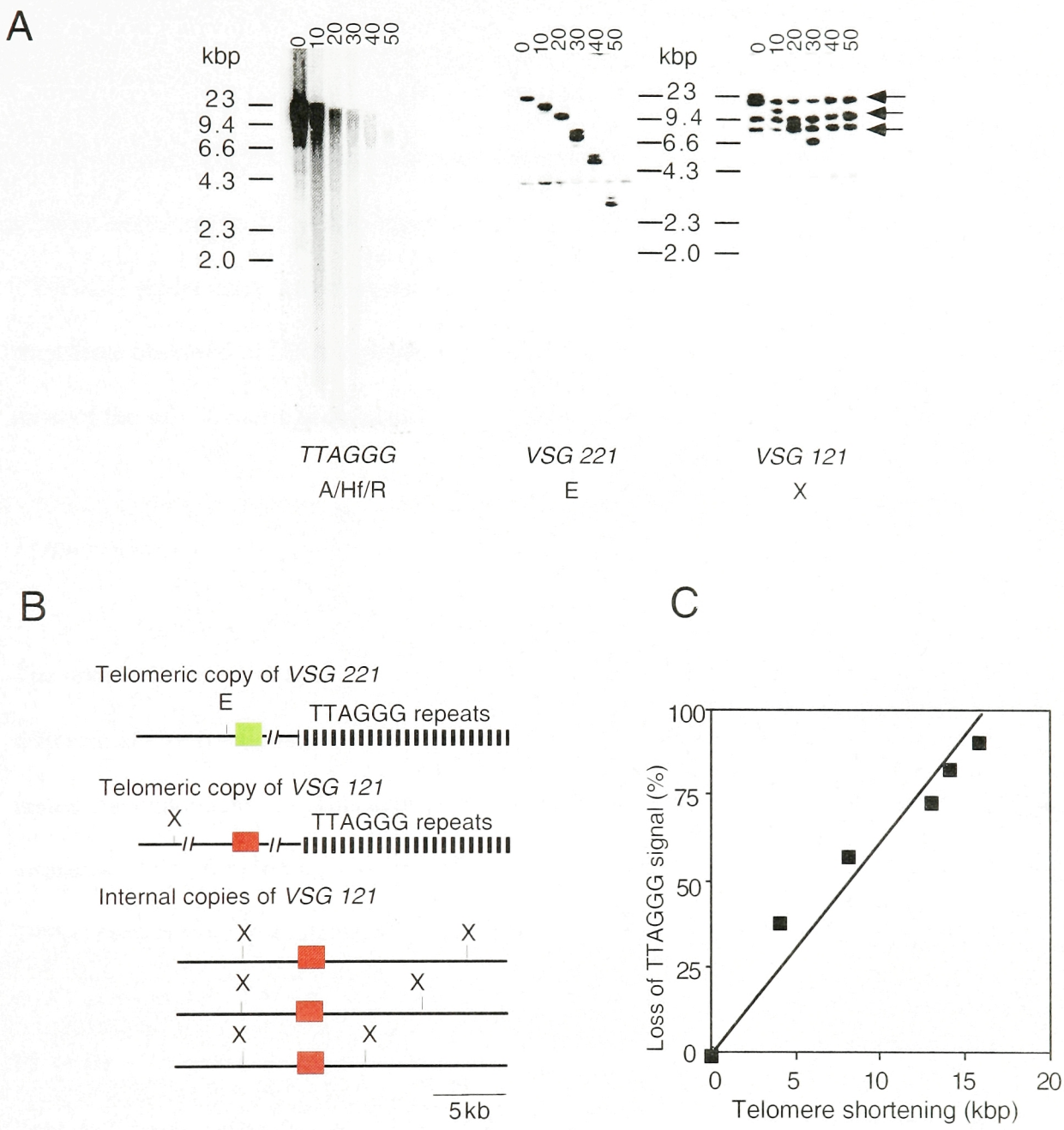


Fig. 13. Measurement of the length of TTAGGG repeats arrays. (A) *Bal*31 digestion of trypanosome telomeric DNA. Intact genomic DNA from bloodstream-form trypanosomes was treated with Bal31 for the indicated times (in min) and digested with *Alu*I/*Hinf*I/*Rsa*I (left panel), *Eco*RI (RI, middle), or *Xmn*I (X, right panel) and probed as indicated below the panels. The arrows indicate three non-telomeric *VSG 121* fragments. (B) Restriction maps of the telomeric *VSG 221* and *121* loci and the chromosome internal *121* genes. (C) Graph of the rate at which *Bal*31 removed the TTAGGG repeat signal plotted against the rate at which the exonuclease shortened the *VSG 221* telomeric *Eco*RI fragment.

such as those carrying non-telomeric copies of *VSG* 121 (also in right panel of Fig. 10A). Comparison of the two rates (Fig. 13C) showed that *Bal*31 removed ~10% of the TTAGGG repeat signal in the time needed to shorten the telomere by 1.5 kb, implying that the average length of the TTAGGG repeat array was ~15 kb. This value for the length of the TTAGGG repeat array was in agreement with the median length for the telomeric fragments observed in DNA digested with *Hinf*I and *Rsa*I, which are expected to remove most of the subtelomeric sequences from the terminal fragments (Fig. 12A).

Trypanosome telomeres end in t-loops

The telomeric repeat arrays of *T. brucei* are sufficiently long to allow their isolation by differential size fractionation after digestion of genomic DNA with frequently cutting restriction endonucleases. This approach was previously employed to isolate human telomeric DNA, for cloning and for EM visualization (de Lange et al., 1990; Griffith et al., 1999). Furthermore, psoralen AMT and UV light crosslink T residues on opposite strands at AT steps in the TTAGGG sequence, potentially stabilizing t-loops during their isolation. PF or BF of *T. brucei* were permeabilized with digitonin and treated with AMT and UV light. Following deproteinization, the DNA was cleaved with *Alu*I/*Hin*fI/*Rsa*I, and size-fractionated on a Bio-Gel A-15m matrix. Fractions containing large DNA fragments were then prepared for EM by spreading on a film of denatured cytochrome C followed by rotary shadowcasting.

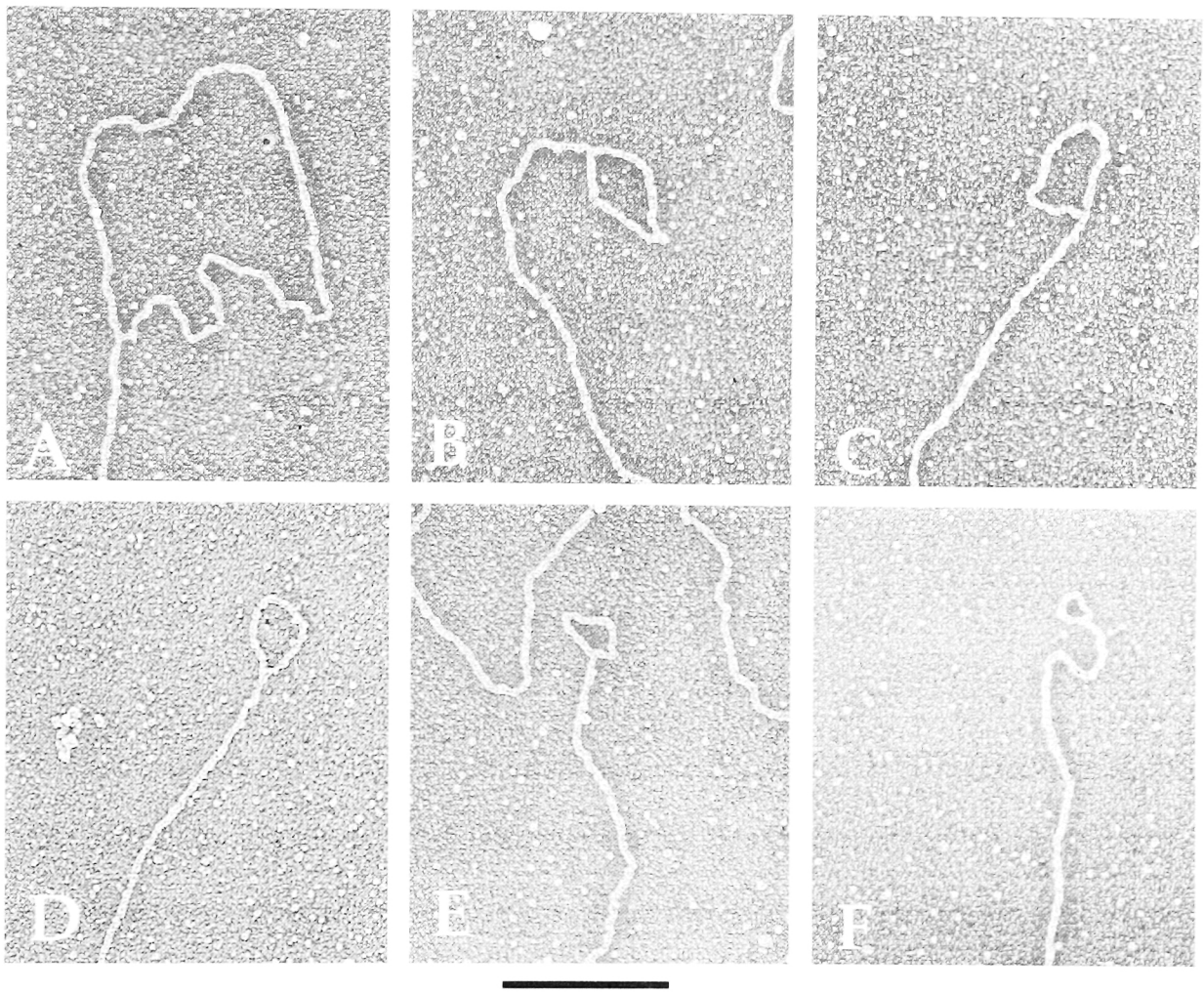


Fig 14. Visualization of t-loops from *T. brucei*, DNA photocrosslinked with AMT and UV light. Trypanosomes were permeabilized with digitonin and treated with AMT and UV light followed by endonuclease cleavage and purification of the telomeric restriction fragments by gel filtration. DNA fragments were prepared for EM by spreading on a denatured film of cytochrome C and rotary shadowcasting with platinum:paladium. Shown in reverse contrast. T-loops shown in A-F measured 6.3, 1.75, 1.5, 1.2, 0.99 and 0.63 kb respectively. Bar is equivalent to 1 kb. Data provided by Jack Griffith.

EM examination revealed the presence of long DNA molecules is the early eluting (high MW) fractions of the Bio-Gel column. In 5 experiments, the fraction of long linear DNAs (10 kb or greater) containing a loop at one end varied from 8 to 25% ($n \geq 100$ for each experiment); a frequency of t-loops similar to that observed for mammalian telomeres. The structure of trypanosome t-loops appeared to be similar to t-loops from mammalian cells, containing a single terminal loop and unforked tails. Examples of trypanosome t-loops are shown in Fig. 14 , with loop sizes varying from 6.3 kb (A) to 0.63 kb (F). No difference was observed in t-loop frequency or structure in DNA from BF and PF trypanosomes.

T-loops and overhangs are present at both ends of the chromosomes

Trypanosome minichromosomes are sufficiently small to allow their visualization as intact molecules, allowing inspection of both ends of each chromosome (Weiden et al., 1991). To isolate minichromosomes for this purpose, permeabilized trypanosomes were treated with psoralen and UV, gently lyzed, protease-treated, and sedimented through a 5-20% sucrose gradient. Gradient fractions containing minichromosomes were identified by gel-electrophoresis, under conditions that separate minichromosomes from larger chromosomes, followed by detection of telomeric DNA with a TTAGGG repeat probe. Using this approach, fractions were identified that were highly enriched for minichromosomal DNA (Fig. 15A and 15B). These fractions appeared to lack DNA derived from the larger chromosomes because they did not contain detectable amounts of an abundant 50-bp repeat element that is present upstream of ESs on the larger chromosomes (compare fractions to total DNA in Fig. 15C).

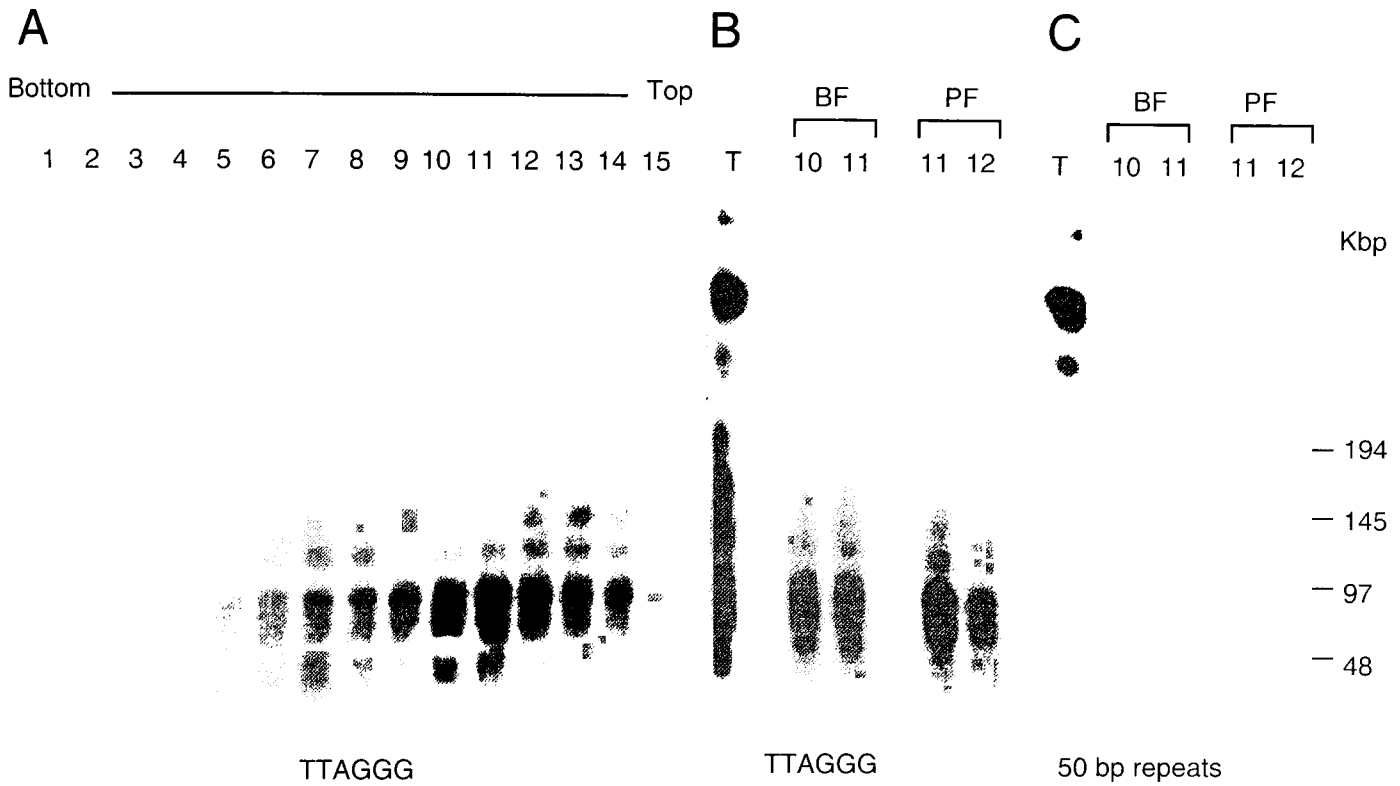


Fig. 15. Isolation and analysis of minichromosomes. (A) Southern blot of fractions from sedimentation of bloodstream-form trypanosome chromosomal DNA through a linear 5 to 20 % sucrose gradient. Fractions were analyzed by RAGE and probed with (TTAGGG)27. Two fractions from (10 and 11) bloodstream forms and two fractions (11 and 12) from a parallel sedimentation of procyclic-form chromosomes are compared alongside input DNA prior to sedimentation (T). Blots were probed with TTAGGG repeats (B) or a 50-bp repeat probe (C).

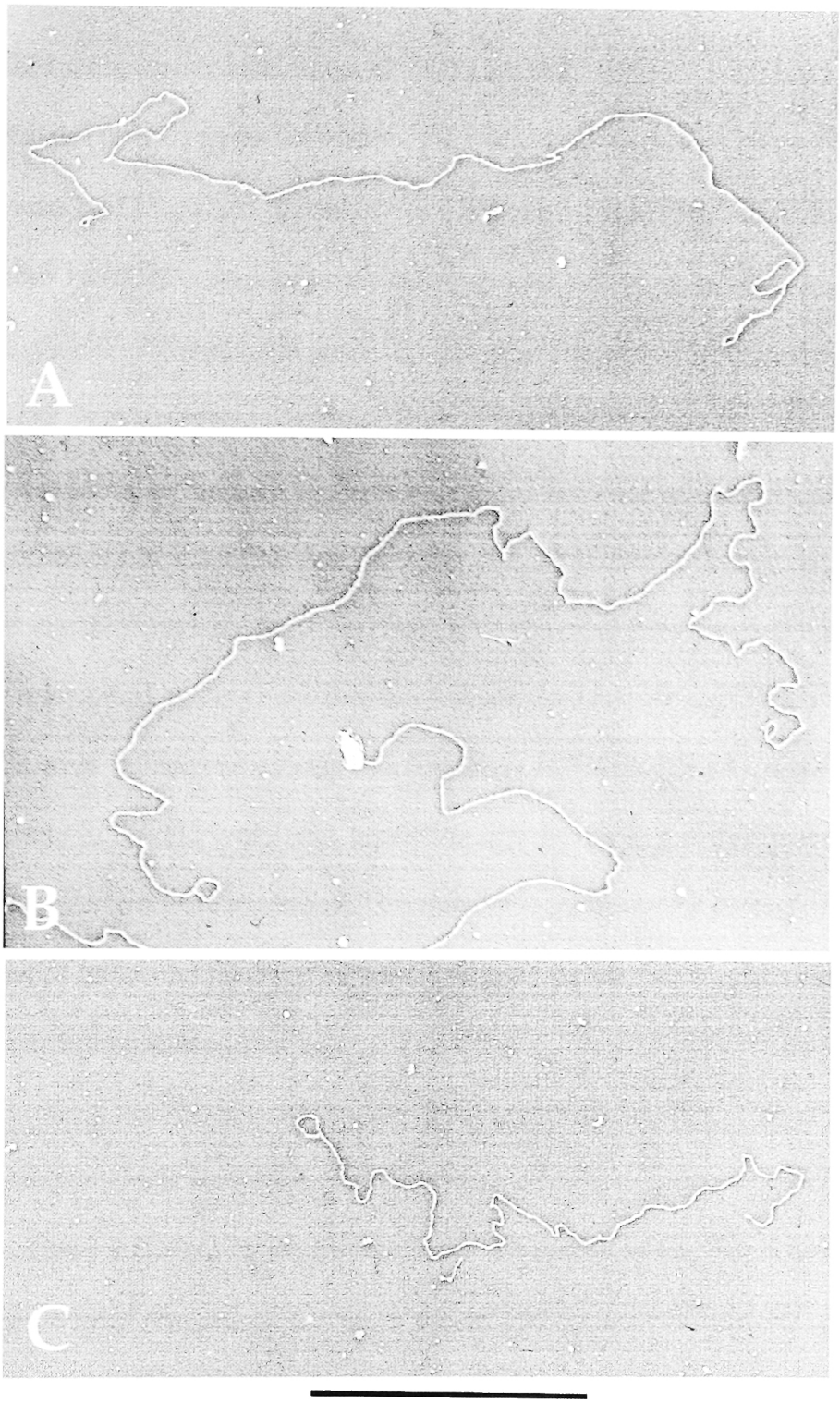


Fig. 16. The two ends of *T. brucei* minichromosomes form t-loops. Minichromosomes enriched by sucrose gradient sedimentation were prepared for EM as described in Materials and Methods. The minichromosome in panel A measures 28.7 kb and the loops at the left and right ends measure 650 and 710 bp respectively. The minichromosome in panel B is 30.5 kb, with loops of 1310 and 790 bp at the left and right ends respectively. The molecule in C is 21.1 kb (most likely a broken minichromosome) and the loop at the left end is 1470 bp. Shown in reverse contrast. Bar is equivalent to 5 kb. Data generated by Jack Griffith.

The enriched minichromosomal fractions were analyzed by EM and found to contain linear DNA molecules ranging from 20 to 50 kb. This size range is about half that expected from measurements by EM and gel-electrophoresis (Fig. 12), suggesting that many of the minichromosomal DNAs were broken during spreading. However, EM analysis showed that 14 out of 144 molecules contained a small t-loop at one end. In a second experiment, 23 out of 115 large molecules showed a t-loop at one end. Thus, overall, approximately 15% of the ends had a t-loop. In these experiments, four minichromosomal DNAs were found that carried t-loops at both ends (Fig. 16) and a fifth double-looped minichromosome was found in a third experiment. If the t-loop frequencies in these preparations were primarily determined by the extent to which t-loops were lost during DNA isolation, due to incomplete cross-linking or breakage, the frequency of double-looped molecules would be approximately 2.25% (15% of 15%), predicting approximately 6 double-looped molecules in the 259 DNAs that were examined. This number is in reasonable agreement with the 4 double-looped molecules that were observed, suggesting that t-loops often occur at both ends of minichromosomes.

A 3´ overhang of single stranded telomeric repeats is likely to be a prerequisite for t-loop formation. However, based on the mechanism of DNA replication, 3´ overhangs are not expected to occur at chromosome ends formed by leading strand DNA synthesis.

E. coli SSB was used to query the status of the DNA at the ends of trypanosome minichromosomes (Fig. 17). *T. brucei* minichromosomes were prepared by lysis of PF cells and sucrose gradient sedimentation in the presence of sarcosyl without AMT and UV treatment. Aliquots were then chromatographed over Bio-Gel A-15m to remove the detergent and the minichromosomes incubated with *E. coli* SSB protein to bind any single stranded DNA. Single tetramers or octamers of SSB bound along the length or at ends of otherwise duplex DNA can be distinguished by EM, and represent the presence of ~75 (single tetramer) or 150 nt (octamer) of single stranded DNA (Wright et al., 1997). The minimum length of a single stranded DNA overhang that will allow binding of an SSB tetramer has not been established. Thus overhangs less than ~75 nt may be missed using this approach. Following preparation of the complexes for EM, examination of 138 minichromosomes judged to be ≥50 kb revealed that 70% showed no SSB on either end, 23% showed SSB binding at one end, and 7% of the minichromosomes had SSB at both ends (Fig. 17). To evaluate the length of the overhang, the number of SSB tetramers bound at an end was counted. For the minichromosomes with SSB bound at just one end or at both ends, 71% and 70% respectively of the ends showed from 1 to 3 tetramers bound, suggestive of overhangs in the range of 75 to 225 nt. The remaining 30% of the ends showed longer SSB bound tracts ranging up to ~500 nt. Thus, consistent with the annealing data in Fig. 12B, trypanosome chromosome ends contain substantial regions of single stranded DNA and these overhangs can occur at both ends of one chromosome.

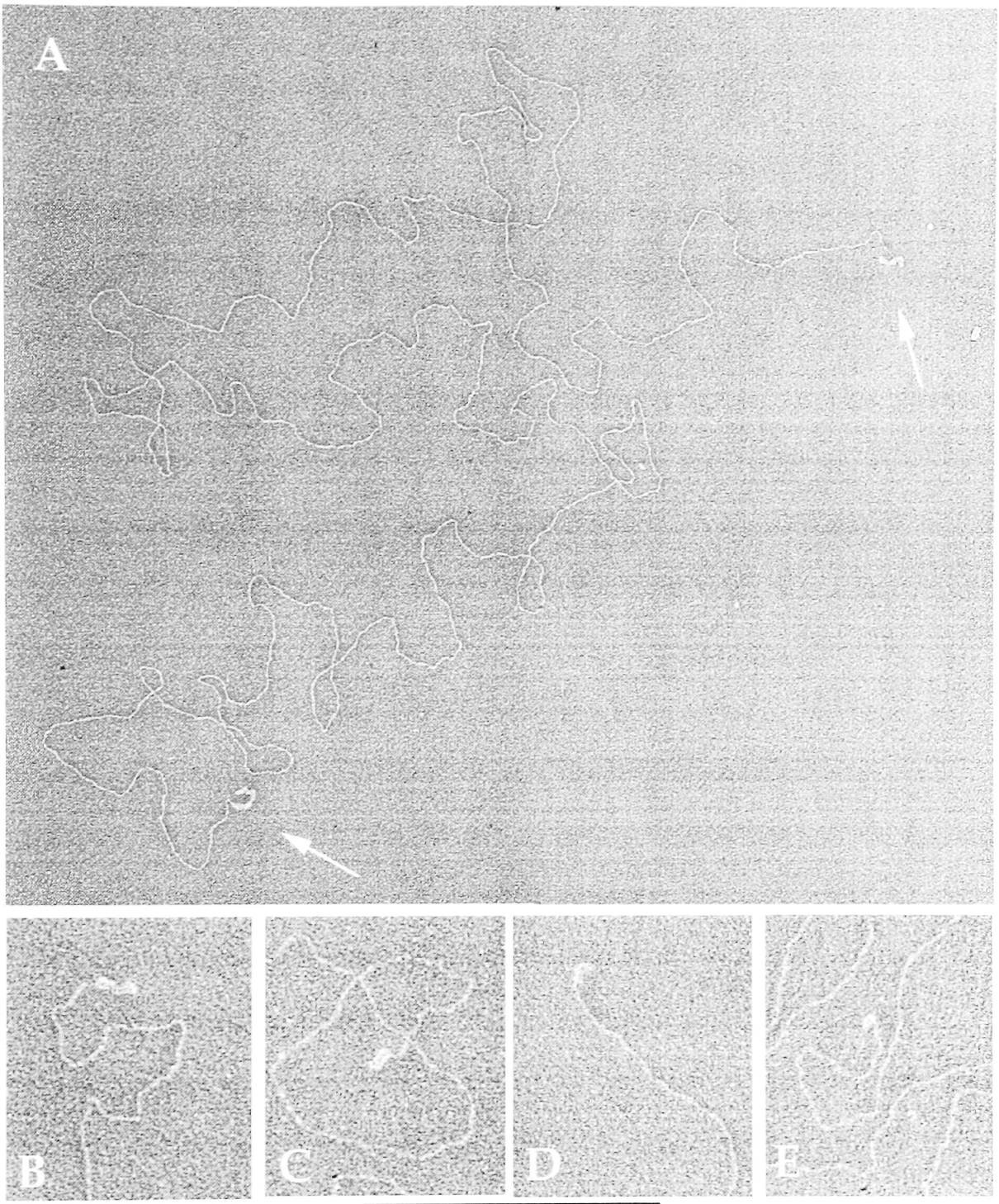


Fig. 17. *T. brucei* minichromosomes with single strand overhangs stained with a single strand DNA-binding protein. Non-crosslinked minichromosomes enriched by sucrose gradient sedimentation were incubated with *E. coli* SSB and prepared for EM as described in Materials and Methods, including adsorption to thin carbon foils, dehydration, and rotary shadowcasting with tungsten. (A) A 31-kb minichromosome with single stranded overhangs at both ends. The overhangs on this molecule are longer than most and were selected for greater visibility at low magnification. (B-E) Individual minichromosome ends with SSB bound. The size of the particle in E corresponds to a single SSB tetramer. Shown in reverse contrast. Bar equals 0.5 microns (A) and 0.27 microns (B-E). Data generated by Jack Griffith.

Trypanosome t-loops are small

The t-loops observed in this study were substantially smaller that the ones observed in mammalian DNA with telomeres of similar length (Griffith et al., 1999). Measurement of loop contour lengths of t-loops in enriched telomeric restriction fragments showed a wide variety of sizes ranging from as small as 0.3 kb to as large as 8 kb. However, greater than 65% of the loops were quite small (< 1.5 kb) and the median length of the 48 t-loops from telomeric restriction fragments was around 1.1 kb. Similarly, t-loops at the ends of isolated minichromosomes showed a range in loop sizes from as small as 570 bp to as large as 8.4 kb, with a median value of 1.0 kb for 21 loops analyzed. The combined data on the size range of the t-loops in both types of DNA preparations are given in Fig. 18. Overall, the median size of the loops was 1.1 kb and 42 out of 69 t-loops analyzed were very small, ranging between 0.5 and 1.0 kb. Furthermore, a number of trypanosome t-loops measured less than 500 bp. Large t-loops (> 3kb) were rare in both the enriched telomeric restriction fragments and in isolated minichromosomes. The level of resolution of the surface spreading method employed here is such that circles of less than 150-200 bp would frequently appear as balls rather than small loops or donuts. However, examination of the minichromosomes by directly adsorbing the samples to carbon supports and rotary shadowcasting allowed a higher resolution inspection of the DNA ends, but no circles smaller than those detected by surface spreading were observed.

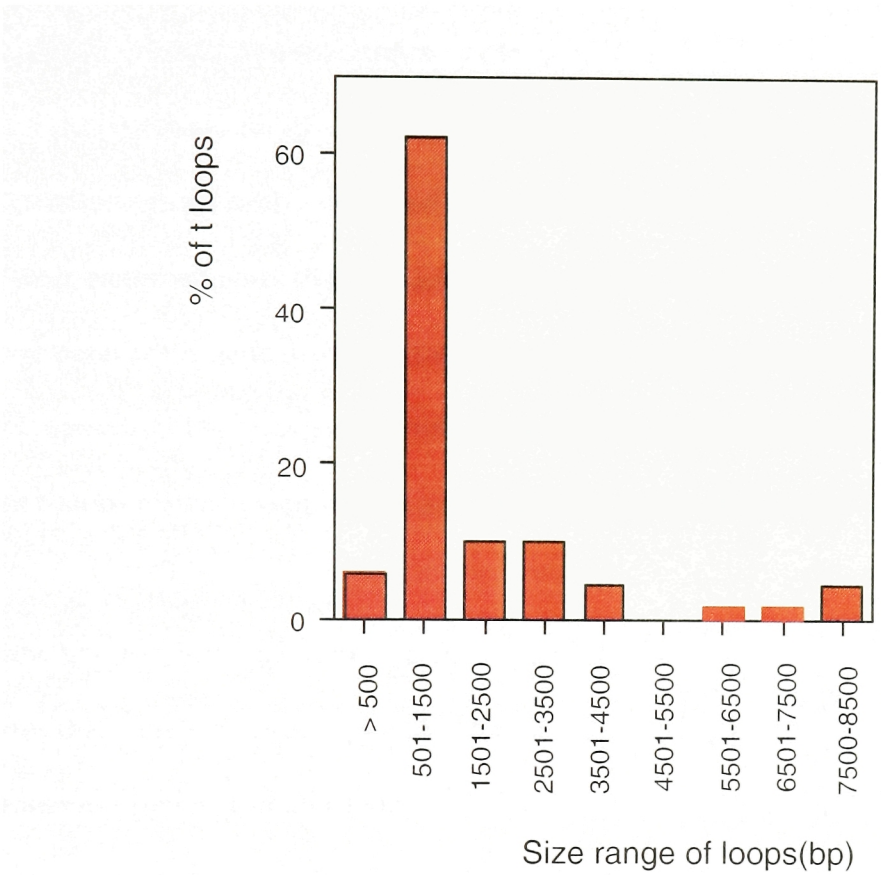


Fig. 18. Size distribution of trypanosome t-loops. Bar graph depicting the size distribution of *T. brucei* t-loops. The data were obtained from measurements of 48 t-loops in enriched telomeric restriction fragments (examples shown in Fig. 14) and from measurements of 21 t-loops at the ends of minichromosomes (examples shown in Fig. 16)

Discussion

This chapter provides a description of the structure of trypanosome telomeres. The data presented demonstrate that 3' overhangs and t-loops are present in trypanosomes. Trypanosomes probably originated more than 500 million years ago, long before the origin of their metazoan hosts (Stevens and Gibson, 1999). As pointed out earlier, the biology of these parasites is quite distinct from the perceived norm, as represented by yeast, plants, and mammals. The conservation of t-loops in these highly divergent organisms predicts that t-loops play an essential role at telomeres in many eukaryotes.

Although they have the same sequence and overall length, trypanosome telomeres had loops that were significantly smaller than those of mammalian telomeres. Trypanosome telomeres often had small t-loops (median size 1.1 kb) whereas human telomeres very rarely showed t-loops in that size range. For instance, HeLa cells with telomeres in the 20-kb range had t-loops with a median size of 14 kb and less than 2% of the t-loops were 1 kb or smaller. The smallest human t-loops were observed in cells with telomeres composed of ~5 kb TTAGGG repeats, but these t-loops were still significantly larger (median 3 kb) than those of trypanosomes. Similarly, the loops observed at the ends of micronuclear chromosomes of *Oxytricha fallax* were much larger (5 to10 kb loops (Murti and Prescott, 1999)) than trypanosome t-loops and more comparable to those of mammalian cells. Although it is not clear what determines the size of the t-loops, the data suggest that there is an active process involved in establishing or maintaining t-loops of a specific size.

The previous demonstration of looped structures at the ends of *Oxytricha fallax* micronuclear chromosomes is in agreement with the proposal that t-loops are highly conserved (Murti and Prescott, 1999). Interestingly, this organism also provides an example of functional telomeres that lack t-loops. The macronuclear DNA of *Oxytricha* and other hypotrichous ciliates is formed by extensive fragmentation and processing of the micronuclear genome, resulting in amplified small DNA fragments each carrying one gene. These gene-sized molecules are all endowed with short telomeres that contain less than 50-bp duplex telomeric DNA and a short single-stranded overhang (Price, 1999). Given their extreme short size, it was anticipated that these telomeres would lack t-loops, a prediction consistent with the EM analysis. Instead, the ends of the macronuclear DNAs may be protected by the tenaciously bound protein complex(Gottschling and Zakian, 1986; Horvath et al., 1998; Price, 1999). Collectively, the presence of t-loops in organisms as diverged as mammals, ciliates and kinetoplastids indicate that this aspect of telomere structure is highly conserved.

The finding of t-loops in diverged eukaryotes has raised the question whether they occur in budding yeast, where telomeres have been characterized extensively. A loop-like structure was proposed by Li and Lustig as an intermediate in the rapid deletions that can occur when yeast telomeres are excessively long (Li and Lustig, 1996). Telomere folding (without strand invasion) was also proposed as a mechanism to explain telomere length regulation in *K. lactis* (McEachern and Blackburn, 1995), and a fold-back structure for telomeres in *S. cerevisiae* has been proposed based on studies of transcriptional regulation of subtelomeric genes (de Bruin et al., 2000a; de Bruin et al., 2000b). Similarly, based on

their ability to cross-link the telomeric DNA-binding protein Rap1p to subtelomeric Y´ elements, Grunstein and co-workers proposed that yeast telomeres form a higher order structure in which the telomere is folded back along the subtelomeric DNA (Grunstein, 1997). Technical limitations of the current t-loop assays have hindered direct examination of yeast telomere structure.

A significant difference between yeast telomeres and those of trypanosomes and mammals is that yeast telomeres appear to lack long single-stranded protrusions (except for a short window late in S-phase) (Wellinger et al., 1993). Such telomere tails are presumed to be required for the strand-invasion that creates the t-loop. Furthermore, t-loop formation in mammals has been proposed to depend on the telomeric protein TRF2 and a recent comparison of the mammalian and yeast telomeric complexes has suggested that the budding yeast has lost the genes encoding both TRFs (Li et al., 2000). Interestingly, the major yeast telomeric DNA binding protein Rap1p has the ability to promote the pairing of single-stranded telomeric DNA with duplex repeat tracts in vitro, an activity that could be indicative of a role in higher order remodeling of telomeric DNA (Gilson et al., 1993).

Several trypanosome minichromosomes showed t-loops at both ends. The frequency of double-looped molecules was high enough to suggest that t-loops are formed at DNA ends created by both lagging and leading strand synthesis. The two modes of DNA synthesis are predicted to generate different ends. Lagging strand synthesis generates a 3´ overhang with a length that depends primarily on the site where primase synthesized the last RNA primer; removal of the RNA primer could contribute an additional 8 to 12 nt to the protrusion. By

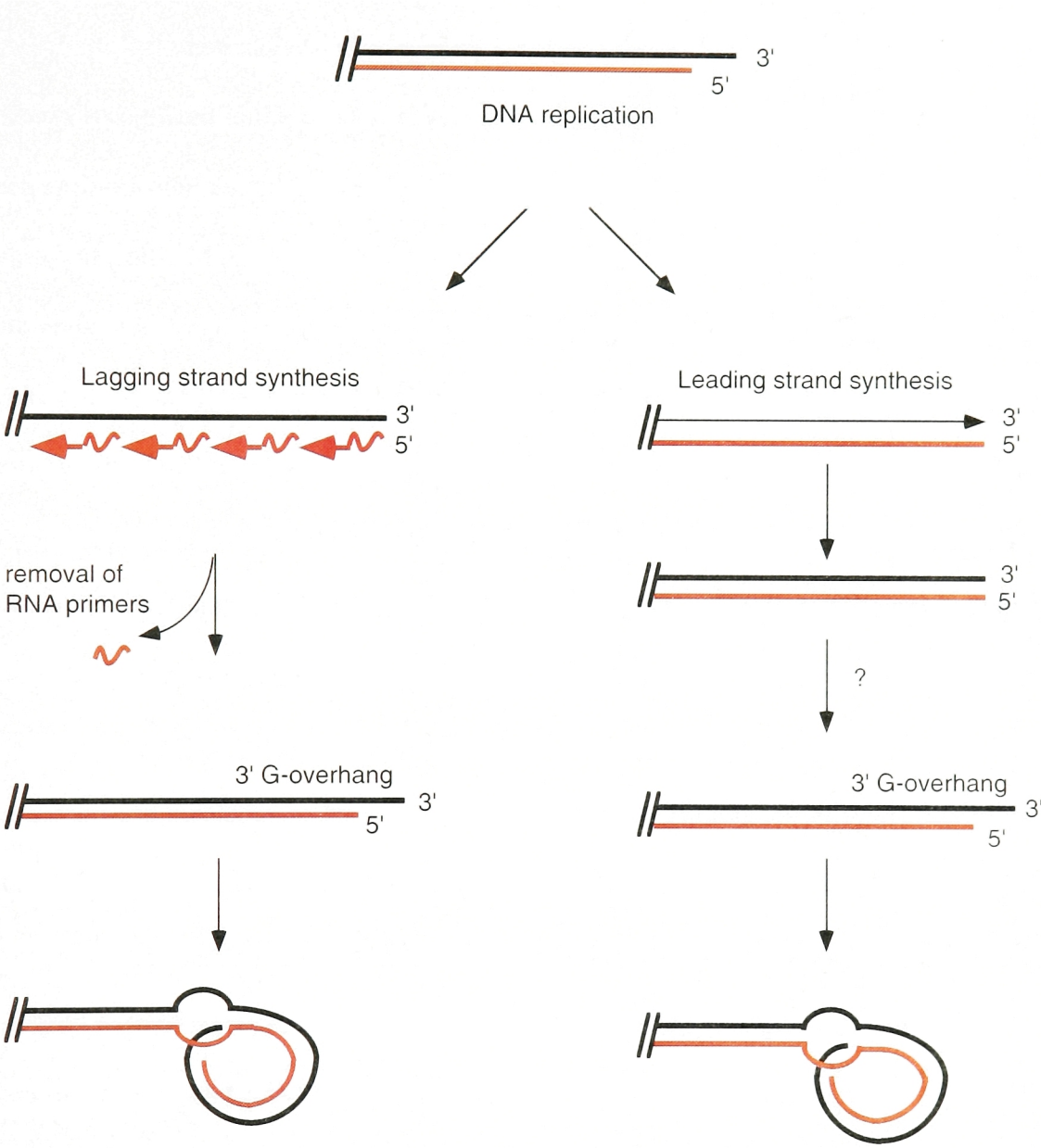


Fig. 19. Current view of how t-loops can be generated at the two ends of a chromosome. At one end of the chromosome, lagging-strand synthesis generates a 3' overhang after removal of RNA primers. At the opposite end, leading strand synthesis replicates DNA continuously to the end of the molecule. How an overhang is generated at this end is not known.

contrast, leading strand synthesis should result in a blunt end, and formation of a 3´ overhang was therefore suggested to require a nuclease (Fig. 19). This dilemma was previously recognized in the context of tests for the presence of 3´ overhangs at both ends of each chromosome and there are conflicting reports on the terminal structure of human chromosome ends (Makarov et al., 1997; Wright et al., 1997). The existence of overhangs at both ends of a *S. cerevisiae* chromosomes had been suggested by the finding that the two opposite ends of artificial plasmids containing 3' overhangs can interact during S phase by a presumed mechanism involving non-Whatson-Crick base pairing (Wellinger et al., 1993). *Streptomyces* linear plasmid pSLA2 is replicated bidirectionally from an internal origin and ends in two 3' overhangs of similar lengths (Chang and Cohen, 1994); but the implication of that finding in prokaryotes is not clear.

There are several mechanisms by which the end created by leading strand synthesis could acquire a 3´ overhang. Telomerase could synthesize the overhangs, but this is contradicted by the finding of overhangs in cells lacking telomeresase. In addition, lack of binding of Cdc13p or Ku70/80 to their respective telomeric DNA targets generates longer 3' overhangs by shortening of the 5' end (Garvik et al., 1995; Driller et al., 2000). It is possible that, in the newly replicated blunt-ends of chromosomes, lack of efficient binding of telomeric proteins can allow access of 5'->3' nucleases to the telomere; a situation that is rapidly corrected by the binding of such proteins once the 3' overhang has been generated, providing a mechanism that regulates formation and length maintenance of the 3' overhang. In mammalian cells, where t-loops have been described and TRF2 has been implicated in forming t-loops (Griffith et al., 1999), the absence of TRF2 generates blunt telomeres (Smogorzewska et al., 2000). It

is possible that TRF2 brings to telomeres a 5' exonuclease activity capable of generating the 3' overhang at the leading strand end of the chromosome. The resent finding of an interaction between TRF2 and Mre11 brings the possibility that the 5' exonuclease activity of Mre11 is responsible for the formation of the 3' overhangs.

Regardless of the mechanism of their formation, the presence of overhangs and t-loops at both ends of trypanosome chromosomes further corroborates the idea that t-loops are required for the protection of all chromosome ends.

The finding of t-loops in trypanosomes suggests the presence of a specific stabilizing protein component at telomeres. The data in this chapter indicate that 3' overhangs are generated and maintained by an active mechanism and not only by lack of DNA replication at telomeres. While mammalian telomeric proteins TRF1 and TRF2 are probably involved in t-loop formation and other proteins have been identified in the mammalian and yeast system, trypanosome telomere factors have not been identified. The finding of telomeric proteins in trypanosomes should propel our understanding of how telomeres are protected in these organisms and could clarify some of the evolutionary questions posed here.

Chapter 3

Expression of TRF1 in *T. brucei*

Homeostatic mechanisms, involving the binding of specific proteins to the duplex part of the telomeric repeats, operate to maintain a telomere length in different species, even in cells with high levels of telomerase activity such as the mammalian germline (Wright et al., 1992). The telomere-binding proteins Rap1p in *S. cerevisiae*, Taz1 in *S. pombe*, TRF1 and TRF2 in animals, bind duplex telomere repeats and are negative regulators of telomere length (Cooper et al., 1997; Marcand et al., 1997; van Steensel and de Lange, 1997; Smogorzewska et al., 2000). The negative regulation of telomere length by TRF1 and TRF2 can be explained by their involvement in t-loop formation (Griffith et al., 1999), which presumably creates a limitation in the accessibility of the 3´ overhang to telomerase.

Several direct and indirect experiments indicate the presence of precise mechanisms of telomere maintenance in trypanosomes. Unlike most organisms, which exhibit considerable telomere length polymorphism, *T. brucei* tightly regulates telomere length; and curiously, telomeres grow by approximately 7-10 bp per cell division (Bernards et al., 1983; Pays et al., 1983; van der Ploeg et al., 1984). Telomerase activity has been described in *T. brucei* (Cano et al., 1999). Position effects within the ES (Horn and Cross, 1995; Rudenko et al., 1995; Horn and Cross, 1997), chromatin remodeling and developmentally regulated repression effects close to telomeres, have been demonstrated (Navarro et al., 1999). In addition, BF but not PF trypanosomes modify a portion of T residues to convert them into the glycosylated base J, the majority of which are located at telomeres (van Leeuwen et al., 1996; van Leeuwen et al., 1998). Trypanosome telomeres redistribute during the cell cycle.

They are situated around the periphery of the nucleus during G1-S phase (Chung et al., 1990) and as cell division progresses, minichromosomes and therefore most of the telomeric DNA cluster in the center of the nucleus during mitosis. In post-mitotic cells, minichromosomes locate at opposite poles of the dividing nuclei (Ersfeld and Gull, 1997; Ogbadoyi et al., 2000).

The accumulating evidence for a precise telomere maintenance mechanism in trypanosomes and the finding of t-loops in this organism, point to the possibility that telomere-binding proteins are present in trypanosomes. Proteins that bind to the duplex part of the telomere, however, remain unidentified in organisms other than yeast and mammals. In an attempt to provide trypanosome protein extracts with a known TTAGGG-binding activity, the human protein TRF1 was expressed in BF and PF *T. brucei*. The effects of TRF1 binding are consistent with the role of TRF1 as a negative regulator of telomere length and with the presence of endogenous telomeric proteins in trypanosomes.

To express TRF1 in *T. brucei*, the inducible, integrating construct pLew111 was employed (Hoek et al., 2000). In this construct, the FLAG-TRF1 fusion was inserted between a T7 promoter and a gene conferring resistance to phleomycin (*BLE*). Fig. 20A depicts this construct and the salient features of TRF1. Plasmid pLew111 containing TRF1 was transfected into PF 29-13 and BF 13-90 cells (Wirtz et al., 1999). Upon low induction with doxycycline (0.1 μg ml^{-1}) and under selection with phleomycin (2.5 μg ml^{-1}), 11 PF clones and 6 BF clones expressing TRF1 were obtained from two independent transfections. All PF and BF clones were analyzed by western blotting, which revealed equal levels of TRF1 in all clones. Comparing with known quantities of baculovirus-expressed TRF1, it was established that, upon induction with high levels of doxycycline (1 μg ml^{-1}), trypanosomes express ~$5x10^5$ molecules of TRF1 per cell. These levels of expression of TRF1 largely exceed those normally present in mammalian cells. Considering that trypanosomes contain 15 kb of telomere tracts, and 230 chromosome ends, they most contain approximately 5.7 x 10^5. Assuming that a TRF1 homodimer is bound every 2 1/2 repeats, it is likely that most of the TRF1 expressed is bound to the trypanosome telomeres. Moreover, trypanosomes expressed high levels of TRF1 for prolonged periods of time (Fig. 20B). An irrelevant cross-reacting band of 50 kDa is seen throughout the blot, and does not vary between samples from induced and non-induced cells.

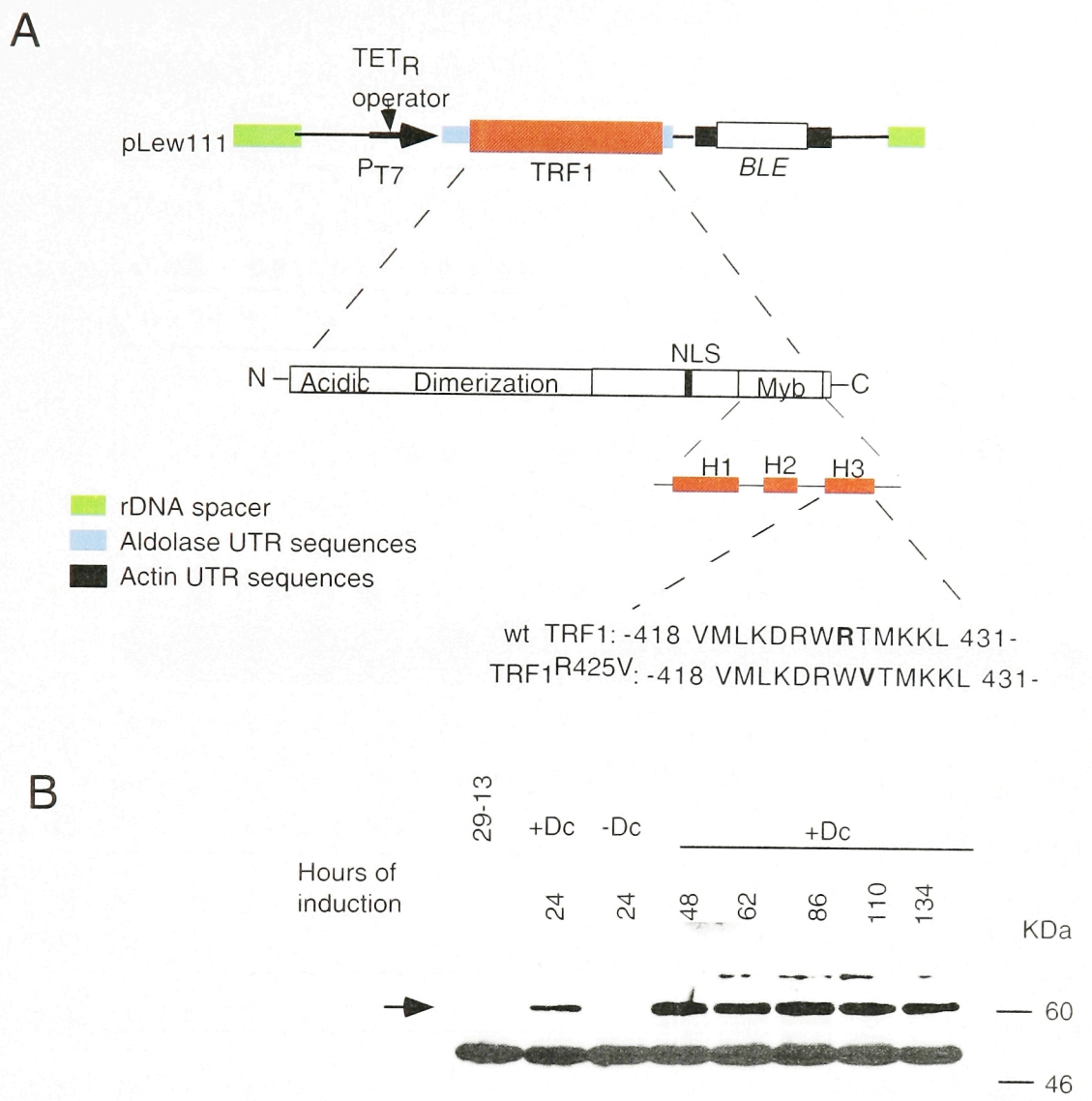


Fig. 20. Inducible expression of TRF1 and TRF1R425V in *T. brucei*. (A) FLAG-tagged *TRF1* was inserted into pLew111 immediately downstream of a T7 promoter containing a TetR operator. The construct was transfected into BF 13-90 or PF 29-13 cell lines, which express T7 RNA polymerase and TetR. The site of insertion is a ribosomal DNA spacer. The untranslated regions flanking *TRF1* and *BLE* are depicted. In the presence of tetracycline (or doxycycline), the T7 promoter is de-repressed and *TRF1* and the bleomycin resistance gene (*BLE*) are transcribed. The salient characteristics of the *TRF1* gene are identified. H1, H2 and H3 are the three helices of the Myb domain. A point mutation was introduced in the third helix of the Myb domain that changes an arginine for a valine at position 425, creating TRF1R425V. (B) A western blot using antibodies for FLAG shows inducible expression of TRF1 in the PF 29-13 strain. As indicated by the arrow, robust expression of TRF1 was obtained 24 h after induction with doxycycline (+Dc) and increased to a constant level after 48 h. The upper band probably corresponds to traces of TRF1 dimers. An endogenous 50-kDa band is also detected by the anti-FLAG antibody.

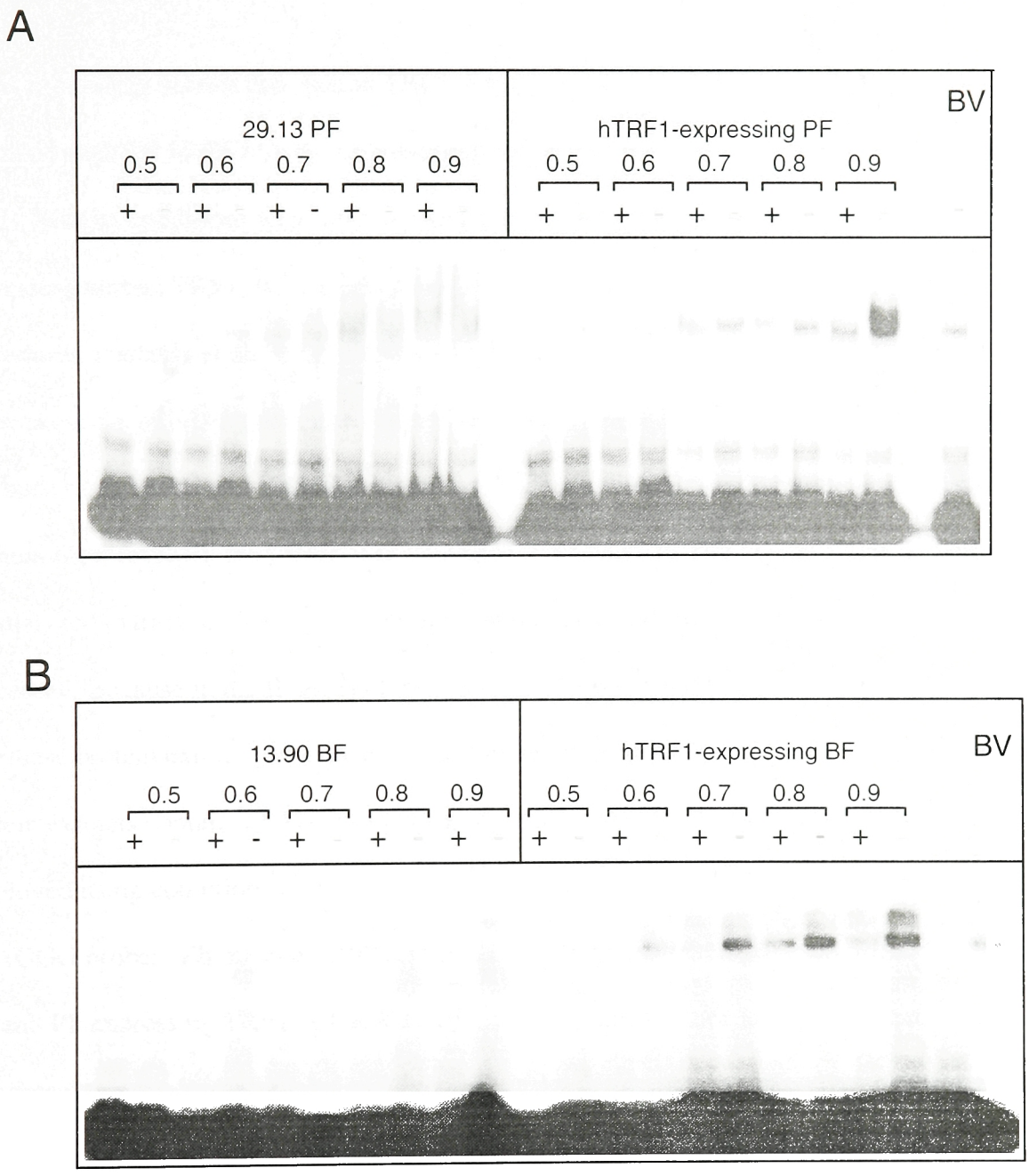


Fig. 21. TRF1 electrophoresis shift assays. Increasing amonts of partially purified proteins (indicated in µg) from 13.90 BF (A) and 29.19 PF (B) trypanosomes expressing or not expressing TRF1 were allowed to bind radiolabeled $(TTAGGG)_{12}$ probe. The reactions were performed in the presence (+) and in the absence (-) of unlabeled $(TTAGGG)_{27}$ as indicated. Baculovirus-purified TRF1 (1 ng) was used as control for the binding reactions.

TRF1-binding activity in trypanosome protein extracts

It was previously known that human TRF1 activity is present in HeLa cell nuclear proteins extracted with 0.4 M KCl (high salt) and dialyzed in 100 mM KCl (low salt) (Zhong et al., 1992). Similar conditions were used to extract proteins from trypanosomes expressing or not expressing human TRF1. Because trypanosome nuclei are easily disrupted by the procedures available at the time of this study, the conditions were adapted to extract proteins in the presence of NP40 (see materials and methods). After lysis and solubilization in high salt buffer, the lysate was ultracentrifuged and the supernatant dialyzed in low salt buffer. Immunoblots revealed the presence of comparable amounts of TRF1in the lysate as well as in the dialyzed extract, indicating that no substantial amount of TRF1 is lost in this simple procedure. Because in the IF assays (see below) TRF1 localized to the nucleus, it is possible that these protein extracts contained substantial amounts of nuclear proteins. To test if these protein extracts contain TTAGGG-binding activities, gel mobility shift assays (EMSA) were employed using conditions previously determined for binding of TRF1and radiolabeled TTAGGG probes (Zhong et al., 1992) (Fig. 21). It was established that protein extracts from BF and PF expressing TRF1 contained TRF1 activity, and that this activity was partially competed by introducing unlabeled TTAGGG probe. With protein extracts not containing TRF1, band shifts were not detected or were not competed by unlabeled probe. In the absence of nonspecific DNA competitor, gel shifts were obtained, indicating that the protein extracts contain DNA binding activities, but no convincing TTAGGG-specific activities under conditions that favor the binding of TRF1.

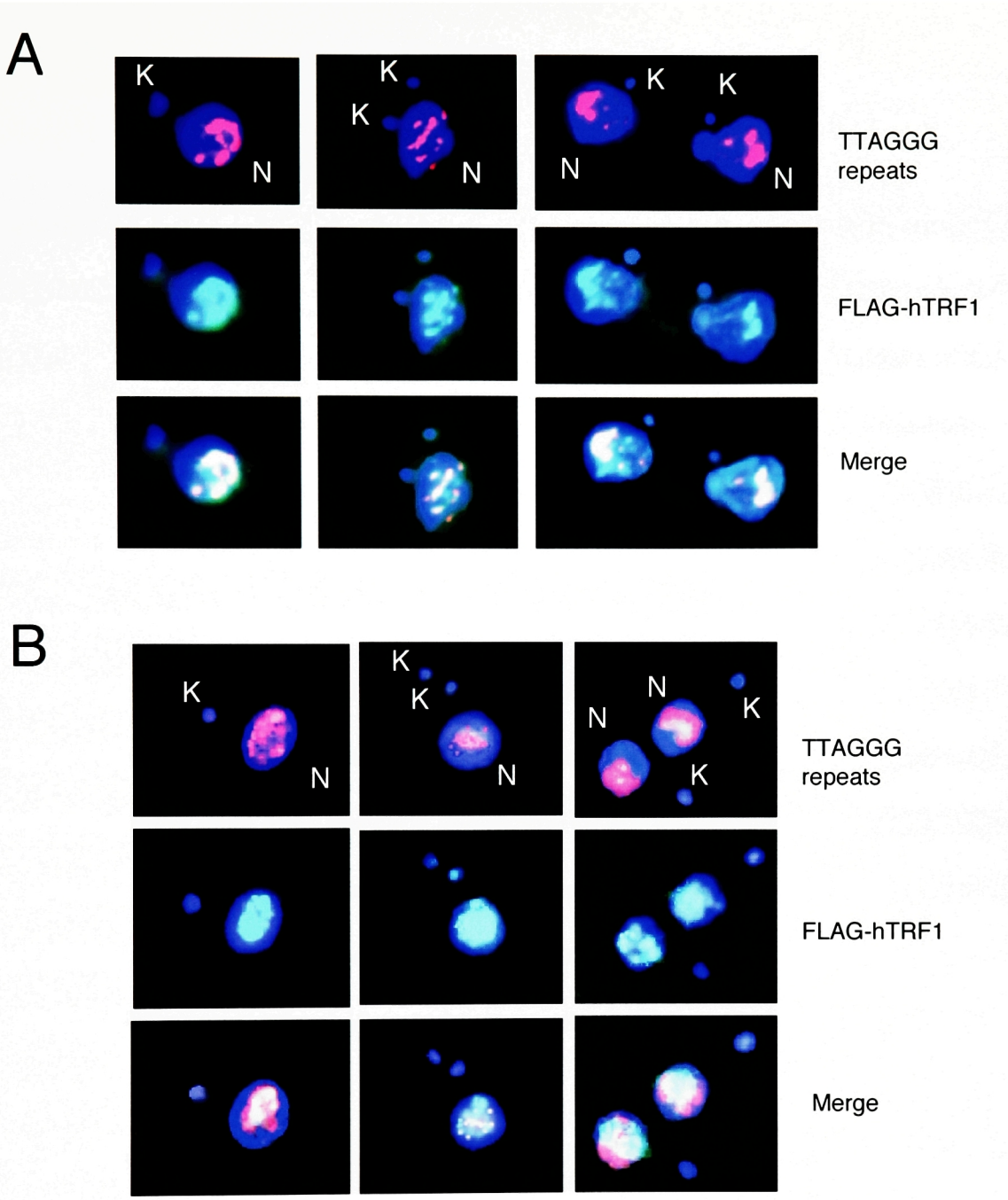


Fig. 22. TRF1 binds to telomeres in PF but not in BF trypanosomes. Nuclear and kinetoplast DNA are detected by DAPI staining (blue fluorescence). An in situ hybridization for TTAGGG repeats (red fluorescence) indicates where the telomeric DNA is located and an indirect immunofluorescence for FLAG-TRF1 (green fluorescence) shows distribution of TRF1in PF (A) and BF (B). The left panels show a trypanosome with one kinetoplast (K) and one nucleus (N). In the middle panels, a trypanosome in S phase with 2 K and 1 N exhibits telomeres aligned in the center of the nuclei. The right panel depicts a post-mitotic trypanosome with 2 K and 2 N, where telomeres are situated at opposite poles of the nuclei. The merge between the red and the green fluorescence (yellow) reveals complete co-localization of TRF1 and telomeric repeats in PF throughout the cell cycle. In contrast, BF exhibited incomplete localization of TRF1 to telomeres.

TRF1 binds to PF telomeres

To examine the possible binding of TRF1 to trypanosome telomeres in vivo, fluorescence in situ hybridization (FISH) coupled with IF were performed on PF and BF trypanosomes expressing TRF1 (Fig. 22A and 22B). The red fluorescence indicated the presence of the digoxygenin-labeled RNA probe specific for telomeric repeats. The green fluorescence reveals the binding of rabbit antibody 371 to TRF1. The merge between the top and middle panels revealed complete co-localization of TRF1 and telomeres in the PF. In contrast, the BF did not exhibit complete localization of TRF1 and telomeres and TRF1 does not condense to punctuate spots as in PF. It was also observed that the division rate of PF expressing TRF1 declined after 48h (see below) in all PF trypanosome clones. In contrast, BF trypanosomes carrying the TRF1 gene grew normally and independently of the presence of TRF1. This indicated a possible correlation between the binding of TRF1 to telomeres and an effect on cell division. In non-transfected as well as in non-induced trypanosomes no immunofluorescence staining is detected using antibody 371.

Binding of TRF1 to PF telomeres arrests cell division

To further study the correlation between the binding of TRF1 to telomeres and the effects of this binding on cell division, the TRF1 mutant allele, $TRF1^{R425V}$, deprived of telomere-binding activity was employed. $TRF1^{R425V}$ carries a point mutation that changes the arginine (R) codon AGG for the valine (V) codon GUG in the DNA-recognition helix of TRF1 (Fig. 20A). This mutation abolishes the binding of TRF1 to duplex TTAGGG repeats in vitro and in mammalian

cells (H. Moss and T. de Lange, manuscript in preparation). By site-directed mutagenesis, the same point mutation was made in the TRF1 copy previously FLAG-tagged and cloned in pLew111. PF were transfected with pLew111 carrying FLAG-TRF1^{R425V} and 10 phleomycin-resistant clones expressing TRF1^{R425V} were obtained. By protein blotting, it was established that the levels of expression of TRF1 and TRF1^{R425V} were identical (data not shown).

In Fig. 23A, the growth rates of induced and non-induced PF carrying TRF1 or TRF1^{R425V}, and the parental procyclic line 29-13 (PF 29-13), are compared. Trypanosomes expressing TRF1 suffered a reduction of their duplication rate. After 62 h expressing TRF1, cell growth was almost completely arrested. Nonetheless, most of the cells in the culture were alive, as evidenced by their motility. After 72 h, when cell density in most of the cultures was 1–2 x 10^7 trypanosomes ml^{-1}, and 5–6 x 10^6 for the cells expressing TRF1, all cultures were diluted in fresh medium to 8 x 10^5 trypanosomes ml^{-1} in order to allow continuous exponential growth. In addition, trypanosome cultures previously growing in the presence of doxycycline were split and grown in the presence and in the absence of doxycycline. Trypanosomes previously expressing TRF1 but deprived of doxycycline at 72 h (rescued), began to divide at a normal rate of 6–8 h, comparable to the normal division rate exhibited by the TRF1^{R425V}-expressing cells. The growth rate of trypanosomes expressing TRF1 also increased gradually after 72 h. Altogether, trypanosomes expressing high levels of TRF1 exhibit a cell division arrest phenotype after 48–62 h of continuous induction (6–8 divisions). This cell division arrest extends for 24–48 h, after which trypanosomes began to divide at a normal rate of 1 duplication per 6–8 h. Similar results were found with two different trypanosomes originating from two independent transfection experiments and expressing similar levels of TRF1.

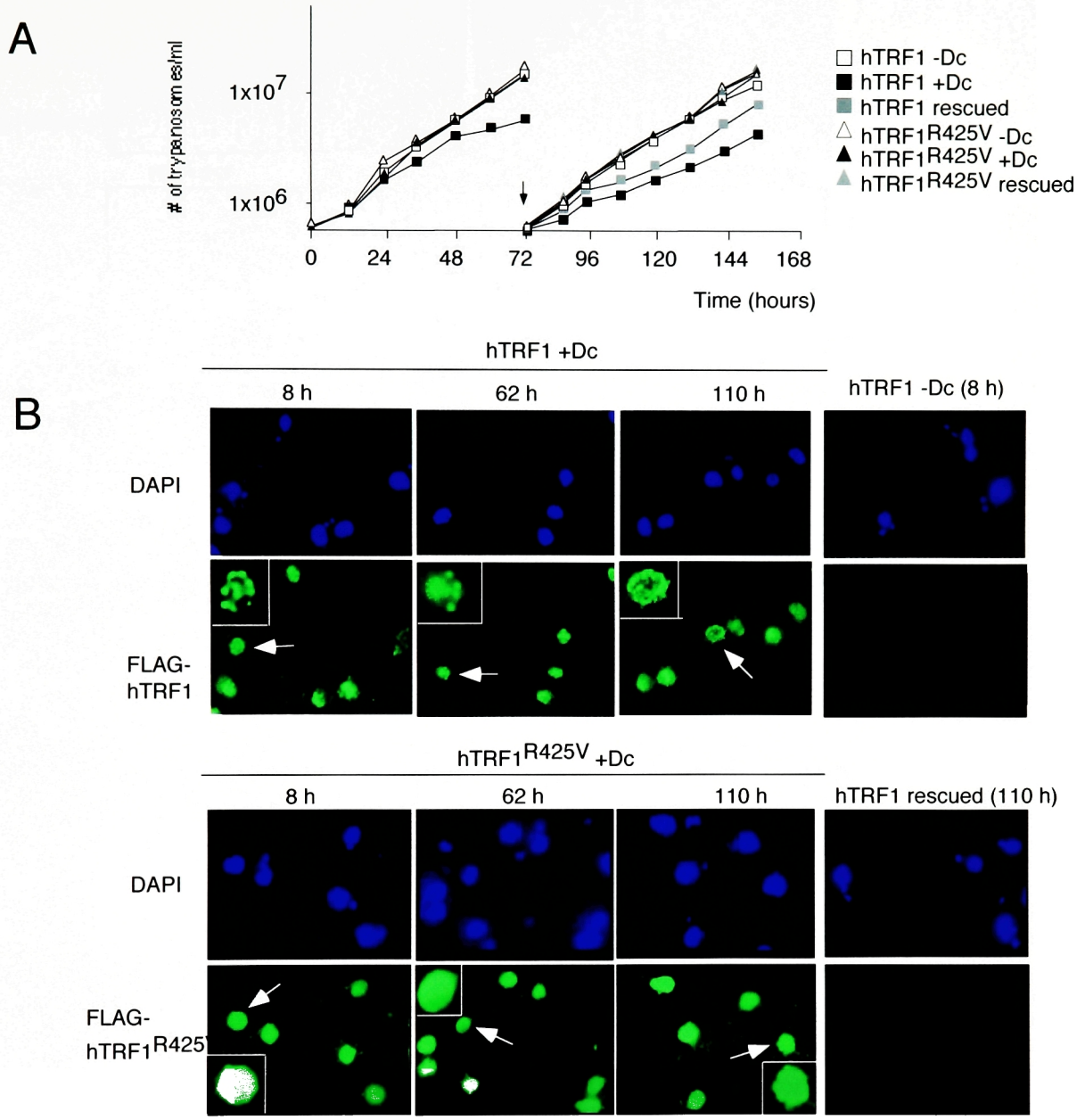
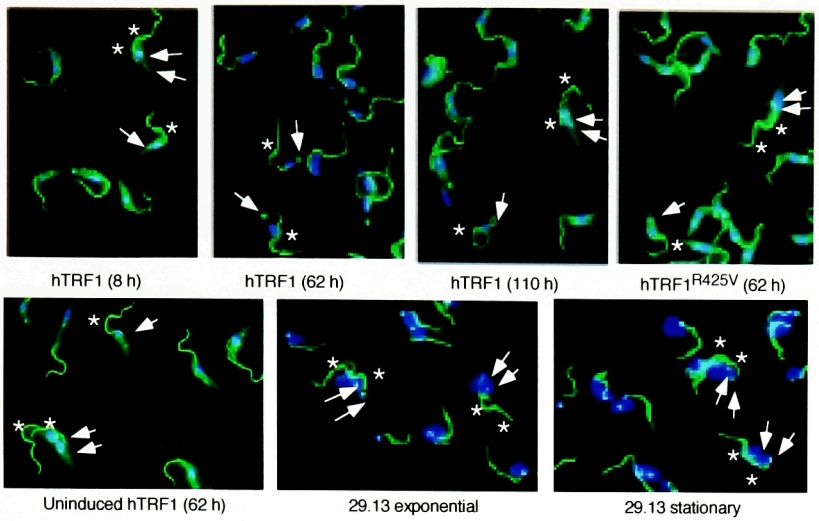


Fig. 23. Binding of TRF1 to telomeres correlates with transient cell division arrest. (A) PF 29-13 carrying TRF1 or TRF1R425V in the presence and in the absence of doxycycline, as indicated, were counted during exponential growth. After 72 h, as indicated by the arrow, cultures were diluted to 8 x 105 trypanosomes ml-1 to continue exponential growth. A portion of trypanosomes previously growing in the presence of doxycycline were then grown in the absence of the drug (rescued). Trypanosomes continuously expressing TRF1 exhibited a transient cell division arrest between 48 and 84 h. (B) At high levels of induction with doxycycline, TRF1 (green fluorescence) localizes to the nucleus (blue fluorescence) in a punctate pattern that reflects its telomeric binding. After 8 h of induction, 85-90% of the cells expressed high levels of TRF1, whereas the rest of the cells expressed low levels. After 110 h, 65% of the cells expressed intermediate levels of TRF1 and 45% exhibited low or undetectable levels of TRF1. In contrast, TRF1R425V exhibited a broad pattern of distribution in the nuclei, which is maintained throughout extended periods of time. Cells not induced with doxycycline, and rescued cells previously induced but deprived of doxycycline at 72 h, do not show detectable levels of TRF1. The inserts show the trypanosomes indicated by the arrows at higher magnification.

A



B

% of cells at a particular phase of the cell cycle		hTRF1			hTRF1^{R425V}			29.13	
		8h	62 h	110 h	8h	62 h	110 h	E	S
G1	+Dc	73	94	74	73	73	74	73	74
	-Dc	74	73	74	73	74	73		
S	+Dc	9	6	9	9	9	9	9	8
	-Dc	8	9	9	9	9	9		
G2	+Dc	8	0	8	8	8	8	8	8
	-Dc	8	9	9	8	8	8		
M PM	+Dc	10	0	10	10	10	9	10	10
	-Dc	10	9	9	10	9	10		

C

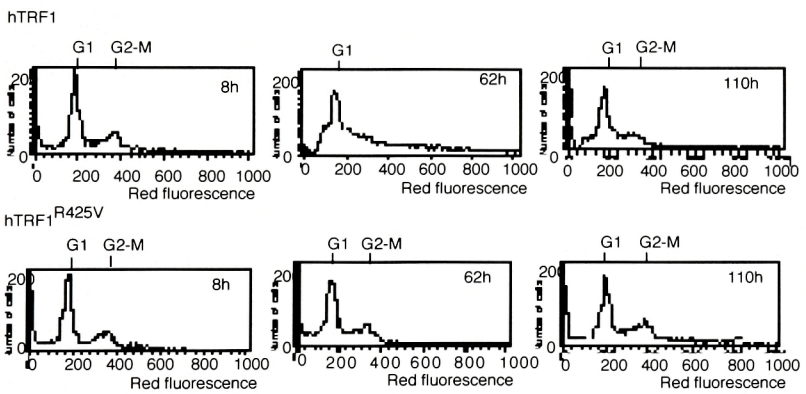


Fig. 24. Cells expressing TRF1 arrest at G1-S. (A) Using antibodies for the flagellar basal body (arrows) and the paraflagellar rod (asterisks), both shown by green fluorescence, trypanosomes at different phases of the cell cycle can be identified. Dividing and non-dividing trypanosomes can be seen after 8 h expressing TRF1. After 62 h, only non-dividing forms are present. After 110 h, dividing forms reappeared. Trypanosomes lacking the TRF1 or TRF1R425V constructs (the parental 29-13 line) were also collected in exponential and stationary phases. Cells growing in the absence of doxycycline, or cells expressing TRF1R425V, or parental cells, can be found as dividing and non-dividing forms at all times. (B) For each time point, 100 cells were counted under the fluorescence microscope. The data are summarized as the percentage of cells at a specific phase of the cell cycle for trypanosomes carrying TRF1 or TRF1R425V either induced (+Dc) or not induced (-Dc). After 62 h, most trypanosomes expressing TRF1 contained one flagellum and 1 basal body (some contain 2 basal bodies), and all contained one nucleus. 29-13 cells in exponential (E) or stationary (S) phase were counted only in the absence of Dc. (C) Cells expressing TRF1 or TRF1R425V were fixed in ethanol and DNA stained with propidium iodide prior to flow cytometry. After 8 h of induction, 2 peaks were detected. The first peak represents a population of cells in G1, whereas the second smaller peak represents dividing cells (G2–M), with twice the intensity of the first peak. After 62 h, cells expressing TRF1 showed only the first peak, whereas cells expressing TRF1R425V showed both peaks. After 110 h both TRF1- and TRF1R425V-expressing cells divided normally.

Cell-division arrest was only observed after high and prolonged induction of TRF1. For this reason, and because a lower resolution camera was used, the punctate pattern of TRF1 is not as easily seen in Fig. 23B as it is in Fig. 22A. However, the nuclear distribution of $TRF1^{R425V}$ was homogeneous and readily distinguishable from that of wild-type TRF1. Some cells expressed less TRF1 than others, and the punctate pattern was more obvious in cells expressing lower levels. After 110 h, when cells have escaped from cell division arrest, more cells expressed lower levels of TRF1 than at previous time points. Trypanosomes not induced, or previously induced but deprived of doxycycline at 72h, do not express detectable levels of TRF1. These findings indicate that the DNA binding of TRF1, and not just the presence of the protein, arrests cell division.

G1-S cell cycle arrest in T. brucei caused by TRF1 binding

Trypanosomes expressing TRF1 or $TRF1^{R425V}$ were investigated to establish whether the cell division arrest occurs at a specific phase of the cell cycle. Trypanosomes carrying TRF1 or $TRF1^{R425V}$ were collected after 8, 62 and 110 h of continuous growth in the presence or in the absence of doxycycline, and IF was performed using the primary antibodies BBA4 for the flagellar basal body and ROD1 for the paraflagellar rod proteins PFR180 and PFR200 (Woods et al., 1989). Trypanosome nuclei were co-stained using the DNA-intercalating dye DAPI. During the exponential growth of PF 29-13, approximately 73% of the cells exhibit one nucleus, one kinetoplast, one flagellar basal body, and one flagellum. These trypanosomes are considered to be in G1. Because nuclear division is anticipated by kinetoplast division, and the flagellar basal body divides before the nucleus, trypanosomes in

nuclear S phase are distinguished by having 2 basal bodies, one flagellum and one nucleus. Towards the end of the nuclear S phase, the new paraflagellar rod becomes visible. Trypanosomes in S phase normally represent 9 % of the cells in culture. Cells in G2, prior to cell division, exhibit an elongated nucleus and an incipient second flagellum. These account for 9% of the cells in the culture. The remaining 10% of the cells consists of mitotic (M) or post-mitotic (PM) cells, with 2 complete flagella and one elongated or completely divided nucleus. These proportions of percentage of cells at each particular phase of the cell cycle are consistent with previous reports (Woodward and Gull, 1990).

As indicated in Fig. 24A, after 8 h induction of TRF1 expression, cells at different phases of the cell cycle are present. After 62 h, when the cells were not dividing, 100 % of the cells were in G1-S. After 110 h of induction, cells in G2 or M reappeared. Cells expressing $TRF1^{R425V}$or not induced with doxycycline can be found in all phases of the cell cycle. The data from these studies are summarized in Fig. 24B. No cells were found that exhibited 2 flagellar rods or 2 nuclei. This contrasts with the normal distribution of 73% G1/ 9% S/ 8% G2/ 10% M-PM. In the absence of doxycycline, or while expressing $TRF1^{R425V}$, trypanosomes divided normally. After 110 h, all cultures contained only cells dividing normally with a normal distribution of cells at different phases of the cell cycle. The DNA content of TRF1- and $TRF1^{R425V}$-espressing cells was also evaluated for the same periods of time by flow cytometry (Fig. 24C). Cells dividing normally showed 2 peaks. The first peak represents approximately 80 % of the cells that are in G1 and therefore contain one nucleus (2n). A second peak represented the remaining 20% of the cells in G2-M, with 2 nuclei (4n). After 8 h expressing TRF1 or $TRF1^{R425V}$, the 2 peaks can be observed. However, after 62 h,

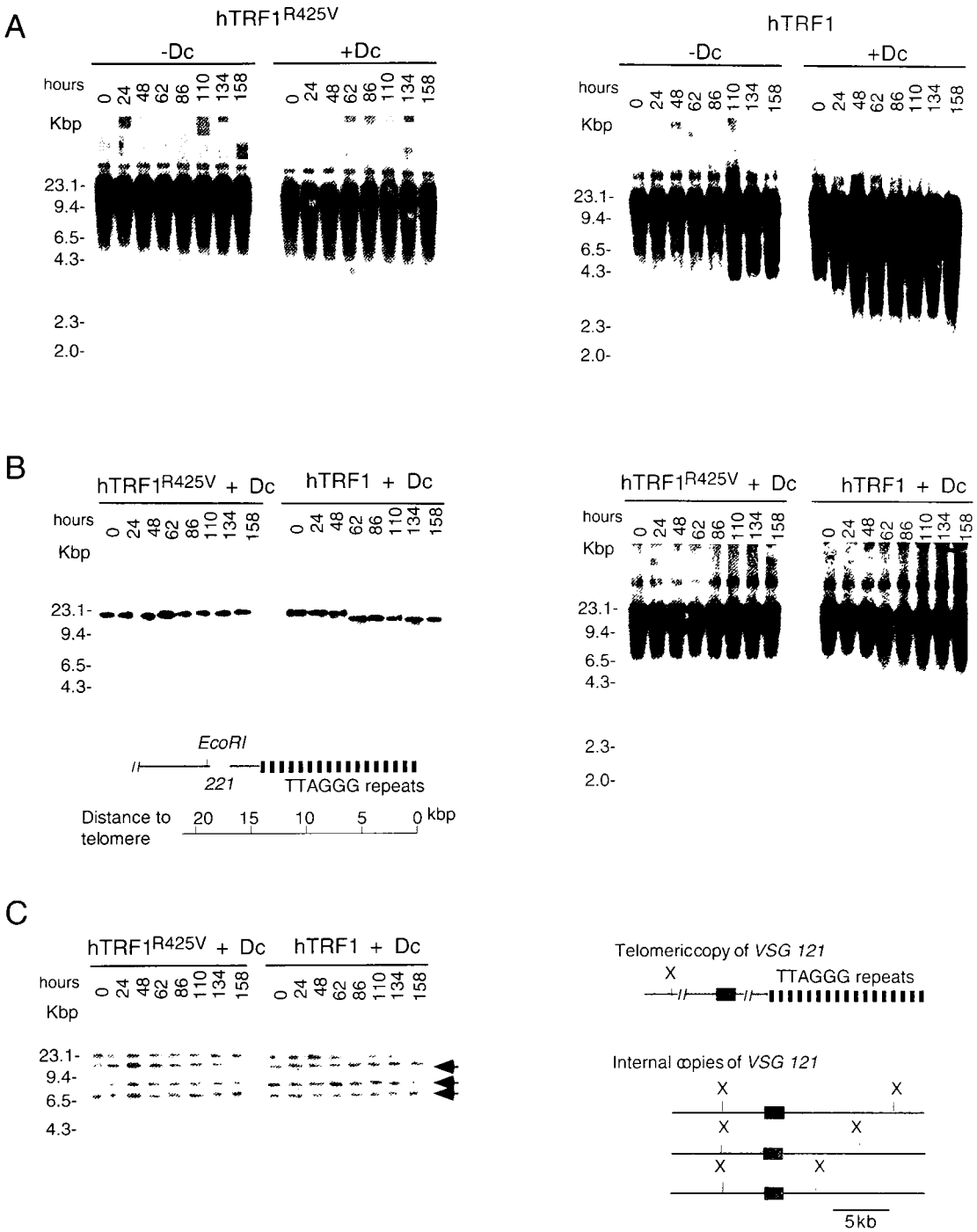


Fig. 25. Telomere shortening in trypanosomes expressing TRF1. (A) Trypanosomes in the presence (+Dc) or in the absence of (-Dc) carrying either TRF1R425V or TRF1 were collected every 24 h as indicated. Genomic blots of DNA digested with endonucleases *Alu* I, *Hinf* I and *Rsa* I were probed with TTAGGG repeats. Cells not induced with doxycycline, as well as cells expressing TRF1R425V. contained telomeres ranging predominantly between 6 and 23 kb. However, after 24–48 h of TRF1 expression, many telomere restriction fragments become shorter. (B) Because an *Eco* RI site is present next to the *VSG 221* gene, a specific telomere fragment can be visualized in Southern blots. (C) Endonuclease *Xmn* I liberates three internal and one telomeric *VSG* 121 fragments, as indicated.

cells expressing TRF1 differ from cells expressing TRF1^{R425V} in that the former exhibit only one peak corresponding to cells in G1. After 110 h, cells in G2-M reappeared. Altogether, the data are consistent with a transient G1-S cell division arrest after 62 h.

TRF1 binding causes telomere shortening

To examine the possible effects of TRF1 on trypanosome telomere maintenance, the length distribution of telomeres from TRF1- and TRF1^{R425V}-expressing cells was investigated. On genomic blots of trypanosome DNA digested with frequent-cutting endonucleases *Alu* I, *Hinf* I and *Rsa* I, telomeric DNA repeats can be visualized between 4 and 23 kb. In Fig. 25A, the distribution of telomeric DNA is compared in trypanosomes induced and not induced carrying either the TRF1 or TRF1^{R425V} constructs. After 62 h, telomeric DNA from trypanosomes expressing TRF1 became increasingly heterogeneous and shorter, whereas telomeres from trypanosomes not expressing TRF1 or expressing TRF1^{R425V} remained unchanged. Using the telomeric *VSG 221* gene as a probe, a specific telomeric fragment of 18 kb can be visualized on a Southern blot of genomic DNA digested with *EcoR* I (Fig. 25B). The telomere carrying *VSG 221* remained invariable in trypanosomes not induced with doxycycline or in trypanosomes expressing TRF1^{R425V}. However, in trypanosomes expressing TRF1, the *VSG 221* telomeric fragment became shorter and heterogeneous. Predominantly, this fragment shortened by 3 kb, and fragments were also detectable that were up to 10 kb shorter than the original *VSG 221* fragment. This corroborates the finding in Fig. 25A, and proves that telomeres in the cells expressing TRF1 get shorter with time, and that this telomere-shortening effect is due to

direct binding of TRF1 to telomeres. To analyze the possibility that telomere shortening was due to internal cleavage and/or degradation of the genome, the status of internal copies of *VSG 121* was analyzed. By digesting DNA with *Xmn* I, three internal copies of *VSG 121* can be detected, in addition to a large fragment containing a telomeric copy of *VSG 121* (Fig. 25C). The sizes of the 3 chromosome internal copies of *VSG 121* remained invariant in DNA from trypanosomes expressing TRF1 and TRF1^{R425V}. Therefore, there is no indication of internal cleavage or degradation of the DNA. The telomeric *VSG 121* fragment is long, and the location of the *Xmn* I site upstream the *VSG 121* is not precisely documented. The size of this telomeric band was maintained in cells expressing either TRF1^{R425V} or TRF1. However, due to its low mobility in the gel, its size remains imprecise. Therefore, both telomere shortening effect and cell division arrest occur at approximately the same time (48-62 h) after induction of TRF1 expression. These results suggest that the binding of TRF1 to trypanosome telomeres destabilizes them.

Discussion

The maintenance of telomeres in a variety of organisms is regulated by proteins that bind the duplex part of telomeres and apparently modulate the effects of telomerase. Lack of protection of telomeres by telomeric proteins also results in DNA-damage responses such as senescence and apoptosis. The human protein TRF1 expressed in trypanosomes binds to PF trypanosome telomeres and produces two distinct effects: transient cell division arrest and telomere shortening.

Whereas TRF1 can bind to TTAGGG repeats, a mutation in the Myb domain of TRF1 ($TRF1^{R425V}$) abolishes this binding. The expression of both alleles in trypanosomes is high and comparable. After few division cycles, PF trypanosomes expressing TRF1 no longer divided and $TRF1^{R425V}$-expressing cells divided normally. Therefore, it is the binding of TRF1 to the telomeres, and not the sole presence of the protein, that causes these effects. In BF, where TRF1 was as highly expressed but not consistently bound to telomeres, changes in cell division were not observed. As demonstrated by DNA staining and cell sorting, and by IF studies, PF arrest occurs in G1-S, and not haphazardly at any stage of the cycle. This indicates that cells completed the cell cycle until they reached a checkpoint that does not allow progress into S and G2. The arrested cells exhibited elongated flagella and nuclei, which may indicate that cell growth continues despite the lack of progress in the cell cycle. These are characteristics typically ascribed to cells undergoing division cycle arrest in other systems (e.g. yeast *cdc* mutants) and not to cells that are simply deprived of nutrients or grown to stationary phase. This has been corroborated by growing 29.13 PF trypanosomes to

stationary phase. After a cell division halt of 24 h, cells can be found in all phases of the cell cycle at proportions similar to those in cells that have been growing at exponential phase. In contrast, trypanosomes expressing TRF1 arrested synchronously in G1-S. Possibly, the binding of TRF1 alters trypanosome telomeres in ways that result in uncapping of the telomere end and concomitant activation of DNA-damage checkpoints. This reasoning is in accordance with the finding that mammalian cells lacking the DNA-binding activity of TRF2 exhibit cell cycle arrest and p53-/ATM-dependent apoptosis (Karlseder et al., 1999). Another possible scenario is that lack of telomere capping could result in covalent fusion of telomeres (van Steensel et al., 1998). This could result in formation of dicentric chromosomes and ultimate disruption of the chromosomes during mitosis. In this case, a DNA-damage response may be generated from internal DNA breaks and not necessarily by unprotected telomere ends. No changes in the telomere restriction pattern were found in the telomere blots that could indicate telomere fusion events. FISH and IF studies showed the patterns of telomere distribution expected during the first 48 h expressing TRF1. During cell division, all the telomeric signal was concentrated in opposite poles of the dividing nuclei, with no evidence of telomeric DNA trapped in the middle that might indicate telomere fusion events.

The cell-cycle division arrest seen in trypanosomes is transient. One possible explanation for this is that some trypanosomes are not as seriously affected as others or onset higher expression of an endogenous telomeric protein that competes with TRF1 for the binding.

For the first time, the tight regulation of telomere length has been lost, and trypanosome telomeres has become heterogeneous and shorter. This effect is protein-mediated and

correlates with similar observations in other organisms, where over-expression of telomeric proteins results in telomere shortening. The telomere binding proteins TRF1 and TRF2 appear to hide telomere ends from telomerase by forming t-loops. In particular, TRF1 has activities consistent with that role, such as its capacity to bend and pair duplex telomeric DNA, acting as a force that promotes formation of t-loops (Bianchi et al., 1997; Griffith et al., 1998). Therefore, the ascribed function of TRF1 as a negative regulator of telomere length could by itself explain some of the effects found in trypanosomes. Reduced accessibility of telomerase normally results in a gradual shortening of telomeres, presumably due to incomplete replication at chromosome ends. Therefore, it could be argued that the high levels of TRF1 result in lack of protection of trypanosome telomeres by removal of endogenous proteins. In this situation, telomere ends might be more vulnerable to degradation and trigger cell division arrest. This type of telomere protection mechanism has been observed in yeast, where lack of Cdc13 binding to the 3' overhang produces longer 3' overhangs and more vulnerable telomeres that consequently become shorter (Wellinger et al., 1996).

In most cases, interference with telomeresase-mediated control of telomere length results in progressive telomere shortening. The telomere shortening effect found in trypanosomes expressing TRF1 is rather abrupt, not gradual as seen in cells expressing high levels of negative regulators of telomerase in other systems. It is possible then that TRF1 is interfering with telomerase-independent maintenance mechanisms, such as those involving intrachromatid recombination machinery. Malfunction of these mechanisms has been shown to trigger rapid telomere deletions (Li and Lustig, 1996).

Protein extracts from trypanosomes expressing TRF1 produced specific band shifts in conditions that favor TRF1-binding. Protein extracts from trypanosomes not expressing TRF1 did not exhibited similar activities under these conditions. It is possible that trypanosome telomeric proteins bind telomeric DNA under different conditions in vitro, or are not as abundant as the expressed TRF1. The trypanosome extracts contained substantial amounts of protein, and under conditions that favor unspecific binding to the TTAGGG probe, substantial DNA-binding activity was found. By obtaining nuclear extracts, it will be possible to concentrate on proteins that localize to the nucleus.

The difference in the binding of TRF1 to telomeres in PF and BF might reflect differences in the telomeric chromatin of these two life-cycle stages. The only identified difference between BF and PF telomeres is the occurrence of J residues (ß-D-glucosyl-hydroxymethyluracil) in BF telomeres that does not occur in PF (van Leeuwen et al., 1998). This results in the presence of bulky glucose residues that might limit the access of telomeric proteins that are specific for TTAGGG repeats. The presence or absence of J could be related to the reported differences in chromatin remodeling between active and inactive ESs (Navarro et al., 1999). Approximately 15 % of the T residues in telomeric repeats is substituted by J. Therefore, one J is expected to occur at least every 2-3 repeats. In vitro, the human telomeric protein TRF1 requires 2 separate sets of one and a half repeats and exhibits a remarkable specificity for TTAGGG, tolerating very few changes in that sequence (Bianchi et al., 1999). Therefore, the presence of J at the observed levels in trypanosome telomeres could account for a substantial

reduction in the binding of TRF1. Differential accessibility of TRF1 to BF and PF telomeres could also reflect differences in chromatin structures in the proximity to telomeres.

Collectively, these results indicate a previously unrecognized function of telomeres in trypanosomes: that of preventing chromosome ends from triggering DNA-damage responses and cell cycle arrest. Because these effects were triggered by the binding of TRF1 specifically to telomeres, it is proposed that a protein with similar function is present in trypanosomes.

Chapter 4

A One-hybrid System to Identify Telomere Binding Proteins

The budding yeast *S. cerevisiae* is amenable for genetic studies. 'Two-hybrid' systems have proved useful in detecting interactions between telomeric proteins. This approach rendered the identification of Rif1p and Rif2p, which interact with the carboxyl-terminus of Rap1p in *S. cerevisiae* and prevent yeast telomeres from growing excessively (Hardy et al., 1992; Wotton and Shore, 1997). The interactions of TIN2 and Tankyrase with TRF1, and of the human RAP1 to TRF2, were identified in yeast two-hybrid systems and subsequently proved to occur at telomeres in human cells (Smith et al., 1998; Kim et al., 1999; Li et al., 2000).

A 'one-hybrid' strategy can be implemented to study or identify proteins that bind directly to a DNA sequence of interest (Dalton and Treisman, 1992; Li and Herskowitz, 1993; Wang and Reed, 1993). DNA-binding proteins fused to a transcriptional activator domain are expressed in a yeast reporter strain. Hybrid proteins that recognize an appropriately placed DNA target sequence position a transcriptional activator domain close to a promoter and activate transcription of a reporter gene. By expressing candidate telomeric proteins fused to a transcriptional activator sequence, in a yeast cell line containing a *HIS3* promoter adjacent to a telomeric sequence, Rif1p, Rif2p, Sir2p, Sir3p, Sir4p, and Cdc13p were demonstrated to bind specifically to telomeric sequence in vivo. Most of these proteins could bind at internal tracts of telomeric repeats, but Cdc13p binding was restricted to the terminal locus (Bourns et al., 1998).

A one-hybrid system led to the identification of the *S. pombe* telomeric protein Taz1p (Cooper et al., 1997). *S. pombe* telomeric repeats were inserted next to a promoter and a LacZ gene (*lacZ*) in the genome of *S. cerevisiae*. In this strain, a library of *S. pombe* cDNAs fused to DNA encoding the GAL4 activation domain (GAD) was expressed. *S. pombe* proteins that bind to the telomeric sequence activate transcription of the *lacZ* reporter gene yielding blue colonies upon exposure to the substrate 5-bromo-4-chloro-3-indoyl-ß-D-galactoside (X-Gal). This screen exhibited considerable specificity and only 4 blue colonies were identified, all of which contained overlapping sequences of the *taz1* gene. Deletion of *taz1* causes telomere elongation, alleviates the telomere position effect, disrupts telomere clustering at the spindle pole bodies and reduces meiotic recombination (Cooper et al., 1997; Cooper et al., 1998).

Like the mammalian proteins TRF1 and TRF2, Taz1p contains a carboxy-terminal Myb domain. No other obvious sequence or structural motives relate TRF1 or TRF2 with Taz1p. A carboxy-terminal Myb domain is also present in *S. cerevisiae* Tbf1p, an essential transcription factor specific for TTAGGG repeats (Liu and Tye, 1991; Brigati et al., 1993). Although X and Y' telomere-associated repeats contain multiple TTAGGG or TTAGGG-like repeats that could serve as the anchor sites for Tbf1p (Fourel et al., 1999; Koering et al., 2000), the association of this protein to telomeres have not been documented in vivo. The major telomere-binding protein in *S. cerevisiae* is Rap1p, whose association with duplex DNA containing poly($C_{1\text{-}3}A$) in vitro and in vivo has been shown (Lustig et al., 1990; Klein et al., 1992; Gilson et al., 1993). Whereas TRF1 and TRF2 form homodimers that bring the Myb domains together for functional binding to the DNA, two consecutive

Myb domains are present in the Rap1p molecule and involved in the binding to telomeric DNA (Konig et al., 1996; Broccoli et al., 1997; van Steensel and de Lange, 1997; van Steensel et al., 1998; Smogorzewska et al., 2000). Yeast Rap1p and human RAP1 do not share significant similarities with TRF1, TRF2 or Taz1p (Li et al., 2000). Within the few identified telomeric proteins with affinity for duplex telomeric DNA, the only common feature is the involvement of Myb-related DNA-binding domains. However, these Myb domains can vary substantially in their sequence, be either in different components of a protein dimer or in the same protein (TRF1/TRF2 or Rap1p), or not be entirely conserved in their roles (yeast Rap1p and human RAP1).

A yeast one-hybrid system has been developed in order to screen for trypanosome telomere-binding proteins. This screen has two components: a *T. brucei* library of GAD-fused cDNAs, and a *S. cerevisiae* reporter strain containing TTAGGG repeats located next to two promoters linked to the *lacZ* and *HIS3* reporters. Trypanosome peptides that bind to the telomeric target sequence are expected to recruit the GAL4 activation domain (GAD) to these promoters and activate transcription of the reporter genes, rendering colonies able to grow in the absence of histidine and to turn blue upon exposure to X-Gal (Fig. 26).

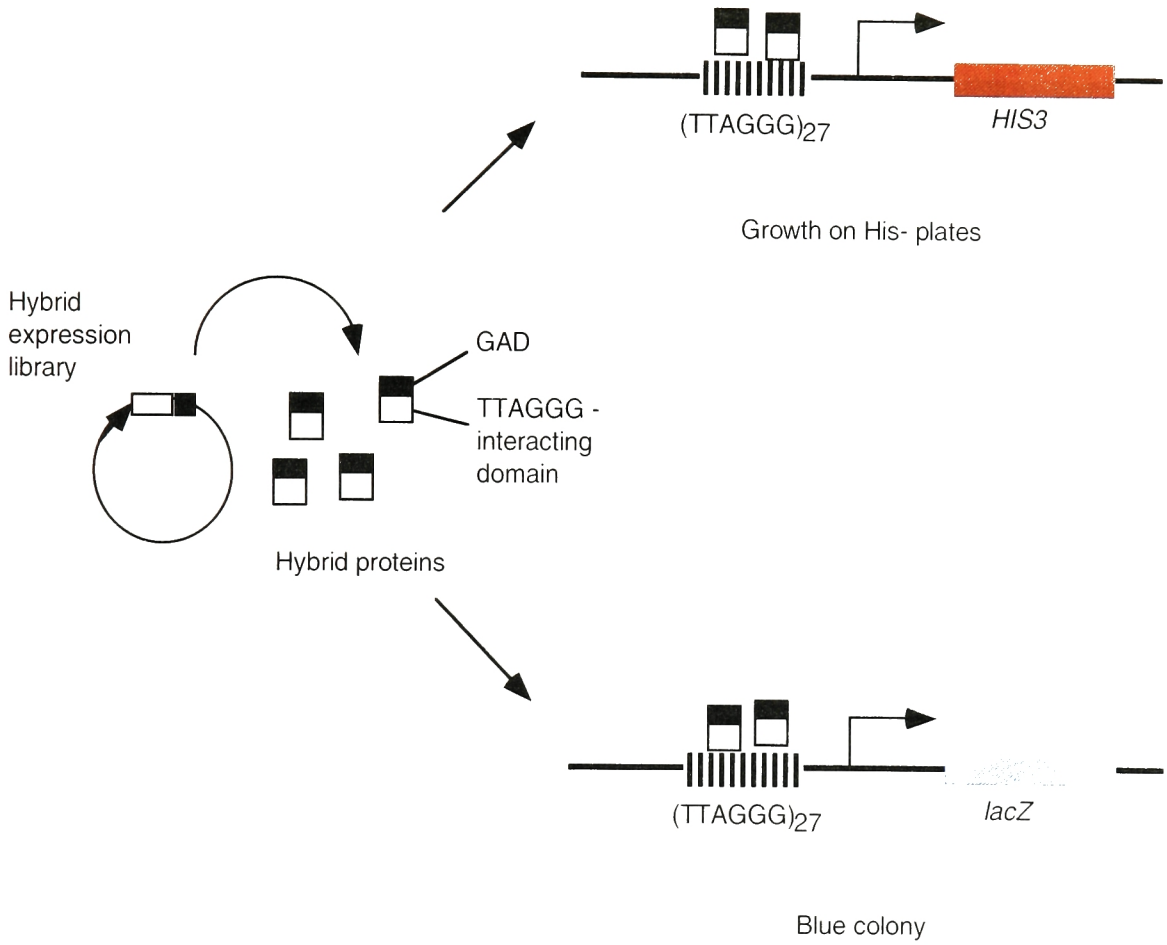


Fig. 26 Schematic representation of a one-hybrid screen for identifying trypanosome TTAGGG-binding proteins. A *T. brucei* expression library of hybrid proteins is transformed into a reporter strain. The hybrids contain protein coding sequences fused to the GAL4 activation domain (GAD). The reporter strain contains *lacZ* and *HIS3* genes immediately upstream of a promoter linked to (TTAGGG)27. Hybrid proteins that bind this DNA sequence act as transcriptional activators of the reporter genes by bringing GAD to the proximity of the promoter, allowing the yeast cells to grow in the absence of histidine and to turn blue in the presence of x-gal.

Engineering of a yeast one-hybrid strain

Plasmid pTH1(Fig. 27A) containing trypanosome telomeric repeats next to the *GAL1* promoter (P_{GAL1}) driving the expression of *HIS3* and the URA3 marker, was inserted in the *LYS2* locus of chromosome 2, and positive clones were selected by their ability to grow in the absence of uracil in the medium. In addition to a region of homology to *LYS2*, a fragment downstream of *LYS2* in chromosome 2 is present in pTH1. Therefore, two copies of the *LYS2* and the downstream region are present in the construct after it is integrated. These two regions provide sites for two possible homologous recombination events. Recombination between the two copies of *LYS2* results in deletion of the *HIS3* marker together with the $(TTAGGG)_{27}$ sequence, and recombination between the two *LYS2*-downtream sequences results only in the loss of *URA3* (Fig. 27B). Using 5-fluoro-orotic acid (5FOA) selection, colonies that lost the *URA3* marker were obtained, and the ones containing the *HIS3* construct were identified by genomic blots. For the second construct employed in the one-hybrid strategy, plasmid pJac13 containing $(TTAGGG)_{27}$ was inserted in the *TRP1* locus of chromosome 2 (Fig. 27C). The final construct inserted in the *TRP1* locus contains all the sequences necessary for induction of LacZ expression upon induction by specific GAD-fused peptides (Fig. 27D). This yeast cell line was named JM 2.10.4. To test the one-hybrid system, JM 2.10.4 cells were transformed with circular plasmids expressing either GAD, GAD-TRF1 or GAD-TRF2 fusion proteins, and the growth on plates without histidine was investigated.

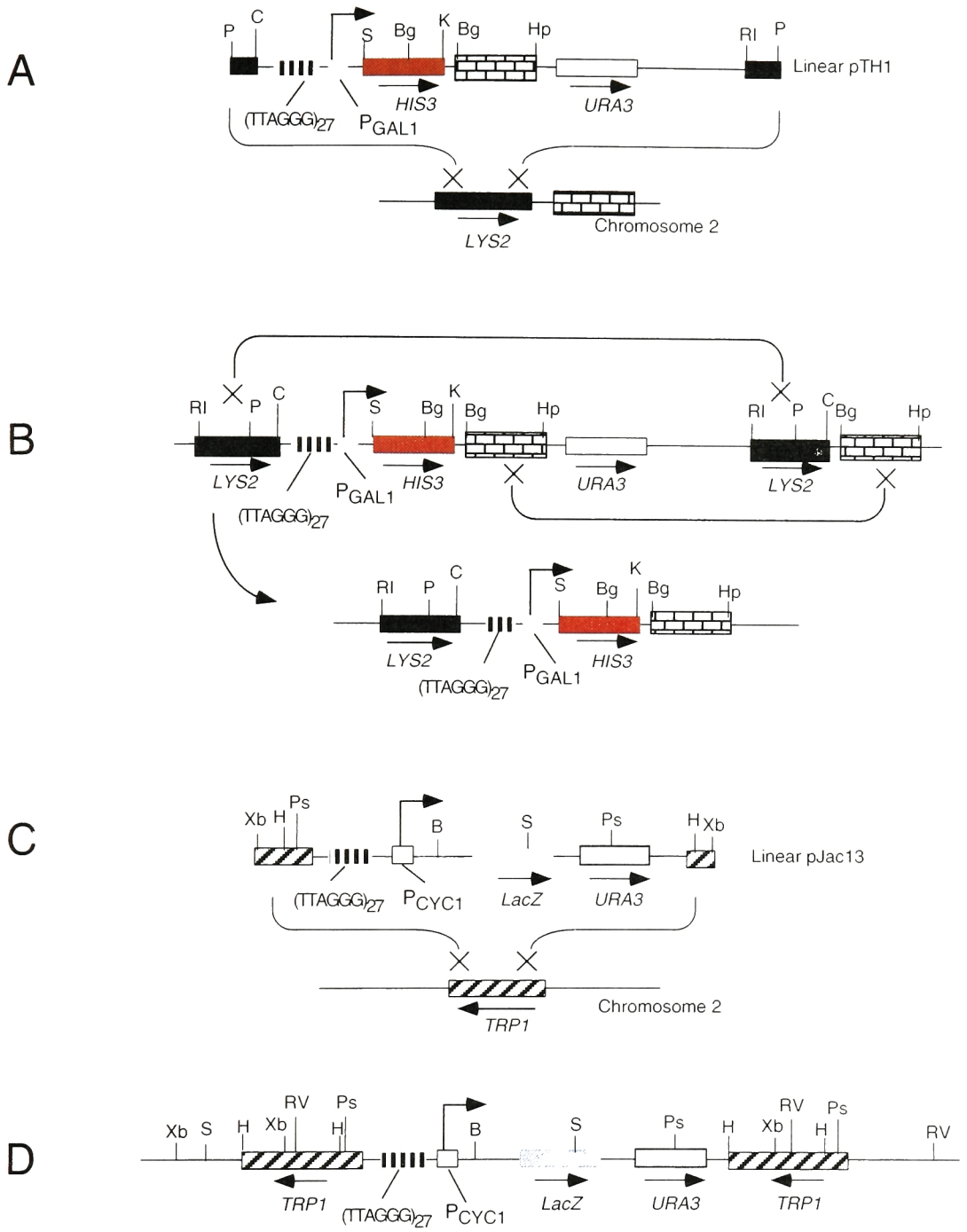


Fig. 27 Integration and restriction maps of *HIS3* (A, B) and *lacZ* (C, D) cassettes in *S. cerevisiae* chromosome 2. **(A)** Plasmid pTH1 containing the (TTAGGG)27 repeats and a *GAL1* promoter (PGAL1) next to the *HIS3* marker was inserted into the *LYS2* locus. A downstream segment (open bricks) is also present in pTH1 together with the *URA3* gene. (B) *LYS2* and the downstream segment provide sites of homologous recombination used in a subsequent genetic screen for cells that lost the *URA3* gene and therefore grow in the presence of 5-fluoro-orotic acid (5FOA). Two types of excision of the URA marker can occur (horizontal brackets). By restriction analysis and genomic blots of DNA from 5FOA-resistant colonies, clones containing *HIS3* were identified. **(C)** Plasmid pJac13 containing (TTAGGG)27 repeats next to a *CYC1* promoter (PCYC1) and *lacZ* was inserted in the *TRP1* locus as indicated. (D) The integrated *lacZ* construct is flanked by two copies of *TRP1*. Abbreviations of restriction sites: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; RI, *Eco*RI; RV, *Eco*RV; H, *Hind*III; Hp, *Hpa*I; K, *Kpn*I; Ps *Pst*I; P, *Pvu*II; S, *Sac*I; Xb, *Xba*I.

Expression of GAD led to some growth in the absence of histidine. This could be due to binding of Tbf1p to the TTAGGG repeats, resulting in low levels of expression of His3p. The growth of yeast expressing GAD in the absence of histidine was kept minimal by adding 0.25 mM of the histidine competitor 3-amino-1,2,4-triozole (3AT). GAD-fused TRF1 and TRF2 had opposite effects. Whereas GAD-TRF2 induced full growth in the absence of histidine, GAD-TRF1 inhibited the growth of JM 2.10.4 cells. This indicates complex interactions between TRF1 with the target DNA sequence or with other proteins bound to the region. For example, TRF1-GAD could be competing with Tbf1p for the binding to TTAGGG repeats but not positioning GAD in the proximity of P_{GAL1}, resulting in lower expression of His3p. Protein blots revealed that the levels of GAD-TRF1 and GAD-TRF2 are the same in JM 2.10.4 crude protein extracts (Fig. 28B), indicating that differences in the growth between cells expressing the fusion proteins is not due to differences in the expression of the proteins. In colony-lift assays for LacZ activity, colonies expressing GAD, GAD-TRF1, or GAD-TRF2, turned blue before 3 h in the presence of X-Gal and no significant differences were observed among the colonies expressing these proteins. This indicates that background levels of LacZ expression are sufficiently high to mask the effects of TRF1 or TRF2 binding to the TTAGGG repeats. This assay was therefore not used during the one-hybrid screen of the *T. brucei* library.

To test the probability of finding a clone expressing a telomere binding protein capable of activating *HIS3* transcription, JM 2.10.4 cells were transformed with mixtures of plasmids carrying GAD and GAD-TRF2 in different ratios. The number of clones that grew in the

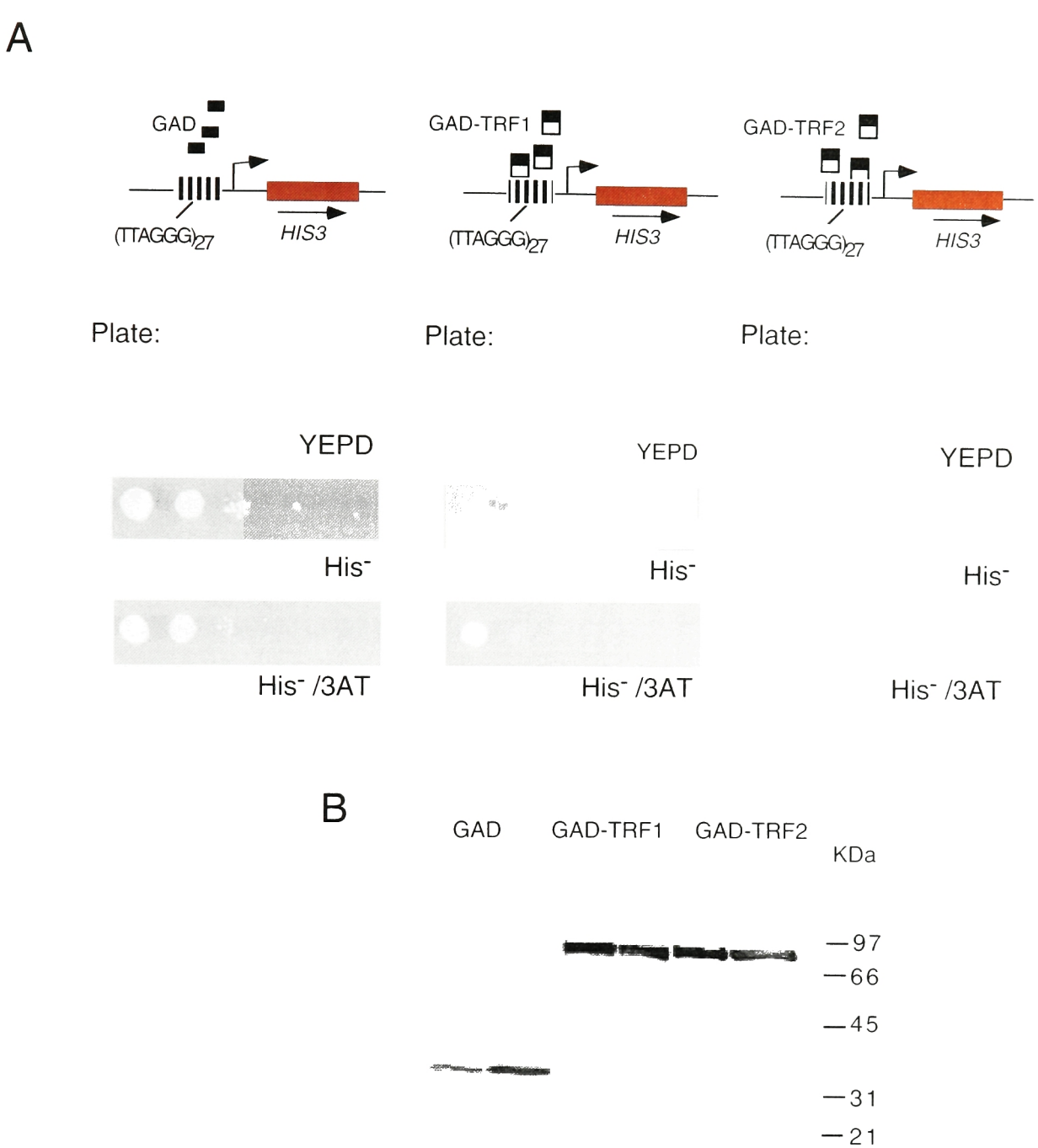


Fig. 28 Induction and repression of *HIS3* expression by GAD fusion proteins. **(A)** GAD (left panel) as well as the fusion proteins GAD-TRF1 (center) and GAD-TRF2 (right) were expressed in the one-hybrid strain JM 2.10.4. Ten-fold serial dilutions of selected colonies were plated on complete medium (yeast extract-peptone-dextrose, or YEPD), in the absence of histidine (-His), and in the presence of 0.25 mM of 3AT. Only yeast cells expressing GAD-TRF2 rendered isolated colonies of equal size in the absence of histidine, whereas growth of GAD-TRF1was repressed. **(B)** Yeast protein extracts from 2 separate colonies of each line (denoted by numbers) were electrophoretically separated in denaturing conditions and blotted on nylon membranes. GAD, GAD-TRF1 and GAD-TRF2 were detected using antibodies for GAD and peroxidase-labeled secondary antibodies. The protein blot reveals equal levels of GAD and the GAD-fused proteins in the samples.

absence of histidine was as predicted, and immunoblots revealed the presence of TRF2 in these clones. A similar experiment using a *T. brucei* library of cDNAs fused to GAD mixed with known amounts of plasmid DNA containing GAD-TRF2 also rendered the predicted number of clones containing and expressing GAD-TRF2.

Yeast one-hybrid screen of a T. brucei library

The yeast strain JM 2.10.4 was next transformed with a GAD-fusion library of *T. brucei* cDNA. Approximately 1.2 million transformants were obtained and 90 clones grew in the absence of histidine and in the presence of 0.25 mM 3AT. Plasmid DNA was obtained from these clones and reintroduced into JM 2.10.4 cells in order to reconfirm the His^- phenotype. Only 3 clones reconfirmed. One of these clones contained a DNA segment of 1.8 kb. However, stop codons were found in frame with GAD, such that a peptide of only 33 aa (mostly acidic) were encoded, many of which were acidic. In the trypanosome genome database, this DNA was found within a region containing stop codons immediately upstream and downstream of the sequence found in the screen, indicating that this 1.8 kb DNA was unlikely to contain a coding sequence. The two remaining positive clones contained overlapping sequences with strong homology to a family of proteins called NAP1 (nucleosomal assembly protein 1) (Fig. 29). These two clones were independent. The size of the cDNA fragments was different and one of them extended in the 5' direction 18 nucleotides further than the other. A *T. brucei* genomic BAC-library was screened using a radioctively-labeled segment of the trypanosome *NAP1* gene, and selected possitive clones were sequenced. This revealed a gene of 349 amino acids. The two *NAP1* segments

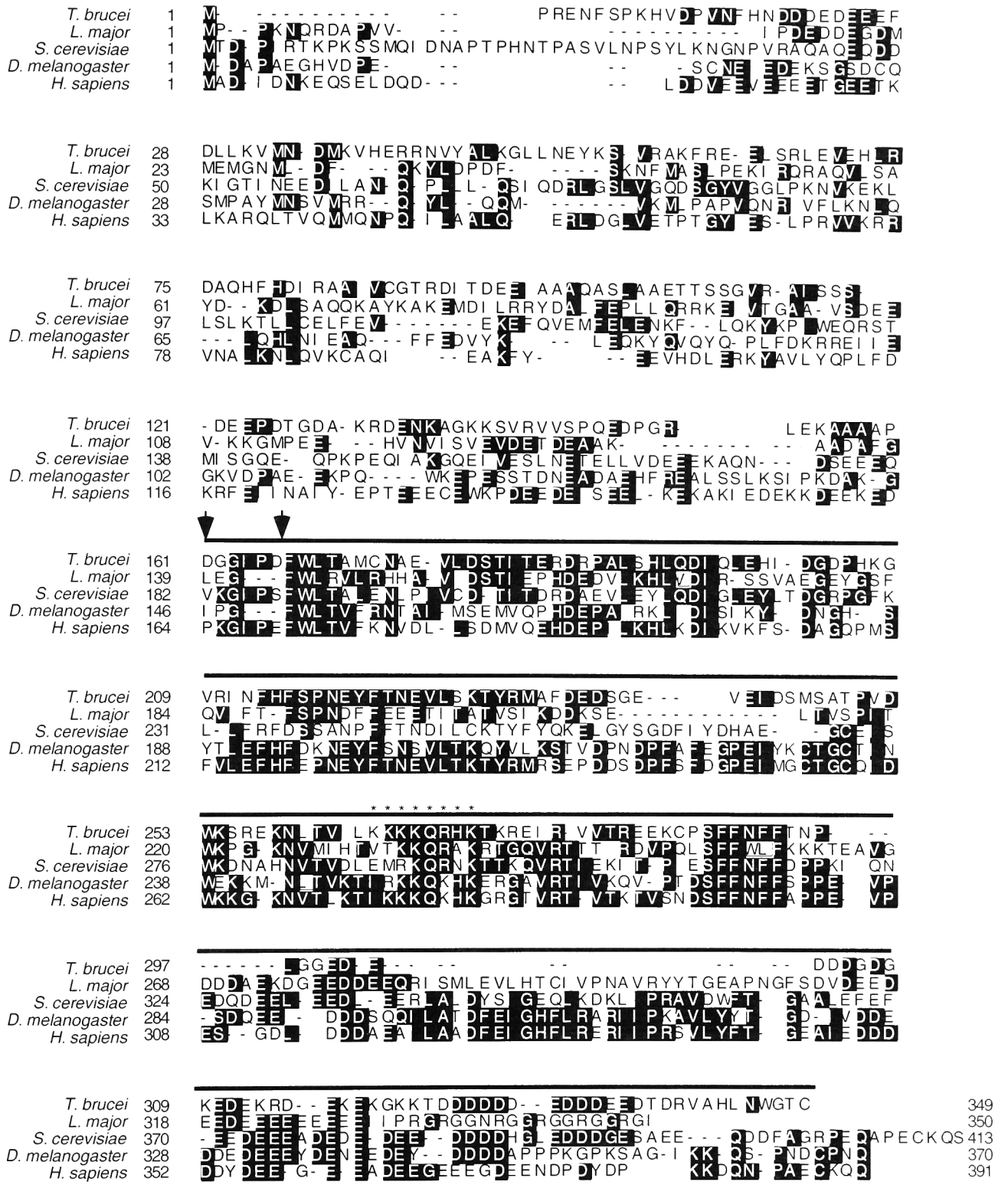


Fig. 29 Sequence comparison of trypanosome NAP1 and different homologous proteins in other species. The alignment was produced by Clustal Method. Gaps (indicated by dashes) are introduced to optimize alignment. Identical aminoacids are indicated by black boxes. The line over the alignment starting at the sites of the two arrowheads indicates the aminoacid sequences of the two NAP1 clones identified by the one-hybrid screen. The asterisks denote a putative nuclear localization signal (NLS).

identified in the screen encoded the 182 or 188 amino-terminal residues. The two clones contained the most conserved regions within the NAP1 family, some of which have been related to the biological function of these proteins (discussed below). No homology with previously reported telomeric binding proteins was found within the trypanosome NAP1. These observations suggested that the GAD-NAP1 fusion proteins are not binding specifically to the TTAGGG repeats.

NAP1 functional assays

To test if the induction of *HIS3* by GAD-NAP1 required TTAGGG repeats, GAD-NAP1 was expressed in two reporter strains: JM 2.10.4 and CG 1945 (Fig. 30). In both strains, the *HIS3* construct has been inserted in the *LYS2* locus of chromosome 2, but CG 1945 contains the HIS3 upstream activator sequence (UAS) instead of TTAGGG repeats. GAD-TRF2 induces expression of histidine in JM 2.10.4 cells through its specific binding to TTAGGG repeats (Fig. 30A) and GAL4 induces histidine expression through binding to the UAS in the CG 1945 strain (Fig. 30B). Induction of *HIS3* by GAD alone was minimal in both strains. Induction by GAD-TRF2 in the CG 1945 strain as well as by GAL4 in JM 2.10.4 cells was minimal. Histidine induction was comparable when GAD-NAP1 was introduced in both strains. This indicates that induction of histidine by NAP1 is likely to be caused by binding of NAP1 to histones in the proximity of the P_{GAL1} promoter rather than by a direct interaction with TTAGGG repeats.

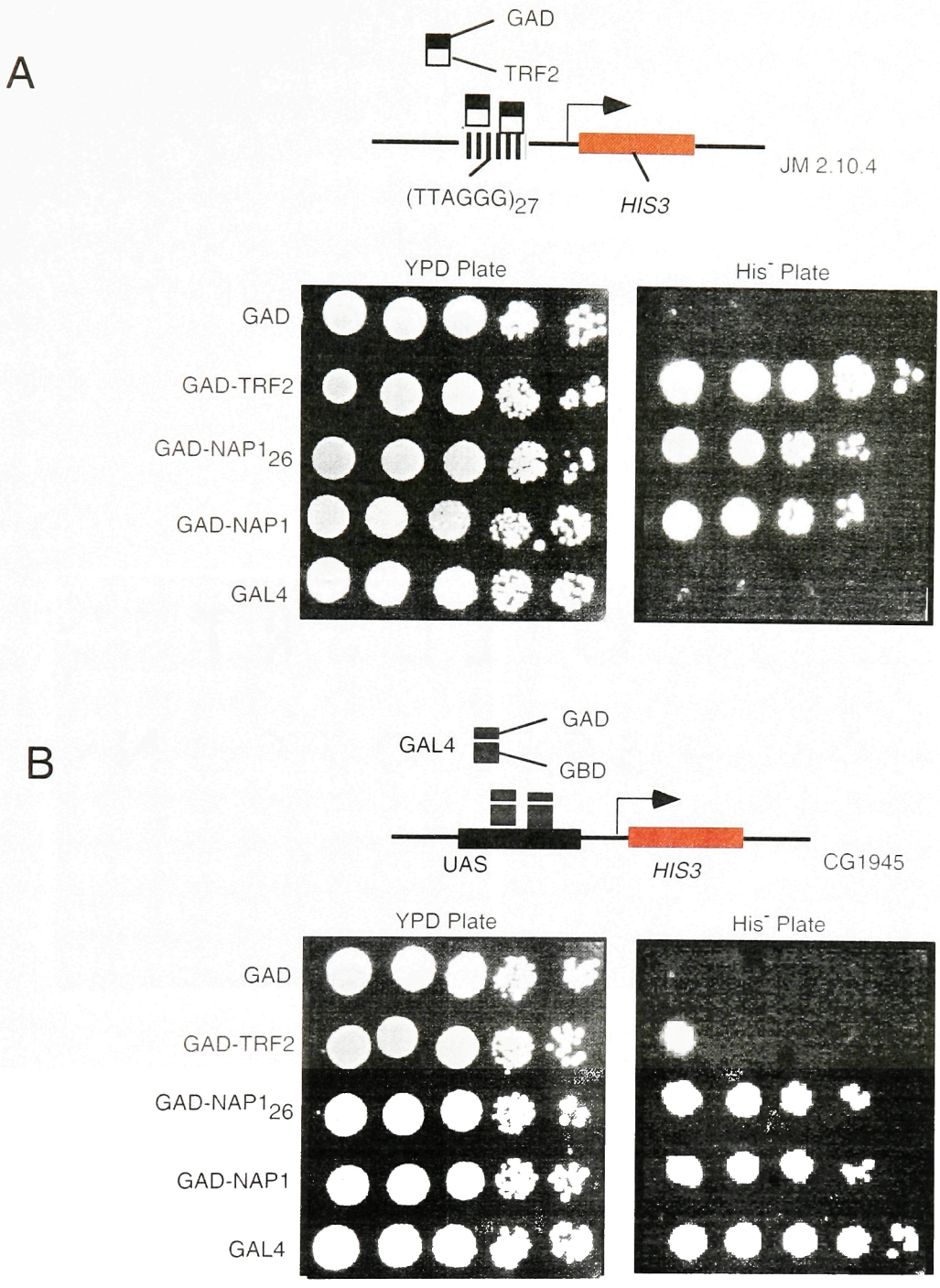


Fig. 30 Viability of yeast clones expressing GAD and GAD-fusion proteins. Strain JM 2.10.4 **(A)** and CG 1945 **(B)** were transformed with plasmids containing GAD or various GAD-fusion genes including TRF2, one of the trypanosome *NAP1* segments from the one hybrid screen (*NAP1*26), the entire trypanosome *NAP1* gene and the entire *GAL4* as indicated. The two schemes depict the interaction of TRF2 with the TTAGGG repeats and of GAL4 with the upstream activator sequence (UAS). Ten-fold serial dilutions from isolated colonies of each strain containing the different constructs were plated on YPD and on His- plates with 0.25 mM of 3AT. Colonies of both strains expressing either GAD-NAP126 or GAD-NAP1 grew equally on His- plates.

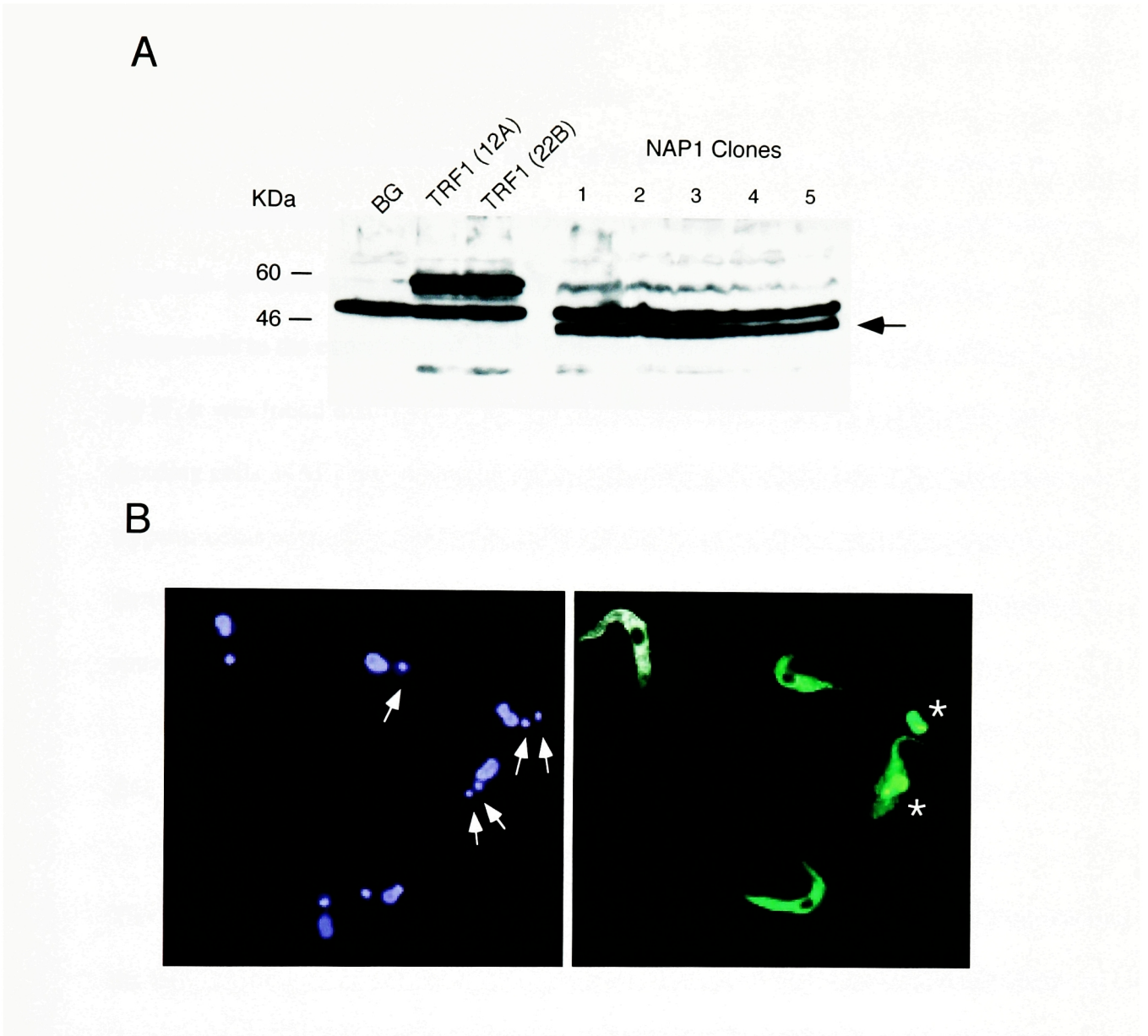


Fig. 31 Expression of FLAG-tagged NAP1 in PF trypanosomes. **(A)** Electrophoretically separated proteins from trypanosome crude extracts were transferred to a nylon membrane. NAP1 from different clones (indicated by numbers) was detected using antibodies for FLAG and a peroxidase-labeled secondary antibody. The arrow indicates a protein of about 40 kDa just below an unspecific band stained by these antibodies. NAP1 is expressed at similar levels to TRF1 from two different clones (12A and 22B). The background strain does not express FLAG-tagged proteins and only shows the unspecific band of approximately 50 kDa. (B) DAPI staining (blue fluorescence) and IF of permeabilized PF trypanosomes expressing NAP1 (green fluorescence). Trypanosomes with two kinetoplasts (indicated by arrows) concentrate NAP1 in the nuclei (asterisks), indicating a cell-cycle-regulated subcellular localization of this protein.

To study the biological function of NAP1 in *T. brucei*, NAP1 was FLAG-tagged at its amino-terminal end and expressed in PF *T. brucei* using the inducible system described in chapter 3. Immunoblots showed that PF trypansosomes expressed NAP1 at levels comparable to the expression of TRF1 in the PF strains described in chapter 3 (Fig. 31A). By IF, it was found that NAP1 is present in the nuclei during S and G2 phases. In non-dividing cells, NAP1 was mainly in the cytoplasm (Fig. 31B). This distribution of NAP1 in trypanosomes suggests a role in cell cycle regulation, possibly in nucleosome remodeling during DNA synthesis prior to cell division, compatible with a role in chromatin binding or remodeling shown in human cells (Rodriguez et al., 1997).

Discussion

The duplex telomeric repeats are the binding sites for proteins with central roles in protecting the integrity of chromosomes. Myb domains seem to be the only common theme among all duplex telomeric binding proteins known, and only TRF1 and TRF2 contain sufficient sequence conservation between each other to allow their finding by homology search (Broccoli et al., 1997). Human RAP1 and its yeast homologue bear some sequence conservation but have diverged in their mode of interaction with telomeres (Li et al., 2000). This hindered the chances of identifying telomeric binding proteins by homology-based methods. While TRF1 is the only duplex telomeric DNA binding protein to have been identified by biochemical purification (Chong et al., 1995), *S. pombe* Taz1p is the only one identified by a yeast one-

hybrid system (Cooper et al., 1997). Given these precedents, and the failure to identify a TRF1 like activity in trypanosome crude extracts (Chapter 3), a one hybrid strategy was tried. The yeast one-hybrid system developed in this chapter presented two problems. GAD-TRF1 and GAD-TRF2 had opposite effects in the yeast reporter strain JM 2.10.4 Whereas GAD-TRF2 was a potent activator of *HIS3* expression, GAD-TRF1 inhibited basal levels of expression of *HIS3*. One possible explanation for this discrepancy is that a yeast protein could interfere with the one-hybrid system in an unpredictable manner. Yeast Tbf1p is an essential binds TTAGGG (Liu and Tye, 1991; Brigati et al., 1993), and the presence of TTAGGG or TTAGGG-related sequences in the boundary of yeast telomeres indicate a potential role of this essential protein in telomere biology (Fourel et al., 1999; Koering et al., 2000). Therefore, Tbf1p could bind directly to the TTAGGG repeats inserted in the two reporter constructs of the one hybrid system and initiate transcription, explaining the low levels of *HIS3* induction in the yeast strain expressing GAD. TRF1 and TRF2 could then be establishing two different modes of competition with Tbf1p for the binding to TTAGGG repeats. Whereas TRF2 is able to bring GAD to the proximity of the promoter and activate transcription, TRF1 might be interfering with Tbf1p-binding without positioning GAD for a functional interaction with the promoter, resulting in less His3p expressed.

A second problem that the one-hybrid strategy presented was that the yeast strain JM 2.10.4 turned blue in the presence of X-Gal when the plasmids containing GAD, fused or not with telomeric proteins, was introduced in the strain. In previous work, the lacZ reporter construct has been useful in reducing the high number of colonies identified in the histidine screen (Chong et al., 1995). However, we were unable to use this feature of our

system due to high levels of *lacZ* induction in the absence of TTAGGG-specific protein binding. This problem could also be attributed to the presence of an endogenous yeast protein that binds to TTAGGG repeats such as Tbf1p. Because, the levels of *lacZ* induction were high, and specific competitors for this assay are not available, differences in the levels of *lacZ* induction by TRF1 and TRF2 were not detectable. Not using the *lacZ* screen could have hindered the chances of identifying a trypanosome proteins with a TTAGGG-specific binding activity. When human libraries were screened for telomere-binding proteins using the JM 2.10.4 strain, similar problems were found, and no specific TTAGGG-binding activities were detected. Curiously, TRF2 was not found in these screens (Diego Loayza, personal communication). Because TRF2 binds efficiently to TTAGGG repeats in a hetorodimeric form, only clones containing both the dimerization domain and the Myb domain are expected to be found in this screening. This could also decrease the chances of identifying a trypanosome telomere-binding protein.

The only gene identified in the one-hybrid screen of the trypanosome library was *NAP1*, and controls in another yeast strain with a target DNA sequence different from TTAGGG showed that the *HIS3* induction caused by NAP1 is sequence-independent . The trypanosome NAP1 protein sequence deduced from the DNA sequence bears 21% amino acid identity and 55% similarity to NAP1 homologs across species. The regions encompassing amino acids 163-199 and 252-295 show approximately 50% identity across species. Trypanosome NAP1 also has the characteristic features of several acidic regions throughout its sequence, including a long carboxyl-terminal region of approximately 40

residues with 9 glutamic and 18 aspartic acids. These regions are conserved in their acidity and location throughout the protein, but not necessarily conserved in their sequence.

Yeast Nap1p facilitates assembly and remodeling of nucleosomes by direct interaction with core histones H2A and H2B (Ishimi and Kikushi, 1991; McQuibban et al., 1998). The involvement of NAP1 and its close homologue NAP2 in ATP-facilitated assembly of equally spaced nucleosomes has been described in *Drosophila* and human cells, where cell-cycle- regulated subcellular localization of these proteins has been shown. The role of NAP1 in nucleosome assembly is therefore conserved, and it has been postulated to reflect a role of NAP1 proteins as histone chaperones, bringing histones H2A/H2B to the nucleus in S phase. In a purified *Drosophila* chromatin assembly system, a chromatin remodeling step requiring ATP and the transcription activator ACF is followed by the binding of transcriptional activators GAL4 and VP16, resulting in enhancement of histone acetylation by p300 and subsequent transfer of acetylated H2A and H2B to NAP1 (Ito et al., 2000). This indicates that NAP1 is involved in histone binding at the site of transcription initiation. In addition, the interaction between yeast Nap1p and B-type cyclin 2 (Clb2) is essential for the ability of Clb2 to carry its full range of functions during mitosis (Kellogg et al., 1995; Kellogg and Murray, 1995). Clb2 initiates a series of events that lead to the mitosis-specific phosphorylation and activation of Gin4 protein kinase. Nap1 is required in vivo for the activation of Gin4 and is able to bind both Gin4 and Clb2 (Kellogg and Altman, 1997).This role of NAP1 also transcends across species (Kellogg et al., 1995). It is not clear whether the nuclear localization of NAP1 during S phase is related to its

association with H2A/H2B or with Clb2 but this localization could be consistent with both roles.

The NAP1 gene family is well conserved. Yeast Nap1p was first identified by its cross-reactivity with monoclonal antibodies for human NAP1 (Ishimi and Kikushi, 1991). It is possible that trypanosome NAP1 can establish a functional interaction with yeast histones. This could explain why NAP1 was detected in the one hybrid system and why it promotes *HIS3* transcription independently of the sequence of the target DNA. Furthermore, the proximity of GAD to the promoter could activate transcription initiation and concomitant acetylation of histones, resulting in more of the histones binding NAP1. Trypanosome NAP1 protein is present in the cytoplasm of PF trypanosomes at the G0/G1 boundary and translocates to the nucleus, where the majority of the protein can be found in S phase. Collectively, the data presented in this chapter indicate that NAP1 has a cell-cycle-regulated function as a chromatin remodeling factor.

CONCLUSIONS

Simultaneous expression of two VSGs in T. brucei

Three different lines of trypanosomes continuously expressing mixed VSG coats were obtained by artificially re-engineering an active ES with two different *VSGs*. These double-expressing trypanosomes replicated normally and were as infectious as the wild-type trypanosomes. The generation of homogeneous cultures of BF expressing equal amounts of two different VSGs demonstrates that the mechanisms normally imposing the expression of only one VSG during infection do not operate at the cell-surface level or by other form of crosstalk or interference between VSGs themselves. Subsequent studies of others showed that homogeneous cultures of double-expressing trypanosomes cannot be obtained by maintaining drug pressure on two different ESs: transcription of the *VSGs* alternates between the 2 ESs. Even when transcription is initiated from different ES promoters, transcription progresses through only one entire ES (the active ES) and declines to undetectable levels towards the telomeres in the other ESs. The singularity of VSG expression is therefore probably influenced by a mechanism involving some kind of telomere position effect in BF trypanosomes. This process is developmentally regulated, and PF trypanosomes maintain fully repressed ESs.

The interest of trypanosome telomeres

Trypanosomes contain a repertoire of approximately 1000 VSGs scattered in the large and small chromosomes at numerous internal loci; but a subset of these genes (approximately 20) is placed at the telomeres of their large chromosomes, the only loci where they are transcribed. The telomeric loci seem to provide the right environment for transcriptional switching and gene replacement events associated with antigenic variation. Trypanosomes are organisms that divide incessantly and do not undergo the processes associated with senescence seen in multicellular organisms, such as down-regulation of telomerase and progressive telomere shortening. Rather, trypanosome telomeres grow at a constant rate. The length of each telomere in the multiplying population of trypanosomes is maintained tightly. The most tangible correlation between the telomere maintenance mechanisms and antigenic variation is the different growth rates observed for active and inactive ESs. In addition to their possible involvement in the process of antigenic variation, trypanosome telomeres contain the conserved sequence TTAGGG. These repeats are very abundant in trypanosomes and make up about 10 % of the trypanosome nuclear DNA, making the analysis of telomeric DNA relatively easy. Because trypanosomes originated 500 million years ago, the study of their telomeres may provide information about the minimal or more conserved requirements for telomere protection and maintenance.

Trypanosome telomeres have conserved features

The sequence TTAGGG is present not only in vertebrates and flagellates, but also in slime molds, and many fungal species. These and most other telomere repeats have the common feature of containing short tracts of G residues (e.g., TTGGGG and TTTTGGGG in ciliates, TTAC/AGGG in *S. pombe*, TTAGGC in nematodes or TTTAGGG in plants). S. *cerevisiae* and *K. lactis* stand out for their unusually heterogeneous telomeric repeats. Despite having an unusually high A-T content throughout its genome, the sporozoan *Plasmodium* has telomeres composed mainly of TT(T/C)AGGG repeats, indicating an evolutionary pressure to conserve the telomere structure. Not only the sequence of the telomeric DNA is considerably conserved in trypanosomes, but also the telomere repeats are polarized such that the G-strand forms the 3' end of the telomere whereas the C-strand forms the 5' end. This sequence conservation, therefore, is probably of some importance. As in other organisms, the trypanosome telomeric G-strand extends beyond the duplex DNA to form a 3' overhang of approximately 75-225 bp or longer. The demonstration of t-loops in species as divergent from each other as mammals, trypanosomatids and ciliates provides a view of how the ends of chromosomes are physically protected. The remarkable sequence and structure conservation of telomeres reflects their important role in preserving the integrity of chromosome ends. Organisms maintain telomeres under a strict control, and changes in the telomeric DNA, even when maintaining their overall length and composition result in the halt of cell division. This intolerance is due to proteins that bind along the stretches of telomeric repeats. Lack of binding of these proteins to their target DNA results in telomere alterations and dangerous cell responses.

Human TRF1 and TRF2 bind to duplex TTAGGG repeats and are probably involved in forming or stabilizing the t-loop, a role consistent with their binding characteristics, and their functions in protecting the chromosome ends. Overexpression of TRF1 in human cells results in progressive telomere shortening, presumably by modulating the access of telomerase to telomeres. In trypanosomes, however, the binding of TRF1 results in abrupt telomere shortening and in a transient cell cycle arrest, as if the maintenance mechanism that normally regulates the length of trypanosome telomeres tightly was lost. These effects are consistent with the lack of adequate capping function. The destabilizing effects produced by the binding of TRF1 indicate that a presumptive trypanosome telomere complex is disrupted. Trypanosomes, therefore, probably have proteins that bind to their conserved telomeric repeats and protect the chromosome termini by mechanisms such as t-loop formation.

The 3' G-overhangs and the symmetry of linear chromosomes

Both telomeres of trypanosome minichromosomes end in 3' overhangs and form t-loops. An active mechanism involving a 5' exonuclease to form the overhang is therefore proposed. In *Streptomyces* bacteria, linear plasmids exhibit identically long overhangs at both ends and these overhangs are folded back on themselves and maintained by unknown mechanisms. What regulates the formation and length of these overhangs ? In *S. cerevisiae*, Cdc13p binds to the 3' overhang and prevents degradation of the 5' end. The failure of this mechanism results in degradation of the 5' end. It is possible that, in newly formed yeast

chromosomes, the blunt ends are not bound by Cdc13p and therefore subject to 5' exonuclease activity. This way, a 3' overhang would be formed and become the substrate of Cdc13p, which then stops the incipient degradation of the 5' end. Similarly, in organisms that form t-loops, the blunt end of the newly synthesized chromosome might not be adequately protected and the 5' end is consequently degraded to a point in which the 3' end can now participate in forming the t-loop. Because trypanosome overhangs are present on both ends of the chromosome and they measure 75-300 bp, an active mechanism of overhang formation most act to generate and maintain trypanosome overhangs. The identification of telomeric proteins is needed in order to study the molecular basis of t-loop formation and telomere maintenance in trypanosomes. The mammalian telomeric TRF2 bring to telomeres Mre11, a protein with recognized 5' exonuclease activity. It is tempting to speculate that this protein might be involved in generating 3' overhangs. This would be consistent with the observation that lack of TRF2 results in shortened telemeres. It would be interesting to observe mammalian and trypanosome telomeres in cells lacking the non-essential Mre11 gene.

The search for telomeric binding proteins

Most effort was centered on trying to identify a trypanosome protein with the ability to bind duplex tracts of TTAGGG repeats. Four proteins with this characteristic had been identified in other organisms: TRF1 and TRF2 in animals, Taz1p in *S. pombe* and Rap1p in *S. cerevisiae*. With the exception of TRF1 and TRF2, which are related, these proteins have little or no sequence conservation. The only homologue of Rap1p is the human protein

hRap1p, whose association to telomeres does not occur by direct binding to DNA, and TRF1 or TRF2 homologues do not seem to be present in yeast. Therefore, it is possible that the function of telomeric proteins is not entirely conserved and that homologues of these proteins are not present in some organisms. The Ku heterodimer, previously known for its role in non-homologous DNA recombination and repair, also binds to the duplex part of the telomeric repeats, probably at the proximity of the overhang in mammalian and yeast telomeres. Recently, a putative Ku80 homologue has been identified in the trypanosome genome (G. Cross, personal communication). This opens the possibility of studying the role of Ku in trypanosomes and its possible association to telomeres.

Among the possible reasons for the failure in identifying duplex telomeric binding proteins by a biochemical approach are the low abundance of these proteins and the unavailability of nuclear purification protocols. Recently, a method to purify trypanosome nuclei has been employed in order to enrich protein extracts with proteins localized in the trypanosome nucleus. In these extracts, the TTAGGG-binding activity of human TRF1 from trypanosomes expressing TRF1 was identified (C. Janzen and G. Cross, personal communication). The yeast one-hybrid screen also has its drawbacks as various domains of the telomeric protein might be needed for functional binding of the protein (e.g. DNA-binding and dimerization domains of TRF1 or TRF2) and interference of yeast endogenous TTAGGG-binding proteins or chromatin-binding proteins such as NAP1 can interfere the screen.

Active and inactive ESs in BF trypanosomes differ in their relative levels of transcription, susceptibility to DNAse I cleavage, J content, and in their rates of telomere growth. These processes are coupled to the remodeling of ES chromatin during transcriptional switching. It is possible that the presence of a differential chromatin structure at telomeres facilitates the remodeling of histones throughout the ESs, generating a zone where transcriptional switches and DNA rearrangements can occur easily. In PF trypanosomes, all the ES are equally repressed and do not exhibit any of the mentioned features present in BF trypanosomes. Differences in telomere chromatin structure exhibited by PF and BF is evidenced by the differential binding of TRF1.

The role of histone acetylation in chromatin remodeling to allow transcription of different genes during development is well documented in other species, but unfortunately nothing is known about histone modification in *T. brucei*. It would be interesting to study the mechanisms related to histone acetylation and transcription initiation in relation to BF-PF differentiation and ES activation/inactivation in BF. The data presented indicates that trypanosome NAP1 likely interacts with nucleosomes at the site of transcription initiation. This protein might facilitate histone acetylation to allow chromatin remodeling in trypanosomes.

Trypanosome cell lines and constructs

All *T. brucei* lines used are derived from strain 427, antigenic type 1.2 (MITat 1.2), clone 221a (Johnson and Cross, 1979). BF trypanosomes were cultured in HMI-9 (Hirumi and Hirumi, 1989) to a maximum cell density of $2x10^6$ trypanosomes/ml. To obtain BF trypanosomes in quantities higher than 10^8, mice were infected by intraperitoneal injection and observed until parasitemia of ~ $5x10^8$ trypanosomes ml^{-1} was reached. A total of 10^9 trypanosomes were obtained from blood, and passed through DEAE cellulose as previously reported (Lanham and Godfrey, 1970). BF trypanosomes were kept on ice in trypanosome dilution buffer, TDB (5 mM KCl, 80 mM NaCl, 1 mM $MgSO_4.7H_2O$, 20 mM Na_2HPO_4, 2 mM $NaH_2PO_4.2H_2O$, 20 mM glucose). PF trypanosomes of the same strain were cultured at 27°C in SDM-79 (Brun and Schonenberger, 1979), supplemented with fetal bovine serum, to a concentration of 10^7 trypanosomes/ml. A total of 10^9 trypanosomes was collected, washed and resuspended in PBS pH 7.3 (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7H_2O$, 1.4 mM KH_2PO_4) and kept on ice. To integrate a second VSG into the active *VSG 221* ES, the pKD4 construct carrying the neomycin-resistance gene was employed (Davies et al., 1997). The Luciferase gene in pKD4 was replaced by *VSGs* MITat 1.5 clone 117, MITat 1.6 clone 121, or ILTat 1.24 (Cross, 1975; Carrington et al., 1991) using appropriate restriction sites. Linear plasmid DNA (5 μg) was used to transfect 2.4 x 10^7 BF trypanosomes by electroporation. Trypanosomes were recovered in HMI-9 and incubated at 37°C for 18 hours. G418 (2 μg ml^{-1}) was added and trypanosomes were

incubated until growth of transfectants was observed (7 to 14 days). Clones were isolated on agarose plates containing G418 (Carruthers and Cross, 1992).

To express TRF1 in trypanosomes the tetracycline (TET)-inducible system was used. Strain 427 had been previously manipulated to create the PF cell line 29-13 and the BF cell line 13-90. These trypanosome lines express T7 RNA polymerase (T7RNAP) from the tubulin locus and TET repressor (TET_R) from the RNP1 locus (Wirtz et al., 1999). PF trypanosomes were cultured at 27°C in SDM-79 supplemented with fetal bovine serum and containing 15 μg ml^{-1} G418 (Sigma) and 25 μg ml^{-1} hygromycin (Sigma), to a maximum cell density of 10^7 trypanosomes/ml. BF were cultured at 37 °C in HMI-9 containing 5 μg ml^{-1} G418 and 5 μg ml^{-1} hygromycin to a maximum cell density of $2x10^6$ trypanosomes/ml. The FLAG-TRF1 fusion was cloned in the *Hind* III-digested plasmid pLew111(Hoek et al., 1999), a derivative of pLew82 (Wirtz et al., 1999). BF and PF trypanosomes were transfected by electroporation. A FLAG-tagged version of $TRF1^{R425V}$ was engineered as below.

TRF1 mutagenesis

A site-directed mutagenesis protocol (Stratagene 'QuickChange' kit) was followed to create FLAG-$TRF1^{R425V}$ from TRF1. Briefly, primers (GTGTCATGTTAAAAGACA GATGG GTGACCATGAGA) and (TCTTCATGGTCACCCATCTGTCTTTTAACAT GACAC), provided by H. Moss and T. de Lange, are complementary to each other and carry the triplets GTG or CAC (underlined), by which a mutation is introduced in the TRF1

sequence that changes codon AGG (Arginine) for GUG (Valine), abolishing the binding of TRF1 to TTAGGG repeats (H. Moss and T. de Lange, unpublished data). Plasmid pLew111 carrying FLAG-TRF1 was used as template. After denaturation of the template and annealing of the primers, extension by Pfu DNA polymerase followed by *Dpn* I digestion of the methylated non-mutated plasmid template were performed. The mutated plasmid was recovered in an *E. coli* recipient cell line.

Protein extracts

A protocol was adapted from previously published procedures (Zhong et al., 1992). Essentially, protein extracts were prepared at 4°C from 10^8 trypanosomes (BF or PF) previously washed two times in TDB or PBS respectively. Trypanosomes were resuspended in 1 ml of high-salt buffer (20 mM Hepes-KOH pH 7.9, 0.42M KCl, 5 mM $MgCl_2$, 0.1 mM ethylenediaminetetraacetate, EDTA, 0.5 mM PMSF, 1 µM Leupeptin; 0.1 µM Apropeptin) supplemented with 0.1 % nonident P 40 (NP40). The lysate was kept on ice for 20 min and centrifuged at 60 krpm for 1 h at 4°C. The supernatant was dialyzed against low-salt buffer (20 mM Hepes-KOH pH 7.9, 100 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM ethylene glycol-bis (ß-aminoethylether)-N,N,N',N'-tetraacetic acid EGTA, 0.5 mM PMSF, 0.5 mM DTT). The procedure yielded approximately 1 ml of protein extract with a protein concentration of 5.8 mg ml^{-1} according to the Bradford assay (Bio-rad) Aliquots of 50 µl kept at -80 °C. These extracts contained TRF1 as evidenced by immunoblots using TRF antibody 371 (see below).

Electrophoresis mobility shift assays (EMSA)

EMSAs were essentially as described (Zhong et al., 1992). Binding reactions were performed in binding buffer (5% glycerol, 4% Ficoll, 20 mM, 150 mM KCl, 1 mM MgCl2, 0.1 mM EDTA, 5 ng ml^{-1} ß-casein, 0.5 mM DTT and 0.1% NP40) at room temperature for 30 min and run on a 5% polyacrylamide gel (29:1) in TBE , at 130 V for 2-3 h. Dried gels were analyzed by autoradiography. DNA probes were gel-purified restriction fragments of 12 TTAGGG repeats labeled by Klenow fill-in (NEB).

VSG-immunoblots

Partially purified preparations of solubilized VSG were obtained by endogenous GPI-PLC digestion essentially as described (Cross, 1984) but on a microscale. Briefly, 1 x 10^8 trypanosomes were pelleted and washed two times with TDB, resuspended in de-ionized water at 0°C and incubated on ice for 5 min. The lysate was centrifuged, resuspended in 100 µl of 10 mM PBS pH 8.0, and incubated for 5 min at 37 °C. Ghosts were pelleted again, and one-tenth of the supernatant was used for polyacrylamide gel electrophoresis (PAGE) in the presence of 0.1 % sodium dodecylsulfate (SDS). The 10 % polyacrylamide gels were blotted onto nitrocellulose, which was incubated in blocking solution (10% fat-free milk powder and 0.5% Tween 20 in PBS) for 2 h at 4 °C. Blots were reacted with rabbit antibodies against individual VSGs in incubation solution (0.1% fat-free milk powder and 0.1% Tween 20 in PBS) at 4 °C over-night. Blots were then washed 3 times for 10 min in incubation solution, then incubated for 1-2 h in the same solution with

Peroxidase-conjugated goat anti-rabbit antibody for 1 h. After 3 more washes of 10 min with incubation solution and a final rinse with PBS, antibody-protein complexes were detected using the ECL chemiluminescence system (Amersham).

Metabolic labeling of VSG

1 x 10^8 BF trypanosomes were grown in vitro, collected during exponential phase, and transferred into 10 ml of methionine-free RPMI (Gibco BRL) supplemented with 10% (v/v) dialyzed fetal bovine serum, 10% dialyzed Serum Plus (JRH Biosciences), and incubated with 50 µCi of ^{35}S-labeled methionine-cysteine mixture for 2 h. Finally, VSGs were extracted as described above and immunoprecipitated with cross-reactive-determinant-depleted rabbit antibody to VSG 221 coupled to protein A-Sepharose (Pharmacia).

TRF1 and NAP1 Immunoblots

10^7 BF or PF were lysed in 100 µl of loading buffer (50mM Tris Cl pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol). 10 µl of these protein extracts (equivalent to 10^6 cells) were separated on a 10% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose filters, which were then incubated in blocking solution. TRF1 protein expressed in Baculovirus (provided by Titia de Lange) was separated under similar conditions. Antibody incubations were performed at 4 °C over-night in incubation buffer. Baculovirus TRF1 or exogenous FLAG-tagged TRF1 expressed

in trypanosomes were detected using antibody 371 (van Steensel and de Lange, 1997). FLAG-tagged TRF1 (Chapter 3) and NAP1 (Chapter 4) were detected using commercially available anti-FLAG monoclonal antibodies M2 (Sigma). To detect TRF1 or NAP1, peroxidase-conjugated anti mouse and ECL system (Amersham) were used.

VSG-immunofluorescence

BF trypanosomes strain 427 expressing VSG 221, trypanosomes co-expressing VSGs 221 and 117, 221 and ILTat 121, or 221 and 121, and trypanosomes 427 of the antigenic type MITat 1.6 clone 121 were grown to a density of 10^6 parasites/ml. Aliquots of 1 ml from each culture were collected, washed twice in TDB, and fixed in 2% formaldehyde dissolved in PBS for 5 min at 4°C. Trypanosomes were centrifuged at 3000 g for 5 min at 4 °C and resuspended 100 µl PBS. Trypanosomes were adhered to microscope slides by desiccation and slides were washed twice for 5 min in PBS. Attached cells were incubated for 30 min in 1%BSA dissolved in PBS. Cells were incubated with chicken anti-VSG 221 either alone or combined with rabbit antibodies against VSG 117, ILTat 1.24 or 121. Trypanosomes expressing VSG 121 were incubated with rabbit antibodies for VSG 121 alone. Incubations proceeded over-night at 4°C in the presence of 0.2% BSA dissolved in PBS. Cells were washed four times with 0.2% BSA in PBS. Goat anti-chicken IgG coupled to FITC and goat anti-rabbit IgG coupled to rhodamine were used as second antibodies. Incubation continued in the same solution for 1 h at room temperature and 3 more washes followed.

In situ hybridization

A protocol was adapted for trypanosomes from previously published procedures (Chong et al., 1995). BF or PF diluted to 10^6 trypanosomes ml^{-1} in TDB or PBS respectively were attached to glass coverslips by centrifugation at 3000 g for 5 min at 4°C. Coverslips were immersed in 2% formaldehyde dissolved in PBS and incubated 5 min at 4°C. Trypanosomes were permeabilized in 4 ml 0.5% NP-40 in PBS for 5 min at 4°C and maintained in PBS at 4°C. For *in situ* hybridization, DNA was denatured by incubating permeabilized trypanosomes in 70% formamide/2xSSC for 5 min at 70°C. Coverslips were washed briefly in 2xSSC at 50°C. DNA denaturation, hybridization and washing conditions were essentially as described (Sibon et al., 1994). The digoxigenin-labeled $(CCCUAA)_{27}$-repeat RNA probe was synthesized by SP6 polymerase from plasmid pTH5 (de Lange et al., 1990), previously digested with *BamH* I, in the presence of digoxigenin-uridine 5´-triphosphate (Boehringer). Cells were incubated with sheep antibody to digoxigenin (anti-digoxigenin) (Boehringer). Either after hybridization or just after permeabilization, the preparations were incubated for 10 min in PBS with 50 mM glycine, and for 15 min in PBG (PBS with 0.1% cold water fish skin gelatin from Sigma and 0.5% BSA).

TRF1-immunofluorescence

FLAG-tagged TRF1 and NAP1 were detected by using the anti-FLAG monoclonal M2 (Sigma) or rabbit antibody 371 (van Steensel and de Lange, 1997). To detect *T. brucei* flagellar basal body and flagellar rod proteins, monoclonal antibodies BBA4 and ROD1 (provided by K. Gull) were used as previously reported (Woods et al., 1989). DNA was stained using DAPI. Antibodies M2, 371, BBA4, and ROD1 were detected with FITC-conjugated donkey anti-mouse or anti-rabbit antibodies respectively (Jackson).

Measurement of DNA content by flow cytometry

Conditions similar to reported protocols were followed (Kanmogne et al., 1997). PF trypanosomes expressing TRF1 or $TRF1^{R425V}$ were washed and resuspended in ice-cold PBS pH 8.0 with 0.5 mM EDTA to a density of 10^6 ml^{-1}. Trypanosomes were fixed by slowly adding ethanol to a final concentration of 70% and storing at 4 °C for periods of 24 h or longer. Fixed trypanosomes were pelleted and resuspended to 10^7 ml^{-1} in PBS pH 8.0 containing 50 μg ml^{-1} propidium iodide and 40 μg ml^{-1} RNAse and incubated for 1 h at room temperature. Intensity of red fluorescence was measured using a FACSort flow cytometer (Beckon-Dickinson & Co).

Sucrose gradients and rotating agarose gel electrophoresis (RAGE) of minichromosome DNA

Sucrose gradients were made according to reported methods (Weiden et al., 1991). Previously permeabilized and cross-linked trypanosomes were resuspended in 2 ml TE (10 mM Tris pH 7.4, 10 mM EDTA) supplemented with 1% sodium lauryl sarcosinate (SLS) and 2 mg proteinase K. Incubation proceeded for 3h at 37 °C. The lysate was overlaid on a 35 ml linear 5-20 % sucrose gradient in 100 mM EDTA, 25 mM Tris (pH 7.5), 1% SLS. Centrifugation proceeded for 16h at 10.000 rpm in an SW28 ultracentrifuge rotor. Fractions of 1ml were collected from the bottom of the gradient. Aliquots of 30 µl from each fraction were mixed with 30 µl of 1.6% low melting point agarose kept at 65 °C. Samples were loaded in a 0.8% agarose pulsed field. Electrophoresis was carried out at 120° at 1-12 sec linear ramp and a constant voltage of 180 V for 15 h.

Genomic blotting

A total of $5x10^8$ trypanosomes were resuspended in TNE (10 mM Tris pH 7.4, 100 mM NaCl, 10 mM EDTA) and lysed in TNES (10 mM Tris pH 7.4, 100 mM NaCl, 10 mM EDTA, 1% SDS) in the presence of 100 µg ml^{-1} of proteinase K. After overnight incubation with proteinase K at 37°C, and phenol/chloroform extractions, DNA was precipitated with isopropanol, and resuspended in TE. Further RNAse A treatment, phenol/chloroform extractions and isopropanol precipitation followed. For telomere blots, DNA was digested

overnight with restriction enzymes *Alu* I/*Hinf* I/*Rsa* I, *Xmn* I or *Eco* RI, under recommended conditions (New England Biolabs). Telomeric restriction fragments were detected using a probe containing TTAGGG repeats and labeled as previously described (de Lange, 1992). The 50-bp repeats probe consisting of one repeat (GTGTACTTGCCTGTACTAAAAG TATTCTTACAGGGG TTGCAGTATCTGT) was synthesized and end-labeled using Klenow DNA polymerase under specified conditions (New England Biolabs). Probes for *VSGs 221* and *121* were labeled as previously reported (Navarro et al., 1999). All probes have been labeled with radioactive 50 μCi α-^{32}P-dCTP per reaction using specified conditions (NEN).

G-strand overhang assays

The non-denaturing hybridization assay was employed to detect G-strand overhangs following previously reported techniques (Makarov et al., 1997). Oligonucleotides $(TTAGGG)_4$ and $(CCCTAA)_4$ were end-labeled using γ-^{32}P-ATP (3000 Ci/mmol, Biorad) and polynucleotide kinase. DNA (10 μg) previously incubated with or without Exonuclease I and digested with *Rsa* I/*Hinf* I/*Alu* I, were ethanol precipitated and resuspended in 21 μl of hybridization buffer (50 mM Tris HCl pH 8.0, 50 mM NaCl, 1 mM EDTA), added to 4 μl labeled probe (10 nM) and incubated for 12 h at 50°C. Following size fractionation by electrophoresis in 0.8% agarose, the gel was dried on Whatman DE-81 filter paper.

Permeabilization, cross-linking, and telomeric DNA preparation for electron microscopy

BF or PF trypanosomes ($5x10^8$) resuspended in TDB or PBS respectively, were incubated in the presence of 40 μM digitonin (Sigma) for 5 min on ice. After spinning at 3000 rpm for 30 sec in a microcentrifuge, trypanosomes were treated for cross-linking and DNA extraction following scaled-down adaptations of previously published protocols (Griffith et al., 1999). Briefly, 50 μl of psoralen 4' aminomethyltrioxalen (AMT) previously dissolved in H_2O to 5μg ml^{-1} were added to 1 ml of permeabilized cells. Trypanosomes were stirred slowly and exposed to 350 nm UV light for 30 min. An equal volume of TNES (10 mM Tris pH 7.4, 100 mM NaCl, 10 mM EDTA, 1% SDS) was added to cross-linked cells and supplemented with 200 μg of proteinase K. The sample was incubated at 55°C for 2 h. Deproteinization was reinforced with 200 μg more of proteinase K and continued for 2 h more. After two phenol/chloroform extractions and isopropanol precipitation, the DNA was resuspended in TNE. Treatment with RNAse A followed for 1 h at 37°C. After 2 more phenol/chloroform treatments, and isopropanol precipitation, the DNA was resuspended in TE, filtered through 2.5 X 100 cm Biogel A5M columns (Biorad), and eluted.

Gel chromatography of T. brucei telomeric restriction fragments

Following restriction of total cross-linked *T. brucei* DNA with *Rsa* I/*Hinf* I/*Alu* I, the DNA was phenol/chloroform-extracted, precipitated with ethanol and resuspended in 10 mM Tris (pH 7.5)/1 mM EDTA at a concentration of ~200 μg ml^{-1} and applied to a 20 ml column of

Bio-Gel A-5m equilibrated in the same buffer. The chromatography was controlled with a Pharmacia Gradifract apparatus. The DNA profile was determined by absorbance readings at 260 nm and fractions containing the telomeric restriction fragments were prepared for EM.

Staining of minichromosomes with SSB

Aliquots of *T. brucei* minichromosomes in sucrose-sarcosyl were chromatographed over 2 ml columns of Bio-Gel A-5m equilibrated in 20 mM Hepes (pH 7.5)/1 mM EDTA. *E. coli* SSB was added to 1 ng ml^{-1} and the sample incubated on ice for 10 min. Glutaraldehyde was then added to 0.6% for 5 min at room temperature and the sample chromatographed over a second Bio-Gel column to remove the free protein and fixative. Minichromosomes in the excluded fractions were prepared for EM by direct adsorption onto glow-charged carbon films in the presence of spermidine, washed, air-dried and rotary shadowcast with tungsten (Griffith and Christiansen, 1978).

Electron microscopy methods

To examine crosslinked *T. brucei* minichromosomal DNA separated by sedimentation, the pooled DNA fractions were chromatographed through 2 ml columns of Biogel A5m (Biorad Inc) equilibrated in 10 mM Tris (pH 7.5), 1 mM EDTA (TE), to remove the sucrose and detergent. Following crosslinking and processing of total *T. brucei* DNA, the DNA was precipitated with ethanol and dissolved in TE. Crosslinked DNA samples were prepared for EM by spreading on a denatured film of cytochrome C protein using the

droplet variation of the method of Kleinschmidt as described in Griffith et al. (1999). The grids were air dried and rotary shadowcast with platinum:paladium (20:1) and examined in a Philips CM12 instrument at 40 KV. DNA lengths were measured by projecting molecule images on EM sheet film onto a Summagraphics digitizing tablet attached to a Macintosh computer programmed with software developed in the Griffith laboratory. Images were scanned using a Nikon LS4500 film scanner. Contrast adjustments and image composition were made using Adobe Photoshop software.

Development of the yeast one-hybrid strain JM 2.10.4

The one-hybrid screening strategy used in Chapter 4 required the insertion of $(TTAGGG)_{27}$ repeats upstream of yeast promoters driving two different reporter genes: *HIS3* and *lacZ*. First, a segment containing the $(TTAGGG)_{27}$ segment was excised from plasmid pTH5 (provided by Titia de Lange, Rockefeller University) using *Bam* HI and *Bgl* II and inserted next to the yeast *GAL1* promoter (P_{GAL1}) in plasmid pTH1 (provided by Dr. S. Field, State University of New York, Stony Brook) previously digested with *BamH* I and dephosphorylated. This plasmid carries *URA3*, allowing growth of yeast on plates lacking the amino acid uracil. In addition, pTH1 contains a *Bgl*II *Hpa*I segment homologous to a region downstream of *LYS2* in chromosome 2. In this plasmid, P_{GAL1} directs the expression of *HIS3*, conferring growth in medium lacking the amino acid histidine during the one hybrid screen (described below). The plasmid was linearized with *Pvu*II and introduced into the yeast W303 (*ade2-101 can1-100 ura3-1 leu2-3, 112 trp1-1, his3-11,15*) strain by the LiAc method (Schestl and Gietz, 1989). Clones that lost the *URA3* gene were selected

by their ability to grow on medium containing 5FOA. To check the presence of the *HIS3* construct after 5FOA selection, genomic DNA was analyzed by restriction analysis with *Bgl*II and *Pvu*II following genomic blots probed with a *Sac*I-*Bgl*II fragment of *HIS3*. The telomeric repeats $(TTAGGG)_{27}$ were also excised from pTH5 using *Eco*RV and *Sma*I and inserted into plasmid pJac13 (provided by Dr. S. Field, State University of Stony Brook) previously digested with *Sal*I, filled-in with DNA polymerase I (New England Biolabs) and dephosphorylated. This positions the $(TTAGGG)_{27}$ repeats upstream of the yeast *CYC1* promoter to regulate expression of the bacterial *lacZ* gene. The reporter plasmid were linearized with *Xba*I and introduced in theW303 containing the *HIS3* construct, creating the strain JM 2.10.4. Integration of pJac13 was confirmed by restriction analysis of genomic DNA with *Sac*I, and a subsequent genomic blot probed with a *Hind*III fragment of *TRP1*.

One hybrid tests and screen

The yeast strains JM 2.10.4 (developed here) and CG1945 (Stratagene), were used as hosts for the *GAL4* activation domain (GAD) plasmid pGAD10 (Stratagene), where TRF1, TRF2 or NAP1 were introduced in frame with GAD. Total RNA was obtained from BF and PF trypanosomes by the guanidinium thiocyanate method (Tel-Test "B") and sent to Clontech. The *T. brucei* cDNA library was made from a mixture of PF and BF mRNA using poly-dT and random primer extension and cloned into pGAD10 plasmid vector (Clontech), creating GAD-cDNA fusion sequences. Plasmid pGAD10 confers growth in the absence of the amino acid leucine. After introduction of the pGAD10 plasmids by the LiAc method, the cells were plated in the absence of leucine. Tests of His3p expression were done on plates

lacking histidine and in the presence of 0.25 mM of the histidine competitor 3-amino-1,2,4-triazole (3AT) (Sigma). After introduction of the cDNA library ($1x10^6$ different cDNAs) into the yeast cells by LiAc method, the cells were plated in the absence of leucine and histidine to select for transcription of the *HIS3* reporter gene. The cDNAs from the clones identified in the screening were purified by standard techniques and amplified in *E. coli.* The purified plasmids were re-introduced into the JM 2.10.4 strain. Positive transformants containing pGAD10 and pGAD10-fusion plasmids were screened for *lacZ* expression by a modification of the filter assay described previously (Bartel et al., 1994). In brief, colonies were spotted onto Whatman filters and the cells made permeable by freeze-thawing the filters twice in liquid nitrogen. The filter was layered onto another filter soaked with buffer containing the substrate 5-bromo-4-chloro-3-indoyl-ß-D-galactoside (X-Gal; Sigma). Filters were incubated at 30 °C and monitored for appearance of blue colonies. The three positive clones that re-tested positive in the $His3^+$ were sequenced. A *NAP1* segment from one of the hybrid clones was used to probe the trypanosome genomic library RPCI-102 (C. Zeng, and P. de Jong, BACPAC Resource Center, Children's Hospital Oakland Research Institute), enabling the identification of clones that contained the full sequence of *NAP1*.

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