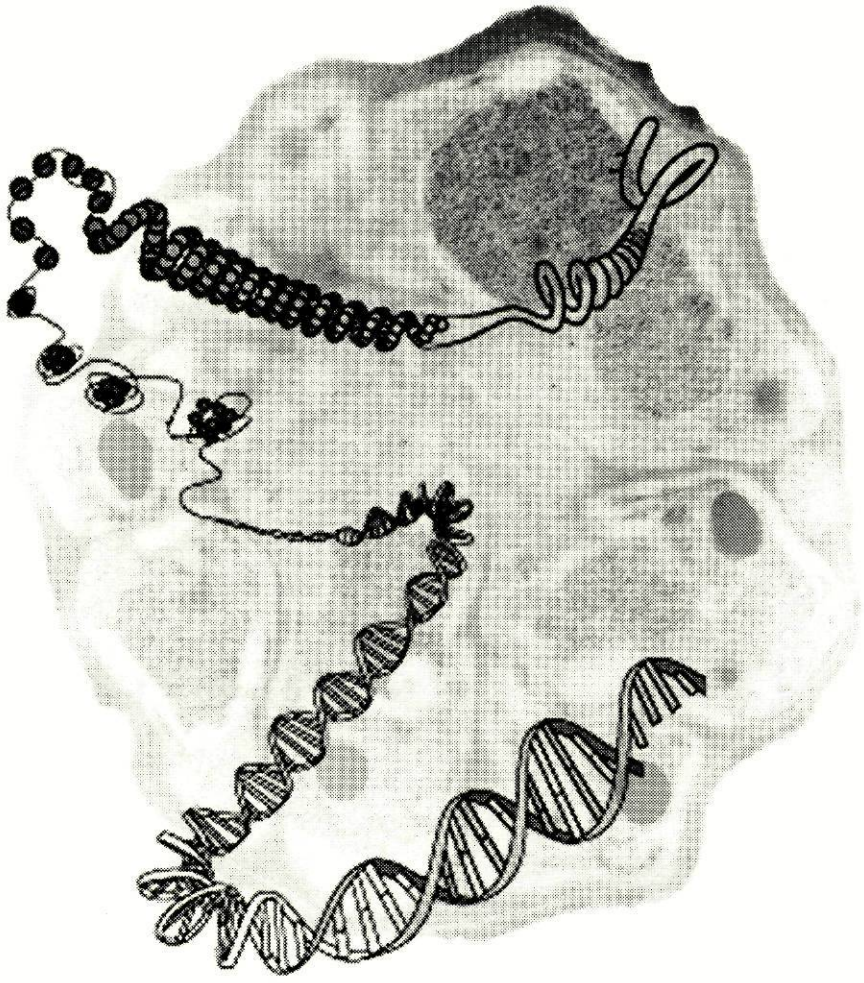
Chromosome ends in *Plasmodium falciparum*:

dynamics of telomeres, roles of subtelomeres and investigation of telomerase as an antimalarial drug target



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Institut Pasteur

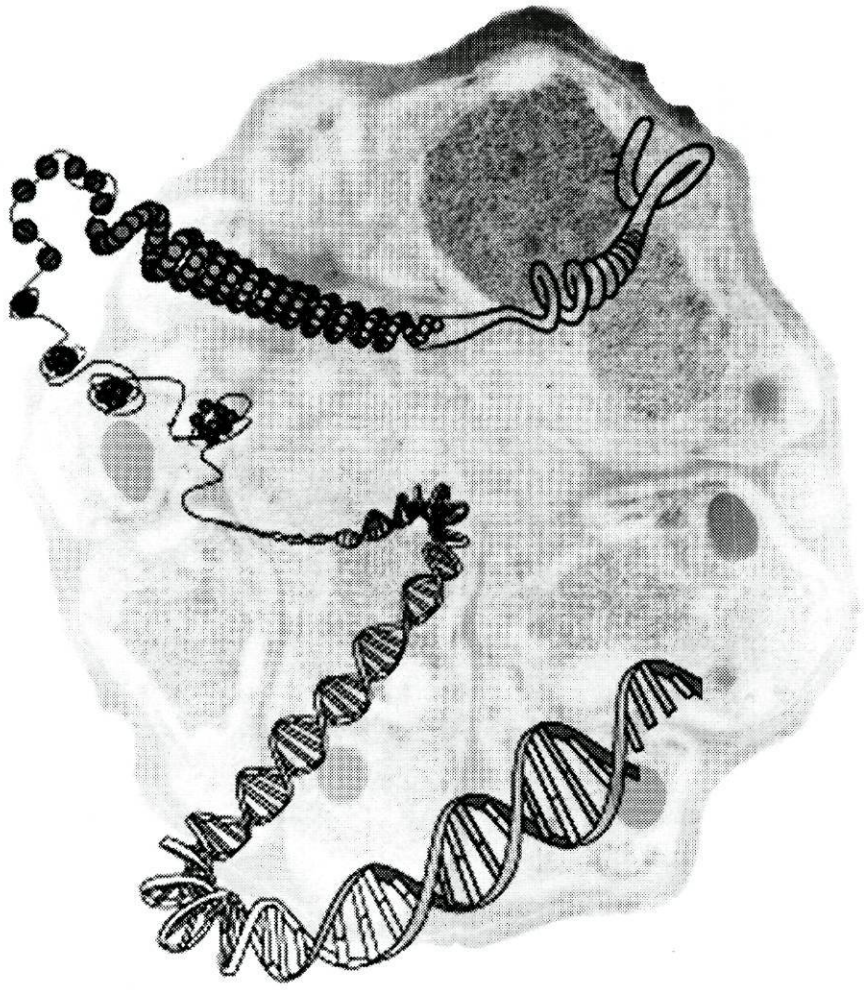
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ICBAS, UP

Luísa Miranda Figueiredo

Porto 2002

Chromosome ends in *Plasmodium falciparum*:

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DISSERTAÇÃO DE CANDIDATURA AO GRAU DE DOUTOR APRESENTADA AO INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR, DA UNIVERSIDADE DO PORTO.

DISSERTATION TO OBTAIN A PH.D. DEGREE IN BIOMEDICAL SCIENCES, MOLECULAR BIOLOGY SPECIALITY, SUBMITTED TO THE INSTITUTO DE CIÊNCIAS BIOMÉDICAS DE ABEL SALAZAR, UNIVERSITY OF PORTO.

NOTA EXPLICATIVA

A Presente dissertação está escrita em inglês na sua quase totalidade, pelo facto de o Doutor. Artur Scherf ter sido o seu orientador.

EXPLANATORY NOTE

This thesis is almost entirely written in English, since Dr. Artur Scherf was its supervisor.

Ao Sr. Professor Melo,
pelo rigor, exigência e entusiasmo.

To Professor Melo,
for his rigour, enthusiasm and
commitment to excellence.

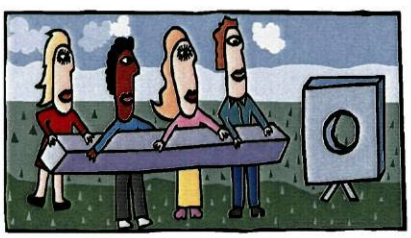
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PAPERS / ARTIGOS

Ao abrigo do Art. 8 ° do Decreto-Lei n° 388/70 fazem parte integrante desta dissertação os seguintes trabalhos já publicados ou em publicação.

Under the law No. 388/70, article No.8, it is stated that the following papers (published or in preparation) are an integral part of this thesis.

- Figueiredo, L.M., Pirrit, L.A. and Scherf, A. (2000) Genomic organisation and chromatin structure of *Plasmodium falciparum* chromosome ends. *Molecular & Biochemical Parasitology*, **106**, 169-174.
- Scherf, A., Figueiredo, L.M. and Freitas-Junior, L.H. (2001) *Plasmodium* telomeres: a pathogen's perspective. *Curr Opin Microbiol*, **4**, 409-414.
- Figueiredo, L.M., Freitas-Junior, L.H., Bottius, E., Olivo-Marin, J.C. and Scherf, A. (2002) A central role for *Plasmodium falciparum* subtelomeric regions in spatial positioning and telomere length regulation. *EMBO Journal*, **21**, 815-824.
- Figueiredo, L.M. and Scherf, A. *Plasmodium* Telomerase Reverse-Transcriptase is unusually large and localises in a discrete subnuclear compartment. *(manuscript in preparation)*
- Scherf, A., Figueiredo, L.M. and Freitas-Junior, L.H. (2002) Chromosome structure and dynamics of *Plasmodium* subtelomeres. In Waters, A. and Jansen, C. (eds.), *Genomes and the Molecular Cell Biology of Malaria Parasites*, in press.

Em cumprimento do disposto no referido Decreto-Lei, declaro ter participado activamente na recolha e estudo do material incluído em todos os trabalhos, tendo redigido os textos com a colaboração dos autores.

According to the aforementioned law, I confirm having actively participated in the research projects listed, as well as writing the papers in association with the co-authors.

ABSTRACT

In this thesis, we have investigated fundamental aspects of the telomere biology in *Plasmodium*, the pathogen responsible for 1-2 million malaria deaths every year. We have also analysed whether telomerase, the enzyme responsible for replicating telomeres, can be used as an anti-malarial drug target. In *Plasmodium* species, telomeres are composed of a tandem array of degenerate G-rich heptameres. The size of telomere tract varies among species: from 960 bp in *P. chabaudi* to 6700 bp in *P. vivax*. In *P. falciparum*, the average length is 1200 bp, although it varies slightly depending on the strain examined. Telomere chromatin in *P. falciparum* is only partially organised in nucleosomes. In several malaria parasites (*P. falciparum, P. berghei, P. yoelii*) the telomere adjacent sequences (TAS), or subtelomeric region, consist of a non-coding region harbouring numerous repetitive motifs. We analysed in detail the organisation of *P. falciparum* TAS and observed that although highly polymorphic in size (15-35 kb), TAS are composed of six different repetitive elements that are present in a conserved higher order structure on virtually all chromosome ends. Upstream from TAS of *P. vivax, P. yoelii* and *P. falciparum* reside members of multigene families associated with virulence. Increasing evidence in *P. falciparum* suggests that the proximity to TAS is important for generation of diversity among those gene-families. Our studies have revealed that TAS are necessary for clustering of chromosome ends, which has been proposed to contribute to the high recombination frequencies among subtelomeric gene-families. We have also shown that TAS are not implicated in anchoring of chromosome ends to the nuclear periphery since chromosome ends in which TAS have been deleted are present at the nuclear periphery. Furthermore, we have shown that TAS play an important role in regulating of the size of the adjacent telomeric DNA tract. The exact mechanism how this happens is not yet clear, but possible models are discussed.

We have identified and characterised a putative Plasmodial telomerase reverse-transcriptase gene, *PfTERT*. *PfTERT* contains the most conserved telomerase motifs, but interestingly is predicted to encode an unusually large TERT. Antibodies raised against the recombinant PfTERT protein revealed a localisation pattern not previously described for telomerase from other organisms where it has been examined. PfTERT is not detectable in early ring forms (G1-like phase); however in parasites that have begun DNA synthesis, PfTERT forms a single discrete spot at the nuclear periphery. Attempts to disrupt *PfTERT* gene failed suggesting that telomerase is eesential for the parasite viability. Rodent malaria *TERT* candidate genes have also been identified.

Taken together, the results of this thesis participate in a better understanding of fundamental telomere biology. In particular, this work opens new perspectives towards the understanding of the nuclear architecture, as well as diversification and regulation of expression of subtelomeric multigene families in *P. falciparum*. Finally, data are presented that support Plasmodial telomerase as a new type of target for anti-malaria treatment.

SUMÁRIO

Nesta tese, interessámo-nos em aspectos fundamentais da biologia dos telómeros de *Plasmodium*, agente da malária, causador da morte de 1-2 milhões de pessoas por ano. Investigámos igualmente se a telomerase, enzima responsável pela replicação dos telómeros, pode ser usada como alvo terapêutico anti-malária. Nas espécies de *Plasmodium*, os telómeros são compostos por heptâmeros degenerados ricos em Guanina repetidos consecutivamente. O tamanho médio dos telómeros varia entre as diferentes espécies: de 960 pb em *P. chabaudi* a 6700 pb em *P. vivax*. Em *P. falciparum*, o tamanho médio é de 1200 pb, embora este varie ligeiramente dependendo da estirpe. A cromatina telomérica de *P. falciparum* organiza-se apenas parcialmente em nucleossomas. Em diversos parasitas da malária (*P. falciparum*, *P. berghei* e *P. yoelii*) as sequências adjacentes ao telómero (TAS: "Telomere Adjacent Sequences") são regiões não codificantes que abrigam numerosos motivos repetitivos. A análise detalhada da organização das TAS em *P. falciparum* revelou que, embora apresentem polimorfismos no tamanho (variando entre 15 a 35 kb), são compostas por seis elementos repetitivos diferentes que formam estrutura altamente conservada na maioria das extremidades dos cromossomas. A montante destas regiões em *P. vivax*, *P. yoelii* e *P. falciparum* residem membros de famílias multigénicas implicadas na virulência do parasita. Recentemente, foi sugerido que em *P. falciparum* a localização subtelomérica é importante para gerar diversidade entre aquelas famílias multigénicas. Os nossos estudos revelaram que as TAS são necessárias à associação das extremidades dos cromossomas em "clusters", estruturas que parecem contribuir para as elevadas frequências de recombinação entre famílias multigénicas subteloméricas. Demonstrámos também que as TAS não participam na ancoragem das extremidades dos cromossomas à periferia do núcleo, já que a localização de cromossomas truncados em que as TAS foram suprimidas é igualmente na periferia nuclear. Além disso, estas regiões subteloméricas têm um papel importante na regulação do tamanho do telómero que lhes é adjacente. O mecanismo exacto ainda não está esclarecido, mas modelos possíveis são discutidos.

Em paralelo, procedemos à identificação e caracterização do gene candidato da transcriptase-reversa da telomerase de *P. falciparum*, *PfTERT*. PfTERT apresenta os motivos mais conservados das telomerases, embora surpreendentemente codifica para uma proteína TERT duas vezes maior que as descritas até hoje. Estudos de imunofluorescência com anticorpos produzidos contra uma proteína recombinante de PfTERT revelaram um padrão de localização nunca anteriormente descrito para as telomerases de outros organismos. Na fase de anel jovem do estado sanguíneo (correspondente à fase de G1 do ciclo celular), PfTERT não é detectável; no entanto, em parasitas que começaram a síntese do DNA (fase S), PfTERT localiza-se em um compartimento único na periferia nuclear. As tentativas de interrupção do gene *PfTERT* revelaram-se infrutíferas sugerindo que a telomerase possa ser essencial para a viabilidade dos parasitas. Genes candidatos *TERT* em espécies de *Plasmodium* que causam a malária em roedores foram identificados.

Em resumo, os resultados desta tese contribuem para um melhor conhecimento da biologia fundamental dos telómeros. Em particular, este trabalho abre novas perspectivas para a compreensão não só da arquitectura nuclear, como também da diversificação e da regulação da expressão de famílias multigénicas subteloméricas em *P. falciparum*. Finalmente, apresentamos resultados que sugerem que a telomerase de *Plasmodium* poderá ser um novo alvo para o tratamento da malária.

RÉSUMÉ

Dans cette thèse, nous avons étudié des aspects fondamentaux des télomères de *Plasmodium*, le parasite du paludisme, responsable de 1 à 2 millions de décès par an. Nous avons également analysé si la télomérase, l'enzyme responsable de la réplication des télomères peut être employée comme cible de traitements anti-paludiques. Chez *Plasmodium*, les télomères sont composés d'un tandem d'heptamères dégénérés riches en Guanine. La taille de la région télomérique varie selon les espèces de *Plasmodium* : de 960 pb chez *P. chabaudi* à 6700 pb chez *P. vivax*. Chez *P. falciparum*, la longueur moyenne est de 1200 pb, bien qu'elle varie légèrement selon l'isolat considéré. La chromatine télomérique de *P. falciparum* n'est que partiellement organisée en nucléosomes. Chez plusieurs espèces de *Plasmodium* (*P. falciparum, P. berghei, P. yoelii*) les séquences adjacentes aux télomères (TAS, « Telomere Associated Sequences ») sont non-codantes et contiennent de nombreux motifs répétés. Nous avons analysé en détail l'organisation des TAS de *P. falciparum* et bien que leur taille soit fortement polymorphe (15-35 kpb), les TAS sont composées de six éléments répétés différents qui forment une structure conservée sur toutes les extrémités des chromosomes analysés. En amont des TAS chez *P. vivax*, *P. yoelii* et *P. falciparum* résident des membres des familles multigéniques qui codent pour des facteurs de virulence importants. Il y a de plus en plus de donnés chez *P. falciparum* qui suggèrent que la proximité des TAS est importante pour la génération de la diversité parmi ces familles multigéniques. Nos études ont indiqué que ces régions sont nécessaires pour grouper les extrémités de chromosomes dans des « clusters », qui semblent contribuer aux fréquences élevées de recombinaison parmi les familles multigéniques subtélomériques. Nous avons également prouvé que les TAS ne sont pas impliquées dans l'ancrage des extrémités des chromosomes à la périphérie nucléaire puisqu'un chromosome tronqué dans lequel les TAS ont été supprimées est toujours placé dans la périphérie nucléaire. En outre, nous avons montré que ces régions subtélomériques jouent un rôle important dans la régulation de la taille du télomère adjacent. Le mécanisme exact n'est pas encore clair, mais des modèles possibles sont discutés.

Nous avons identifié et caractérisé un gène putatif de la transcriptase-reverse de la télomérase, *PfTERT*. PfTERT contient les motifs les plus conservés des télomérases, mais en revanche ce gène semble coder une protéine TERT exceptionnellement grande. Des études d'immunofluorescence avec des anticorps produits contre une protéine recombinante de PfTERT ont indiqué une localisation dans la cellule qui n'a pas été décrite pour les télomérases d'autres organismes. Chez des anneaux jeunes des stades sanguins (phase équivalente à G1 du cycle cellulaire), PfTERT n'est pas détectable; cependant dans les parasites qui ont commencé la synthèse d'ADN, PfTERT apparaît localisé dans un seul compartiment nucléaire à la périphérie nucléaire. Les tentatives de knock-out du gène *PfTERT* ont échoué suggérant que le télomérase est essentielle à la viabilité des parasites. Nous avons également identifié des gènes potentiels *TERT* chez des espèces de *Plasmodium* responsables du paludisme chez les rongeurs.

Les résultats de cette thèse contribuent à une meilleure compréhension de la biologie fondamentale des télomères. En particulier, ce travail ouvre de nouvelles perspectives vers l'étude de l'architecture nucléaire, de la diversification et de la régulation de l'expression des familles multigéniques subtélomériques chez *Plasmodium*. En conclusion, nous présentons des données qui soutiennent que la télomérase plasmodiale peut être une nouvelle cible pour le traitement anti-paludique.

CONTENTS

ABBREVIATIONS **19**

INTRODUCTION **21**

I. Malaria: A Global Picture *23*

1. What is malaria and what is its impact? 23
2. *Plasmodium*: the infectious agent 25
3. Efforts to Combat Malaria 26

II. Telomere Biology *31*

1. Telomere structure 31
2. Subtelomeric sequences 34
3. End replication problem 38
4. Telomere functions 39
5. Telomeric chromatin 41
6. Telomerase: telomere replication and chromosome repair 43
7. Structure of telomerase subunits: TERT and TER 45
8. Telomere maintenance without telomerase 47
9. Telomere length regulation 49
10. Telomere loss and senescence 50
11. Telomerase Inhibition 52
12. TRAP: an in vitro assay used to measure telomerase activity 53

III. Genome Organisation in Plasmodia *55*

1. Chromosome structure 55
2. Chromosome polymorphism 57
3. Chromatin organisation 57
4. Nuclear architecture in *P. falciparum* 58

OBJECTIVES **61**

RESULTS **65**

Genomic Organisation and Chromatin Structure of Plasmodium falciparum Chromosome Ends *69*

A Central Role For Plasmodium falciparum Subtelomeric Regions in Spatial Positioning and Telomere Length Regulation *77*

Plasmodium Telomerase Reverse-Transcriptase Is Unusually Large and Localises in a Discrete Subnuclear Compartment *89*

Organisation of Subtelomeres in Human and Rodent Malaria Species *107*

REVIEW ARTICLES **117**

Plasmodium Telomeres: a Pathogen's Perspective *121*

Chromosome Structure and Dynamics of Plasmodium Subtelomeres *129*

CONCLUDING REMARKS **147**

1. Subtelomeric Regions: Conserved Higher-Order Structure *150*

2. Roles of P. falciparum Subtelomeric Regions *151*

2.1 Telomere length regulation 152

2.2 Nuclear architecture 153

3. P. falciparum Telomerase: A Drug Target *157*

REFERENCES **161**

ABBREVIATIONS

TAS: Telomere Associated Sequences

TAREs: Telomere Associated Repetitive Elements

TRAP: Telomere Repeat Amplification Protocol

TERT: Telomerase Reverse Transcriptase

TR (TER): Telomerase RNA

TPE: Telomere Positioning Effect

ALT: Alternative Lengthening of Telomeres

FISH: Fluorescent In situ Hybridisation

DHFR: Dihydrofolate Reductase

WHO: World Health Organisation

kb: kilobase

bp: base pair

nt: nucleotide

INTRODUCTION

I. MALARIA: A GLOBAL PICTURE

1. What is malaria and what is its impact?

Malaria is an infectious disease transmitted by mosquitoes. It is caused by single-celled parasitic protozoa of the genus *Plasmodium*, which alternatively infect vertebrate and insect hosts. According to the WHO, human malaria is by far the world's most important tropical parasitic disease and causes 1-2 million deaths each year, more people than any other communicable disease except AIDS and tuberculosis. It is a public health problem today in more than 90 countries, affecting 40% of the world's population. The worldwide prevalence of the disease is estimated by the World Health Organisation to be on the order of 300-500 million clinical cases each year, of which more than 90% are in sub-Saharan Africa (Marsh, 1999). Mortality due to malaria is especially high among children under five years old. Other high-risk groups are pregnant women, non-immune travellers, refugees, and labourers entering endemic areas. Indigenous adults are protected by a form of partial immunity, which develops slowly as a result of continuous exposure to *Plasmodium falciparum* malaria (Snow *et al.*, 1997).

After an initial period of several days (10-15) of irregular fevers and gastro-intestinal troubles, clinical episodes of malaria may comprise intermittent fevers. A classical attack of malaria begins with a feeling of unbearable cold, followed by a quick rise of the body temperature to 40-41°C that lasts for 2-3 hours. Finally, profuse sweating ends the attack, restoring the body temperature (Fig.1).

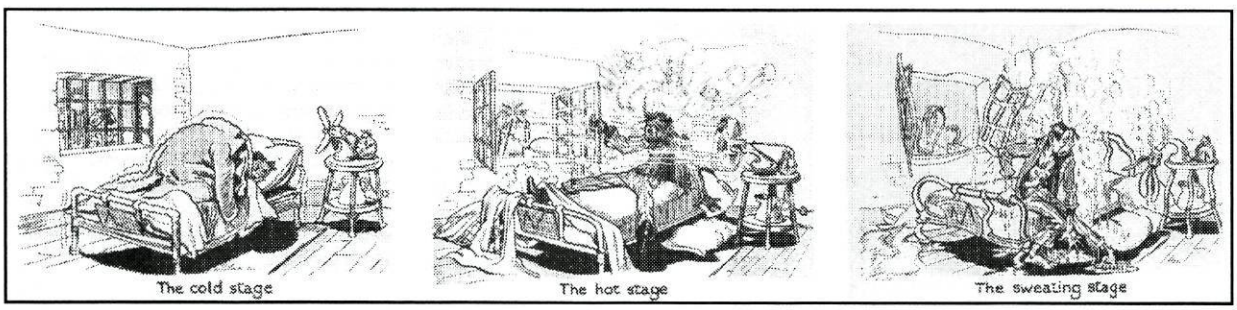
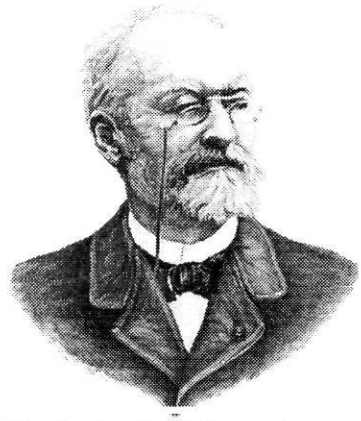


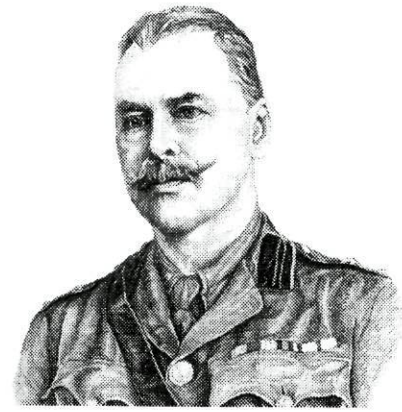
Figure 1. The three stages of intermitent fevers of a typical attack of malaria. Cartoons by a British servicemen in North Africa during the war (Royal Air Force Institute of Pathology & Tropical Medicine, Halton).

Each crisis is abrupt and often severe lasting 6-8 hours, recurring with a periodicity that depends on the Plasmodial infection. Other symptoms of malaria include shivering, pain in the joints, headache, repeated vomiting and convulsions. Severe anaemia (exacerbated by malaria) is often the attributable cause of death in areas with intense malaria transmission. If not treated, the disease may progress at any time to severe malaria, which is often lethal (for a detailed review see (Miller *et al.*, 2002)).

Transmission of malaria from one vertebrate host to another is achieved by a mosquito. It was Sir Ronald Ross (Fig. 2), who observed for the first time human *Plasmodium* parasites on the outer wall of the mosquito stomach. This mosquito was later classified as belonging to the genus *Anopheles*. They are common in most temperate and tropical countries, provided that there are suitable breeding sites. They are active at night, sheltering themselves in shaded humid places during the day. They are relatively small insects, up to 8 mm long, and only females are capable of transmission. The number and type of mosquitoes determine the extent of transmission in a given area. Transmission of malaria is affected by climate and geography, and often coincides with the rainy season.



Charles Louis Alphonse Laveran
(1845-1922)



Ronald Ross
(1857-1932)

Figure 2. Nobel laureates for major contributions in the understanding of malaria. Laveran was born in Paris and while serving the French Army in Algeria he observed the presence of malaria parasites in the blood of patients with febrile symptoms (1880). His discoveries were initially viewed with scepticism, but were later acknowleged as one of profound importance. Laveran was awarded the Nobel Prize in Medicine in 1907 as "initiator and pioneer of the pathology of protozoa". Laveran left the army in 1896 to continue his studies at the Pasteur Institute in Paris. Ross, an English army surgeon in India, made his first breakthrough on August 20th 1897 (known afterwards as the "Mosquito Day"), when he discovered pigmented cysts on the stomach wall of a mosquito later classified belonging to the genus *Anopheles*. By 1889 he had elucidated the entire life-cycle of bird malaria, which was confirmed by Grassi for human malaria. Ross was awarded the Nobel Prize in Medicine in 1902.

2. Plasmodium: the infectious agent

The malaria-causing organisms are obligate intracellular parasites of the genus *Plasmodium*, order Haemosporida, class Hematozoa, phylum Apicomplexa and kingdom Protozoa (Ayala *et al.*, 1998). First observed under a rudimentary microscope by Alphonse Laveran (Fig. 2), more than hundred Plasmodia species have been described today (Garnham, 1966). There are four species that infect man: three can cause severe illness but are rarely fatal: *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae*. The fourth causes much more serious and progressive illness, often leading to coma and death within a few days: *Plasmodium falciparum*. *P. falciparum* accounts for the majority of infections and mortality as a result of its prevalence, virulence and drug resistance.

The general life cycle of Plasmodia species is quite complex (for details see (Fujioka and Aikawa, 1999)). Fig.3 shows the life cycle of *Plasmodium falciparum*, which can be considered as a sequence of four phases: one sexual stage and three highly proliferative asexual stages. *Plasmodium* is haploid for the majority of its life cycle, although a brief diploid stage exists during its sexual stage. The three asexual stages each begin with an intracellular growth form that enlarges rapidly before maturing into invasive forms (by a process of internal segmentation). The invasive form at the end of each phase is extracellular, mobile, and able to enter host cells.

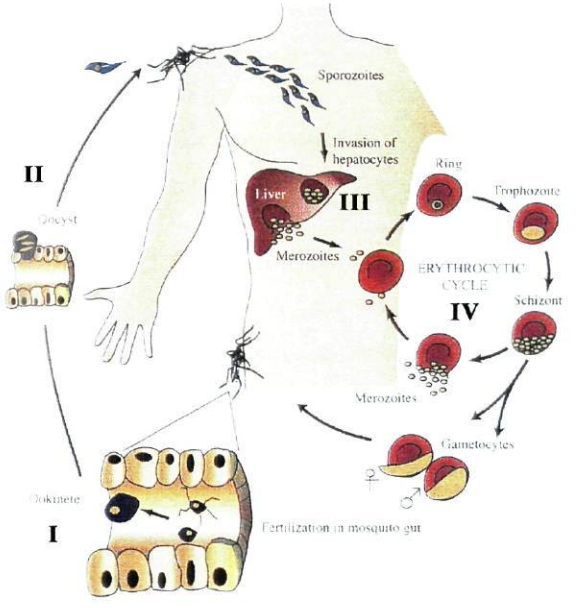


Figure 3. The life cycle of *Plasmodium falciparum*. The four mains phases are shown: I is the sexual stage and II-IV are the asexual highly proliferative stages. I and II happen in the mosquito host, III and IV in man (courtesy from Andreas Heddini, Sweden).

The size and shape of the invasive forms vary according to the phase, but some features are in common: an apical complex (an apparatus necessary for invasion of host cells), a thick three-layer pellicle and a cytoskeleton of microtubules (responsible for maintaining the pear shape of the organism and contributing to its mobility).

The sexual and first asexual phases occur in the Anopheles mosquitoes, leading to the release of around 10 000 sporozoites from a single sexual form. During the blood meal, sporozoites enter in the bloodstream of the human host, where they infect the liver. It is here that the second asexual phase occurs. In 5-15 days, one sporozoite develops into an exoerythrocytic schizont, each containing between 10 000 - 30 000 merozoites. Sporozoites of some malaria species do not develop immediately. Instead, once in the liver, they differentiate into dormant parasitic forms, named hypnozoites, responsible for delayed malaria relapses. Merozoites are released into the bloodstream, where the third asexual phase occurs. A merozoite invades an erythrocyte and matures from ring to trophozoite stage. Within 48 hours, it divides asexually to form 8-32 new merozoites. These burst from the infected erythrocyte and invade new ones, a process that may be repeated many times. The erythrocytic stage is the only stage responsible for the clinical manifestations of malaria. Some merozoites differentiate into gametocytes (the sexual forms), which are taken up by a feeding mosquito.

3. Efforts to Combat Malaria

Prevention of malaria encompasses a variety of measures that may protect against infection or against the development of disease in infected individuals. Measures that **protect against infection** are directed against the mosquito vector. These can be personal protection measures (e.g., protective clothing, repellents, bed nets), or community/population protection measures (e.g., use of insecticides, environmental management to control transmission) (for review see (Greenwood and Mutabingwa, 2002)). Because mosquitoes are becoming increasingly resistant to insecticides, and in many cases have adapted so as to avoid insecticide-treated surfaces altogether, more attention is being given to measures that protect against disease: **vaccines** and **chemoprophylaxis.**

3.1 Vaccine Development

Attacking a chronic infection such as malaria is not an easy task given the complex immune response developed by the host. Nevertheless, considerable progress has been made in the search for a malaria vaccine. Several lines of evidence suggest that malaria vaccines are feasible. First, immunisation with irradiated sporozoites protects or partially protects humans from being infected by sporozoites (Egan *et al.*, 1993), (Rieckmann *et al.*, 1979). Second, people infected repeatedly by malaria develop "naturally acquired immunity" (NAI), which protects them against clinical disease if continuously exposed to infection (Baird, 1995). Third, immunisation studies show that vaccines already in hand can protect against infection in animal models and in humans, as reported for a recombinant anti-sporozoite vaccine based on the cirscumsporozoite protein (Kester *et al.*, 2001). Finally, studies have shown success in protecting mosquitoes against infection by *P. falciparum* and *P. vivax* (Hisaeda *et al.*, 2000). In these studies, the mosquito was feeded with a gametocyte-containing blood mixed with serum from animals immunised with prototype human anti-mosquito stage (transmission-blocking) vaccin.

An effective vaccine would constitute a powerful addition to malaria control. More than a dozen candidate vaccines are currently in development, some of them are in clinical trials. According to a recent WHO report, the hope is that an effective vaccine will be available within the next 7-15 years (WHO, 2000).

Three main types of vaccine are being developed:

- **"Anti-infection"** vaccines, aimed at protecting malaria-naïve travellers or residents of low endemic areas; targeted to sporozoites.
- **"Anti-disease / anti-parasite"** vaccines, aimed at children, pregnant woman and migrants living in endemic areas; designed to reduce the parasite dosage and severe and complicated manifestations of the disease, by targeting asexual blood stages.
- **"Anti-mosquito-stage"** vaccines, designed to arrest the development of the parasite in the mosquito, thereby reducing or eliminating transmission of the disease.

3.2 Antimalarial drugs

Antimalarial drugs are used to prevent the onset of disease, to treat clinical cases in individuals, and to prevent disease transmission within populations. Quinine, in the form of Chinchona bark, was used as early as 1640 to treat ague, as malaria was then known . All other antimalarials have come from pharmacological research in the 20th century, often stimulated by the medical problems of war. The number of compounds synthesised and tested as antimalarials exceeds a quarter of a million, but only a handful have proved suitable for general use (for review see (Rosenthal, 2001) and (Ridley, 2002)).

The existing antimalarials belong to four different classes of chemical compounds and are summarised in Table 1. **Quinoline derivatives** inhibit heme polymerase activity resulting in the accumulation of free heme, which is toxic to the parasites in all asexual stages (Sullivan *et al.*, 1998). **Antifolates** act on erythrocytic stages and intrahepatic stages, but not hypnozoites. Pyrimethamine also acts on gametocytes. The combination of sulphadoxine / pyrimethamine has an inhibitory effect on dihydrofolate reductase (DHFR), an enzyme involved in the folic acid metabolism, thus fatally impairing the development of the parasite, causing it to arrest in a non-mature, inviable form. maturation and producing large non-viable parasites (Wolfe *et al.*, 2001). Atovaquone interferes with mitochondrial electron transport (Fry and Beesley, 1991). The addition of proguanil to atovaquone results in a synergistic activity. **Artemisinin derivatives** are effective against gametocytes, the sexual stage, and act by binding iron in the malarial pigment (Haemozoin) damaging the parasite oxidatively (Meshnick *et al.*, 1996). **Antimicrobials** act synergistically with quinoline derivatives to kill erythrocytic schizonts (2001). They are thought to inhibit parasite growth through the inhibition of prokaryote-like protein biosynthesis in the apicoplast – an organelle that is unique to apicomplexan parasites such as *Plasmodium* (Fichera and Roos, 1997).

Other areas have been investigated in an effort to identify new drug targets. Lactate dehydrogenase, proteases involved in erythrocyte invasion, and channels responsible for transporting solutes and nutrients are targets of high potential (Dunn *et al.*, 1996), (Blackman, 2000), (Desai *et al.*, 2000). Recently, a team from Montpelier (France) found that pyrrolidine compounds, such as G25, that inhibit phosphatidylcholine biosynthesis *de novo* from choline have excellent antimalarial properties, curing monkeys infected with *P. falciparum* and *P. cynomolgi* with very low doses. (Wengelnik *et al.*, 2002).

Class of Drug	Drug	Main Limitations
Quinoline Derivatives	Quinine	Compliance / Safety / Resistance
	Chloroquine	Resistance
	Mefloquine	Resistance / Cost / Safety
	Halofantrine	Safety / Resistance / Cost
	Amodiaquine	Safety / Resitance
Antifolates	Sulphadoxine / Pyrimethamine	Resistance
	Proguanil /Atovaquone	Resistance / Cost
Artemisinin Derivatives	Artemisinin	Compliance / Safety / Cost
	Artemether	Compliance / Safety / Cost
	Artesunate	Compliance / Safety / Cost
Antimicrobials	Tetracycline	Resistance / Potential / Cost / Compliance
	Clindamycin	Resistance / Potential / Cost / Compliance

Table 1. Principal antimalarials in use, according to a recent WHO report (WHO, 2001). The main inconvenients of each drug is indicated in decreasing order of importance (adapted from (Ridley, 2002)). Proguanil is an inhibitor of the mitochondrial respiratory chain and is used in association with other drugs, such as Atovaquone and Chloroquine.

Resistance to antimalarial drugs is a major problem in most tropical areas. Drug-resistant strains of *P. falciparum* are spreading rapidly and, more recently, there have been reports of drug-resistance in people infected with *P. vivax*. Thus, adequate treatment of malaria is becoming increasingly difficult. New, especially inexpensive and affordable drugs, and more practical formulations of existing drugs/compounds are badly needed.

The erythrocytic stage of the parasite life, which gives rise to the clinical symptoms of the disease, is characterised by a high proliferative capacity. In 48 hr, *P. falciparum* undergoes 4-5 mitotic divisions, which leads to a rapid increase of the parasite number in the blood stream (varying from 0,5-5% parasitemia, which represents around $1,5\text{-}15x10^{13}$ parasites). Thus, inhibition of the mitotic replication machinery of the parasite should have drastic consequences in the parasites capacity to multiply and thus inhibit the progress of the disease. One enzyme that is probably crucial for the continual proliferation of the parasite is telomerase. This enzyme, extensively studied in yeast, ciliates and humans, is responsible for the replication of the very end of the chromosomes: the telomere. Telomerase activity has been detected in *P. falciparum* by Bottius et al. To test if Plasmodial telomerase is a valuable drug target, it is first necessary to understand the fundamental principles underlying the telomere biology of malaria parasites.

II. TELOMERE BIOLOGY

Telomeres are DNA-protein complexes that form the ends of linear eukaryotic chromosomes. The first evidence that eukaryotic chromosomes ended with a peculiar structure was obtained from cytological studies, in the late thirties of last century by Hermann J. Muller (Muller, 1938). He found that after X-ray irradiation, terminally deleted and inverted chromosomes were not recovered in *Drosophila*, while interstitial deletions or inversions could be detected. This observation suggested that a specialised structure required for chromosome stability was present at the end of the chromosomes. This structure was named telomere, from the Greek words telos and meros, that is "end" and "part". In 1941, many years before any tool to identify the molecular structure of telomeres were available, Barbara McClintock observed that maize broken chromosomes tended to fuse with each other forming dicentric chromosomes (McClintock, 1941). Since normal chromosomes did not behave in such a way, she proposed that telomeres protected chromosome ends from recombination and allowed them to be distinguished from double strand breaks.

1. Telomere structure

The molecular nature of telomeres was elucidated in the 1970s. Much of the initial molecular description of telomeres was performed in Elizabeth Blackburn's lab on the ciliated protozoa, *Tetrahymena*. This organism is an ideal system to study telomeres because *Tetrahymena* contain in their macronucleus millions of single-gene containing DNA molecules, each capped by telomeres. Since then, telomeric DNA has been sequenced in numerous organisms (Table.2) and, even among distantly related eukaryotes, telomeric organisation is well-conserved. It was shown that in most eukaryotes, telomeres consist of tandemly repeated simple sequences characterised by a G-rich strand (G-strand) forming the 3' end of each terminus and a C-rich complementary strand (C-strand). However, some organisms display a different organisation of telomeres. In *Drosophila*, for example, telomeres are composed of a complex mosaic of primarily two types of transposable elements: HeT-A and TART (for review see (Pardue *et al.*, 1996)). Ironically, the organism that first provided cytological evidence for the existence of telomeres has atypical chromosome ends!

Organism	Species	Sequence	Reference
Ciliates	*Tetrahymena thermophila*	GGGGTT	(Blackburn and Gall, 1978) (Challoner and Blackburn, 1986)
	Stylonichia pustulata	GGGGTTTT	(Oka *et al.*, 1980)
	Oxytricha fallax	GGGGTTTT	(Klobutcher *et al.*, 1981), (Pluta *et al.*, 1984)
	Euplotes adiculata	GGGGTTTT	(Klobutcher *et al.*, 1981)
	Paramecium primaurelia	GGGGTT or GGGTTT	(Baroin *et al.*, 1987)
Yeast	*Saccharomyces cerevisiae (budding yeast)*	$G_{1-3}T(GT)_{1-3}$	(Szostak and Blackburn, 1982), (Wang and Zakian, 1990)
	Schizosaccharomyces pombe (fission yeast)	$GGTTACAA_{0-1}C_{0-1}G_{0-6}$	(cited in reference (Matsumoto *et al.*, 1987))
	Candida albicans	ACGGATGTCTAACTTCT TGGTGT	(McEachern and Hicks, 1993)
	Kluyveromyces lactis	ACGGATTTGATTAGGTATG TGGTGT	(McEachern and Blackburn, 1994)
Higher eukaryots	*Arabidopsis thaliana (plant)*	GGGTTTA or GAGTTTA	(Richards and Ausubel, 1988)
	Homo sapiens	GGGTTA	(Moyzis *et al.*, 1988), (Meyne *et al.*, 1989)
	Mus musculus (mouse)	GGGTTA	(Starling *et al.*, 1990)
	Drosophila melanogaster	Retrotransposon sequence repeats (HeT-A and TART)	(Levis *et al.*, 1993)
	Xenopus laevis	GGGTTA	(Bassham *et al.*, 1998)
Kinetoplastideae	*Trypanosoma brucei*	GGGTTA	(Blackburn and Challoner, 1984), (Van der Ploeg *et al.*, 1984)
	Trypansoma cruzi	GGGTTA	(Freitas-Junior *et al.*, 1999), (Chiurillo *et al.*, 1999)
	Leishmania brazilensis, L. major, L. donovani	GGGTTA	(Fu and Barker, 1998), (Chiurillo *et al.*, 2000)
Apicomplexa	*Plasmodium berghei*	GGGTTTA or GGGTTCA	(Ponzi *et al.*, 1985)
	Plasmodium falciparum	GGGTTTA or GGGTTCA, and other related sequences	(Vernick and McCutchan, 1988)
	Plasmodium vivax	GGGTTTA or GGGTTCA	(del Portillo *et al.*, 2001)

Table 2. Telomeric DNA sequences. The first studies were performed in ciliates which, as a result of their unusual genomic organisation, provided an abundant source of telomeres. In the majority of organisms, telomeric DNA is a tandem array of short repeats that are G-rich in one strand.

Telomeric regions from midge *Chironomus pallividittatus* (Saiga and Edstrom, 1985), the mosquito *Anopheles gambiae* (Biessmann *et al.*, 1996) and plant *Allium cepa* (onion) (Pich and Schubert, 1998) contain complex-sequence tandem repeats.

The fact that a telomeric organisation consisting of tandem arrays of short G-rich repetitive motifs is conserved among evolutionary distant organisms strongly suggests that these particular arrays of nucleotides play an important role in the recognition of specific DNA binding proteins and/or in the formation of specialised structures, which are important for chromosome stability.

Telomeres display other common features:

- most of the telomeric DNA is duplex, but the extreme tip of the DNA is in the form of a 3' single-strand overhang (a consequence of semi-conserved replication) (Klobutcher *et al.*, 1981), (Henderson and Blackburn, 1989), (Wellinger *et al.*, 1993), (Wright *et al.*, 1997) ;
- heterogeneous in length because of the presence of varying numbers of tandem repeats (references from Table), probably a result of the mechanism of synthesis and loss of telomere repeats.

It has been speculated that the protruding G-rich end is folded into a G-quartet: a four-stranded structure, in which each guanine is the acceptor and donor of two hydrogen bonds to generate a square plan (for detailed description see (Rhodes and Giraldo, 1995)). However, the relevance of this structure to the situation *in vivo* remains controversial.

Recently, telomeres from some organisms were shown to end in a large loop resembling a lasso, termed the t-loop. Telomeric t-loops have been isolated from mice, humans, ciliates and African trypanosomes, and may be an evolutionarily conserved structure (Griffith *et al.*, 1999), (Murti and Prescott, 1999), (Munoz-Jordan *et al.*, 2001). In mice and humans, the size of the t-loop correlates with telomere length, ranging from ~3 kb to 18 kb. *T. brucei*, by contrast, has smaller t-loops (~1 kb), despite telomere lengths of 10-20 kb, suggesting that the size of t-loop can be regulated. Indirect evidence suggests the telomeric loops are formed by invasion of the 3'-overhang into the duplex part of the telomeric repeat array (Griffith *et al.*, 1999) (Fig.4). Thus the t-loop may provide the telomere with a structure that is devoid of recognizable DNA "ends" and hence does not resemble a double-strand break. It could also protect the 3'-overhang from degradation and limit the ability of

telomerase to access its substrate. Disruption of t-loop is thought to signal a cellular response that, at least in some regards, resembles the cellular response to double-strand break in the genome.

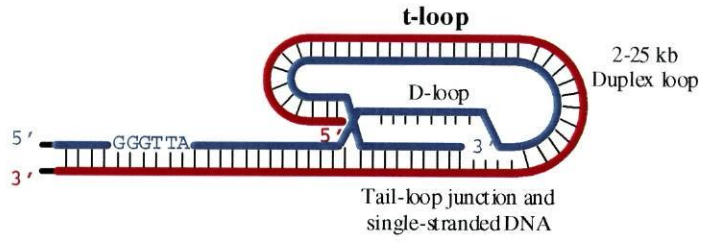


Figure 4. Model of a t-loop. The 3'-telomeric overhang invades the duplex telomeric array in mammals, ciliates and AfricanTrypanosomes.

In yeast, biochemical (Strahl-Bolsinger *et al.*, 1997) and genetic (de Bruin *et al.*, 2001) data suggest that telomeres form a folded structure, in which the telomere loops back, to come closer to subtelomeric DNA. Whether looping in yeast involves base pairing of the telomere with subtelomeric DNA remains to be determined. The current model for telomere length regulation proposes that the telomere binding protein, Rap1p, modulates the sequestration of the chromosome terminus in a fold-back structure, thereby regulating telomere length by inhibiting access of the telomerase enzyme to the telomere end (reviewed in (Evans and Lundblad, 2000)).

2. Subtelomeric sequences

The DNA sequences adjacent to the simple telomeric repeats are called subtelomeric regions or telomere-associated sequences (TAS). Generally, they are extremely polymorphic in size and contain species-specific repeated motifs (Fig. 5).

The organisation of TAS in ***Homo sapiens*** is complex and includes several families of low-copy elements, which can extend up to a few hundred kb. *In situ* hybridisation experiments have shown that some of these sequences can be shared between numerous chromosome ends, indicating that different combinations of repeated elements compose the subtelomeric regions (reviewed in (Mefford and Trask, 2002)). ***Drosophila*** chromosome ends have variable tandem repeats flanking the telomere-specific retrotransposons. Some repeats are common between chromosomes, but others are chromosome-specific (Mason and Biessmann, 1995).

In ***Saccharomyces cerevisiae***, telomeric sequences are flanked on one side by by 0-4 copies of tandemly arrayed Y' elements. These repeats are either 6,7 or 5,2 kb and are exclusively present in subtelomeric regions (Walmsley *et al.*, 1984). Y' elements can recombine between each other or excise and move to new positions (Louis and Haber, 1990). Internally proximal to Y' elements, all chromosomes have a small core X element (Chan and Tye, 1983) that is less conserved than Y' elements and more heterogeneous in size (0,3-3,7 kb). Multigene families involved in the use of carbon sources follow the X element (eg. *MAL, MEL, SUC*, etc). Tracts of 50-130 bp telomeric repeats are found between X and Y' and between tandem copies of Y'.

The first malaria species in which the structure of chromosome ends was studied was ***Plasmodium berghei***. The majority of subtelomeric regions consist of a tandem array of an unknown number of copies of a unique 2.3 kb unit (Pace *et al.*, 1987), (Dore *et al.*, 1990). The 2.3 kb units are found exclusively in subtelomeres and contain ~160 bp stretch of telomeric sequences. This subtelomeric organisation appears to be important for recombinational events that disperse subtelomeric sequences/elements from one chromosome end to another, thereby contributing to chromosome-size polymorphism (Pace *et al.*, 1990). *P. berghei* subtelomeric regions display an AT content of 78%, matching the average of its genome. No gene families have been described at subtelomeric loci. As described in detail in the Results chapter of this thesis, plasmodial species present different subtelomeric organisations. Namely, ***Plasmodium falciparum*** displays a pattern of subtelomeric repeats highly conserved among 14 chromosomes (Fig. 1 of Results IV).

In ***Trypanosoma brucei***, upstream from telomeres, two blocks of repetitive elements separate telomeric DNA from the VSG genes, a multigene-family that encode an important virulence factor. Centromere-proximal to the VSG there is an array of 70 bp repeats (reviewed in (Cano, 2001)). Subtelomeric regions of ***Trypanosoma cruzi*** do not appear to harbour arrays of tandem repeats. Instead they contain a conserved 189 bp sequence, members of the Gp85/sialidase gene-family (which is important for the virulence of the parasite) and at some chromosomes, a SIRE element (a retrotransposon-like element) (Chiurillo *et al.*, 1999). Recently, telomeres and subtelomeric regions were cloned from ***Trypanosoma rangeli***. A multiple sequence alignment of six chromosome ends showed that next to the hexameric telomeric repeats, a homologous region of ~600bp and 50-57% GC-rich is present. Subtelomeres from *Leishmania* species contain conserved repetitive elements (called LCTAS,

Leishmania conserved telomere-associated sequences), which are organised in tandem arrays in ***Leishmania major***, but are found in only a single copy in ***Leishmania braziliensis***. In addition, both organisms harbour species-specific repetitive elements (Fu and Barker, 1998). In ***Leishmania donovani***, the block organisation of subtelomeric sequences is similar to the one observed in other *Leishmania* species. Aditionally, some chromosomes contain non-repeated subtelomeric sequences (LSTS).

The location of important multigene families in subtelomeres has prompted several authors to suggest that these regions of the chromosome provide a mechanism for permanently increasing the diversity of genes nearby, thus allowing a rapid adaptive evolution (Corcoran *et al.*, 1988), (Pologe and Ravetch, 1988), (Hinterberg *et al.*, 1994), (de Bruin *et al.*, 1994), (Louis, 1995), (Trask *et al.*, 1998). In *P. falciparum*, for example, higher frequencies of ectopic recombination have been detected among the subtelomeric members of the *var* gene-family, potentially promoting antigenic variability, which is essential for the survival of the parasite in its human host (Freitas-Junior *et al.*, 1999).

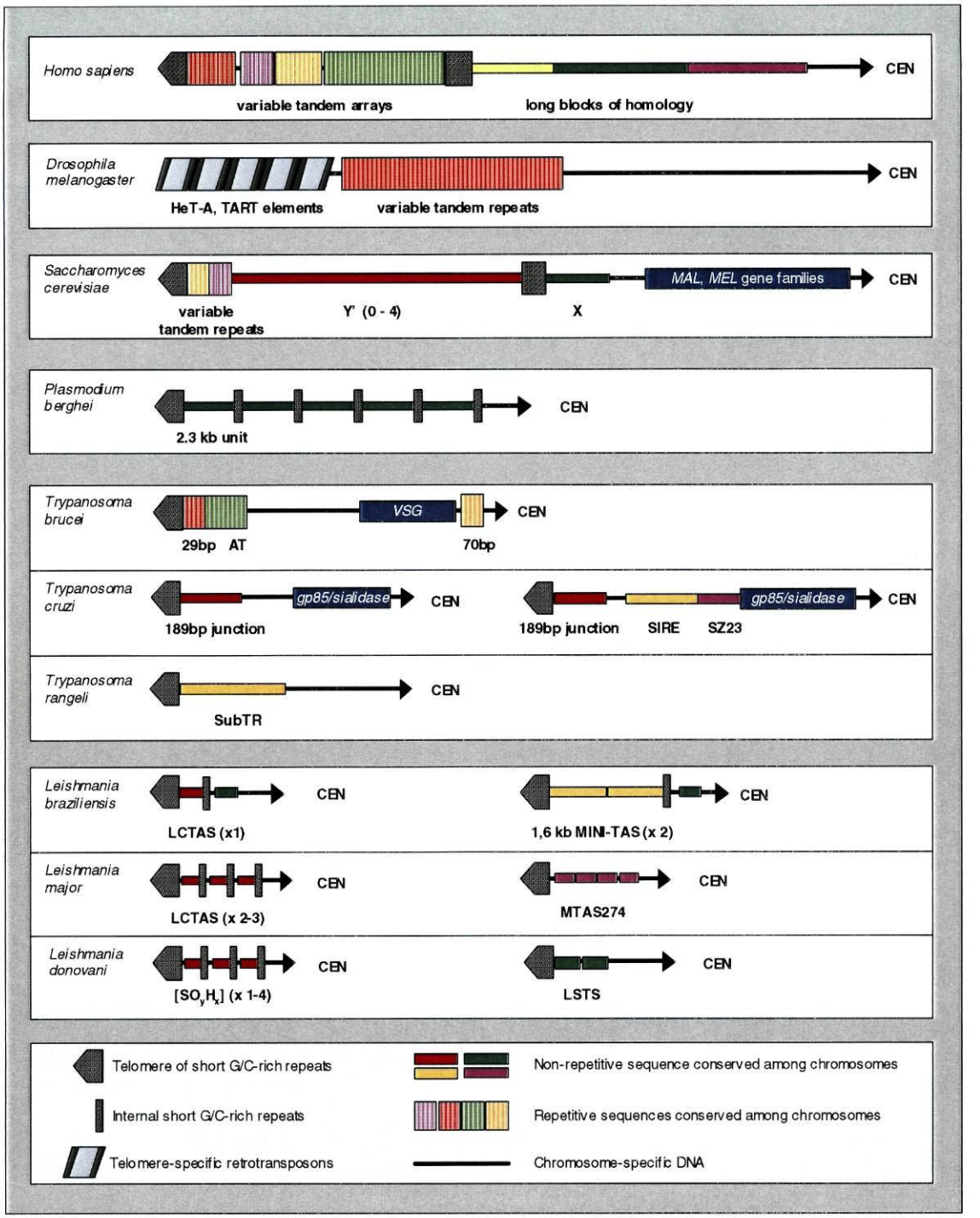


Figure 5. Schematic diagram of the DNA organisation at chromosome ends of a variety of eukaryotes. Canonical telomeres are composed of tandem arrays of a short, tandemly repeated sequences in which guanine residues are sequestered to one strand (eg. GGGTTA in *H. sapiens*), except in *Drosophila*, in which the ends are maintained by retrotransposition of the HeT-A and TART elements. Subtelomeric regions are unusually dynamic and contain variable mosaics of sequence, sometimes composed partially of short tracts of telomeric repeats. For the purpose of this figure, one "block" is either a repetitive element (dashed squares) or a non-repetitive element (plain bars). Some blocks are conserved among all chromosomes, while others are only shared by a restricted group of chromosomes. As shown here, for *T. cruzi*, and *Leishmania* spp, subtelomeric regions can be grouped in two different classes. LCTAS in *L. braziliensis* and *L. major* corresponds to $[SO_yH_x]$ in *L. donovani*. In *S. cerevisiae, T. brucei* and *T. cruzi*, multigene families are located within subtelomeric regions, which is probably a reflection of the high frequency of recombination that occurs at these regions of the chromosome. Blocks are not drawn to scale. (For organisation of TAS in other Plasmodia species, refer to RESULTS IV in his thesis.)

3. End replication problem

One of the main functions of telomeres is to overcome the problem of replicating the 3' end of linear DNA molecules. The "end-replication problem" derives from the two well-known properties of all DNA polymerases: the requirement of a 3'-OH primer and the ability to synthesise only in the 5' to 3' direction. In the lagging strand, short RNA primers are required to start synthesis of the Okazaki fragments (Watson, 1972). Therefore, when the last primer complementary to the 3'end is removed, a gap remains that cannot be replenished in by DNA polymerase (Fig. 6).

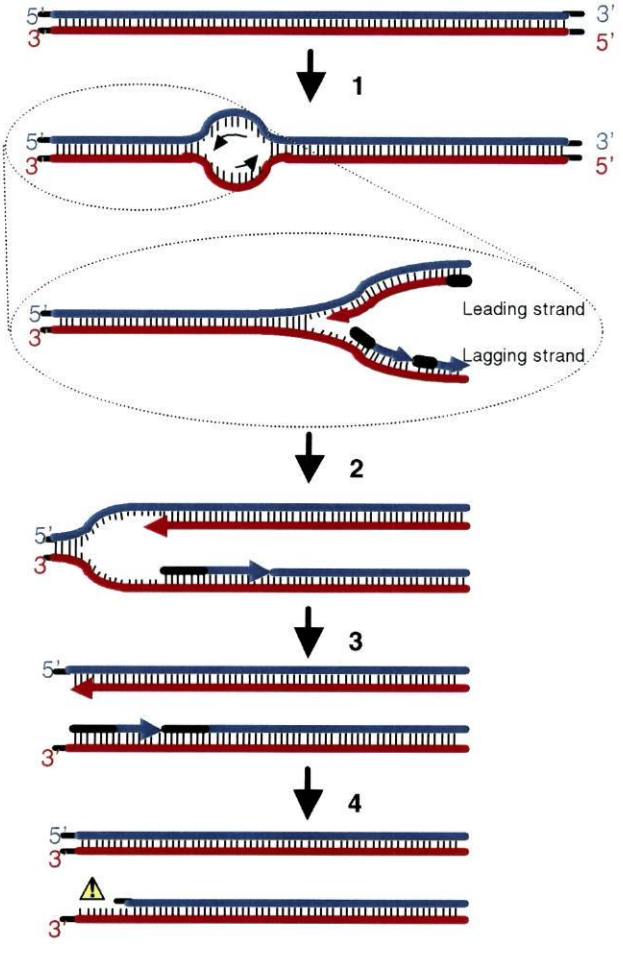


Figure 6. The end-replication problem. 1) The replication fork is established. Zoom in one end of the double-strand DNA molecule. 2) The leading strand is synthesised continuously up to the 3' end. The lagging strand is synthesised discontinuously starting from the RNA primers (black boxes), which are lengthened to form the Okazaki fragments (blue arrows). 3-4) When the primers are removed, the Okazaki fragments are extended and ligated togther. The degradation of the RNA primer from the terminal Okazaki fragment is not replenished, generating a single-strand overhang.

If the "end-replication problem" is not solved, DNA sequences are lost in every round of replication (50-200 nt in human cells (Harley *et al.*, 1990), 75 nt per fly generation (Biessmann *et al.*, 1990) and ~4 nt in *S. cerevisiae* (Lundblad and Szostak, 1989)) and eventually cells lose viability. Several "solutions" to this "problem" exist in different organisms (reviewed in (Zakian, 1989)):

- circular chromosomes / genomes (eg. in bacterial plasmids, several viruses);
- particular terminal sequences that allow the formation of hairpins, concatameresor circles during the replication (some viruses and organelles with linear genomes);
- an aminoacidic –OH group of a protein bound to the terminal DNA is used as a primer (in some viruses such as the bacteriophage ϕ29 or the adeno group of animal viruses);
- transposition events (for example, *Drosphila*)
- recombination events (for example, in *Anopheles gambiae* (Biessmann *et al.*, 1996));
- short repeated sequences at the telomeres (eg. GGGTTA in humans), which are replicated by a specific enzyme: telomerase (solution adopted by the majority of eukaryotes).

4. Telomere functions

Telomeres are specialised protein-DNA complexes that protect the ends of eukaryotic chromosomes by assuring that chromosome ends are fully replicated (as discussed above) and thereby preventing the loss of sequence information and by preventing end-to-end fusions. This latter form of protection, known as 'capping' was originally described by Muller and McClintock, and is crucial to preventing deleterious genomic instability (Levis, 1989). More specifically, the telomere provides a structure that allows the cell to distinguish a chromosome end from a broken chromosome, and thus avoids eliciting the downstream events that are normally set in motion by the cellular "alarm bells" that sound when double-stranded breaks in DNA are incurred. Experimentally, telomeres were shown to be essential for chromosome stability by protecting chromosomes from end fusion and nucleolytic degradation of the termini (Gottschling and Zakian, 1986). The Zakian lab observed that removal of a telomere from a dispensable chromosome in yeast led to the following: (i) transient arrest of the cell cycle and activation of mechanisms of repair of broken chromosome ends, and (ii) frequent loss of telomereless chromosomes (Sandell and Zakian, 1993).

In addition to their essential functions, telomeres are also involved in regulation of the transcription of genes located at subtelomeric positions. Initially described in *Saccharomyces cerevisiae* (Gottschling *et al.*, 1990), telomeres were shown to repress the expression of genes placed in subtelomeric locations – a phenomenon called Telomere Position Effect (TPE). TPE occurs virtually in all truncated telomeres, but not in all native (intact) telomeres (Pryde and Louis, 1999). It is still unclear why different chromosome ends have different silencing properties. It was recently shown that TPE is correlated to the positioning of the telomeres to specific regions within the nucleus (reviewed in (Dubrana *et al.*, 2001)). TPE was also described in *Drosophila* (Henikoff, 1992), *Schizosaccharomyces pombe* (Nimmo *et al.*, 1994) and *Homo sapiens* (Baur *et al.*, 2001).

Telomeres have been shown to associate with the nuclear envelope during meiosis in several organisms, suggesting that they can play a role in chromosome pairing and recombination (reviewed in (Cooper, 2000)). In yeast and *P. falciparum*, interphase mitotic telomeres cluster at the nuclear periphery, which might be important for chromosome positioning in the nucleus (Gotta *et al.*, 1996), (Freitas-Junior *et al.*, 2000) (discussed below).

When chromosomes undergo spontaneous or programmed breakage, *de novo* addition of a new telomere to the truncation site "heals" the chromosome and prevents the activation of the Non-Homologous End-Joining (NHEJ) repair mechanism, which would result in end-to-end fusions.

5. Telomeric chromatin

The unique DNA sequence of telomeres and their ability to fold into tertiary structures strongly suggests that telomeres should interact with specific proteins. Indeed, a variety of studies have shown that in protozoa and fungi, unlike the bulk of the genome, telomeric chromatin is not organised in nucleosomes. At least partially, telomeres are complexed with proteins other than histones (Blackburn and Chiou, 1981), (Cheung *et al.*, 1981), (Gottschling and Cech, 1984). In higher eukaryotes, the telomeres are much more longer than in protozoa and fungi. In the rat and other vertebrates, telomere chromatin is packaged in nucleosomes that are ~40bp smaller than bulk chromatin (Makarov *et al.*, 1993), (Lejnine *et al.*, 1995). Another study suggested that human telomeres are only partially organised in nucleosomes (Tommerup *et al.*, 1994). An unusual chromatin structure was found in relatively short human telomeres (2-7 kb), leading the authors to propose that in higher eukaryotes, similarly to lower eukaryotes, the chromatin adopts a non-nucleosomal structure at the very end of chromosomes, which they called the telosome, while the more centromere proximal repeats are organised in canonical nucleosomes.

There are a considerable number of proteins that interact with telomeres: specialised G-strand overhang binding proteins, dsDNA-binding proteins, their interacting partners, telomerase, telomerase-recruiting proteins, and proteins involved in double-strand break repair. Table 3 is a summary of those proteins that directly bind to telomeric DNA or that are involved with telomerase recruitment. Characterisation of these proteins has shown that they are often implicated in more than one process at the telomeres (reviewed in (McEachern *et al.*, 2000)), such as telomere length regulation, telomeric silencing, telomere localisation within the nucleus, regulation of access to telomerase, and protection from breakage-repair mechanisms.





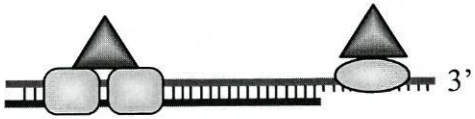


S. cerevisiae	Cdc13p	(Nugent *et al.*, 1996)	Rap1p Tel2p Ku70/80	(Berman *et al.*, 1986), (Runge and Zakian, 1996) (Gravel *et al.*, 1998)	Rif1-2 Sir2-4	(Hardy *et al.*, 1992), (Aparicio *et al.*, 1991),
S. pombe	Pot1p	(Baumann and Cech, 2001)	Taz1	(Cooper *et al.*, 1997)	Rap1p Rif1p (TAP1)	(Kanoh and Ishikawa, (Kanoh and Ishikawa,
K. lactis	Unknown	Unknown	Rap1p	(Larson *et al.*, 1994)	Unknown	Unknown
Ciliates	α/β	(Froelich-Ammon *et al.*, 1998)	Unknown	Unknown	Unknown	Unknown
H. sapiens	Pot1p	(Baumann and Cech, 2001)	TRF1 TRF2	(Bilaud *et al.*, 1996), (Broccoli *et al.*, 1997)	Tankyrase TIN2 Rap1p Ku70/80 RMN complex (RAD50+MRE11+NBS1)	(Smith *et al.*, 1998) (Kim *et al.*, 1999) (Li *et al.*, 2000) (Hsu *et al.*, 2000) (Wu *et al.*, 2000), (Zhu *et al.*, 2000)

Table 3. Proteins interacting with telomeres in yeast (*Saccharomyces cerevisiae, Schizosaccharomyces pombe* and *Kluyveromyces lactis*), ciliates (*Tetrahymena thermophila* and *Oxytricha trifallax*), and man. Three categories of proteins are represented: (i) proteins that specifically bind to the 3'-single-strand G-rich overhang, (ii) proteins that specifically bind to dsDNA and (iii) proteins that directly interact with the previous two. As Tom Cech once said "We wonder how there is even room for so many proteins!"







Organism	3'-single-strand overhang	References	Double-strand	References	Telomere-associated	References
S. cerevisiae	Cdc13p	(Nugent *et al.*, 1996)	Rap1p Tel2p Ku70/80	(Berman *et al.*, 1986), (Runge and Zakian, 1996) (Gravel *et al.*, 1998)	Rif1-2 Sir2-4	(Hardy *et al.*, 1992), (Aparicio *et al.*, 1991),
S. pombe	Pot1p	(Baumann and Cech, 2001)	Taz1	(Cooper *et al.*, 1997)	Rap1p Rif1p (TAP1)	(Kanoh and Ishikawa, (Kanoh and Ishikawa,
K. lactis	Unknown	Unknown	Rap1p	(Larson *et al.*, 1994)	Unknown	Unknown
Ciliates	α/β	(Froelich-Ammon *et al.*, 1998)	Unknown	Unknown	Unknown	Unknown
H. sapiens	Pot1p	(Baumann and Cech, 2001)	TRF1 TRF2	(Bilaud *et al.*, 1996), (Broccoli *et al.*, 1997)	Tankyrase TIN2 Rap1p Ku70/80 RMN complex (RAD50+MRE11+NBS1)	(Smith *et al.*, 1998) (Kim *et al.*, 1999) (Li *et al.*, 2000) (Hsu *et al.*, 2000) (Wu *et al.*, 2000), (Zhu *et al.*, 2000)

Table 3. Proteins interacting with telomeres in yeast (*Saccharomyces cerevisiae, Schizosaccharomyces pombe* and *Kluyveromyces lactis*), ciliates (*Tetrahymena thermophila* and *Oxytricha trifallax*), and man. Three categories of proteins are represented: (i) proteins that specifically bind to the 3'-single-strand G-rich overhang, (ii) proteins that specifically bind to dsDNA and (iii) proteins that directly interact with the previous two. As Tom Cech once said "We wonder how there is even room for so many proteins!"

6. Telomerase: telomere replication and chromosome repair

In most organisms, telomere maintenance is assured by a specialised enzyme called telomere terminal transferase or, more commonly, telomerase. Telomerase is an RNA dependent DNA polymerase. The essential core components of telomerase include a protein, named telomerase reverse transcriptase (TERT), and an RNA molecule, named telomerase RNA (TER; also called TR in humans and TLC1 in yeast) (Greider and Blackburn, 1987). Replication of telomeres by telomerase is schematically represented in Fig. 7. The 3' end of the G-strand overhang of the telomere functions as a primer in the polymerisation reaction, while the RNA component of telomerase provides the template sequence. Once the RNA template is copied, telomerase is repositioned with respect to the substrate and can initiate a further round of synthesis (Greider and Blackburn, 1989). The DNA primer binds at two sites in the enzyme: the anchor site, which resides in the protein moiety, and the template site, which corresponds to the RNA templating region. The primer binding to the anchor site avoids the dissociation of the DNA during the translocation step and allows the processive addition of new tandem telomeric repeats. After elongation of the G-strand, the C-strand can be synthesised by conventional DNA replication (Greider, 1995).

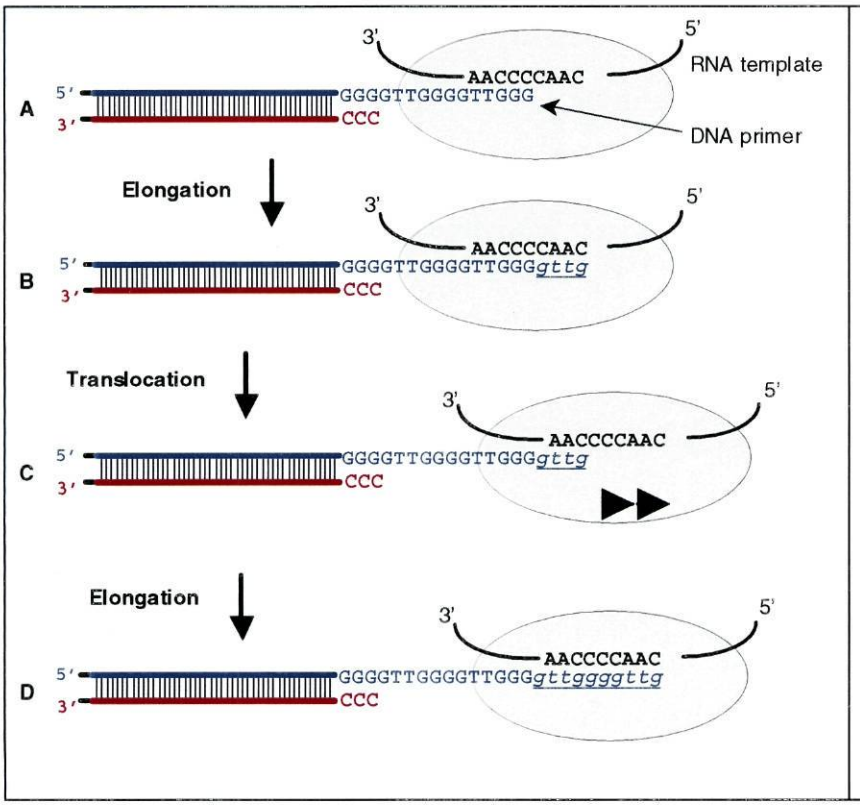


Figure 7. Schematic representation of telomere replication by telomerase in *Tetrahymena thermopila*. Telomerase interacts with telomere (A) and, using the 3' end as the primer, elongates the G-rich strand by copying the RNA template of the enzyme (B). After the addition of each repeat, telomerase becomes repositioned relative to the substrate (C) and another round of synthesis can commence (D).

Telomerase activity has been detected in extracts from several organisms that have telomeric repeats of different lengths (regular or irregular), indicating that telomerase can account for replication of virtually all telomeric sequences. It was first described in *Tetrahymena* cells during macronuclear development (a stage in which many new telomeres are formed and high levels of telomerase are present) (Greider and Blackburn, 1985). Subsequently, it was also found in extracts from other ciliates (Shippen-Lentz and Blackburn, 1989), human (Morin, 1989) and mouse cells (Prowse *et al.*, 1993), Xenopus (Mantell and Greider, 1994), yeast (Cohn and Blackburn, 1995), (Lin and Zakian, 1995), plants (Fitzgerald *et al.*, 1996), and kinetoplastids (Cano *et al.*, 1999). Telomerase activity has been detected in semi-purified nuclear extracts of *P. falciparum* asexual bloodstages, suggesting that *P. falciparum* canonical telomeres are probably maintained by this enzyme (Bottius *et al.*, 1998).

Telomerase is implicated not only in telomere maintenance, but also in a process called chromosome breakage and healing. When chromosomes undergo spontaneous or programmed chromosomes breakage, in some instances they can be repaired or "healed" by the addition of a telomere to the truncation point. *De novo* telomere formation can be a programmed event such as during ciliate macronucleus formation (for review see (Prescott, 1994)), or can occur occasionally on chromosomes broken accidentally. In humans, for example, a terminal chromosome deletion on chromosome 16p is responsible for a genetic disease, α-thalassemia (Wilkie *et al.*, 1990), but genomic stability was maintained by addition of a new telomere at that truncation site.

Characterisation of polymorphic chromosomes in *P. falciparum* strains revealed the presence of large deletions, ranging from 50 to 300 kb, that arise mitotically during the asexual life cycle of the parasite, in culture and very rarely in field isolates (Van der Ploeg *et al.*, 1985), (Pologe and Ravetch, 1986). Pologe & Ravetch suggested that such chromosome deletions could result from breakage and healing events (Pologe and Ravetch, 1988). Addition of telomere repeats is probably mediated by Plasmodial telomerase, since oligonucleotides mimicking truncation sites, are good substrates in an *in vitro* assay of telomerase activity (Bottius *et al.*, 1998).

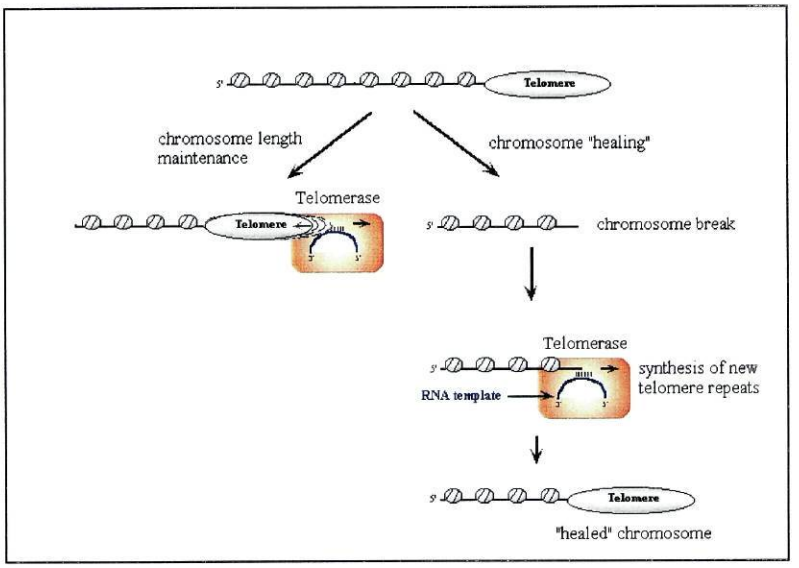


Figure 8. Model showing the two probable roles of telomerase in *P. falciparum* : maintenance of telomere length and healing of truncated chromosomes (Scherf *et al.*, 1999).

7. Structure of telomerase subunits: TERT and TER

The protein component of telomerase (TERT - telomerase reverse transcriptase) was first purified from *Euplotes aediculatus* (Lingner *et al.*, 1997). By sequence homology, homologues have been identified in yeasts (Meyerson *et al.*, 1997), (Counter *et al.*, 1997), (Nakamura *et al.*, 1997), (Metz *et al.*, 2001), other ciliates (Bryan *et al.*, 1998), (Collins and Gandhi, 1998), mouse (Greenberg *et al.*, 1998), plants (Oguchi *et al.*, 1999), *Giardia lamblia* and *Caenorhabditis elegans* (Malik *et al.*, 2000), and *Xenopus laevis* (Kuramoto *et al.*, 2001). They share common features: a high molecular weight (~100-130 kDa), an isoelectric point superior or equal to 10 and structural and enzymatic motifs in common with reverse transcriptases (RT). The RT motifs, located within the C-terminus, are critical structural elements as single amino acid substitutions have been shown to abolish telomerase activity (Lingner *et al.*, 1997). Additional studies have revealed the presence of conserved motifs in the N-terminus half of TERT (Friedman and Cech, 1999), (Xia *et al.*, 2000), (Miller *et al.*, 2000), (Malik *et al.*, 2000): motifs T2 (also called region I, motif N or motif GQ), CP (previously described as a ciliate-specific motif, but later shown to be common to all TERTs) and QFP (also termed region III). While the RT domain of TERT is essential for catalytic activity, the N-termini are implicated in efficient binding of RNA molecule, definition of 5' RNA template boundary, functional multimerisation with other TERT and interaction with associated proteins (reviewed in (Liu, 2002)).

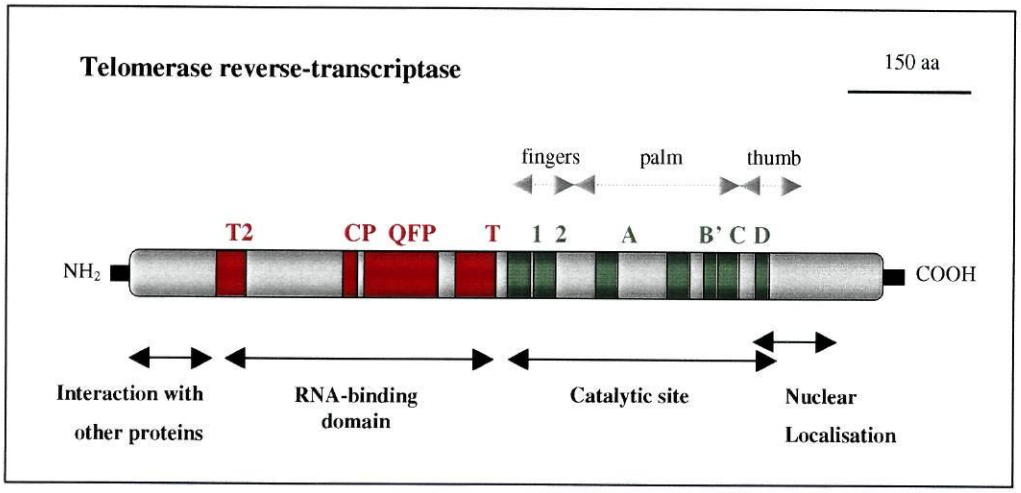


Figure 9. The primary structure of *S. cerevisiae* telomerase reverse-transcriptase (Est2p). Motifs in red are telomerase-specific; motifs in green are conserved with other reverse-transcriptases, such as the p66 subunit in HIV-1 reverse-transcriptase. When Est2p is modeled after the crystal structure of p66, its active site is proposed to adopt a quaternary structure similar to a right hand cupped with the RNA template in the cleft of thumb, palm and fingers (shown by grey arrows). Functional regions, as mapped in yeast, humans and ciliate TERTs, are represented by black arrows.

The other essential component for core telomerase activity is an RNA molecule (TER). Perhaps surprisingly, with the exception of the template region itself, the TER nucleotide sequence is not conserved even among species whose telomeres are composed of the same repeat (Greider, 1995). Moreover, the RNA polymerase responsible for transcribing TER differs from one organism to another. For example, ciliate TER is 150-190 nt molecule transcribed by RNA polymerase III, whereas TER from budding yeast is an enormous ~1300 nt transcript transcribed by RNA polymerase II (McEachern and Blackburn, 1995). Human telomerase RNA (hTR), 451 nt in length, is also transcribed by pol II (Chen *et al.*, 2000). *In vitro* mutagenesis studies have shown that besides serving as a template for reverse transcription in telomeric DNA elongation, hTR is also involved in the catalytic site, probably by specific nucleotides interacting with structural components of the DNA substrate primer and protein subunits (Roy *et al.*, 1998). Despite the divergence in nucleotide sequences, the secondary structure of this ribonucleotide molecule is conserved between ciliates and vertebrates (Romero and Blackburn, 1991), (Lingner *et al.*, 1994), (Chen *et al.*, 2000), including a pseudoknot essential for its activity and stable assembly with TERT (Gilley and Blackburn, 1999). In contrast, yeast telomerase RNA does not display a conserved secondary structure (as discussed in (Chen *et al.*, 2000)).

In yeast, several proteins that are required for telomerase activity *in vivo* are known to directly or indirectly interact with the RNA or reverse-transcriptase components of telomerase (Est1p (Lin and Zakian, 1995) and Cdc13p (Evans and Lundblad, 1999)). In humans, a

number of proteins have been found to be associated to telomerase activity in cell extracts: hsp90, p23, TP1, dysterin, L22 and hStau (Holt *et al.*, 1999), (Harrington *et al.*, 1997), (Mitchell, 1999), (Le *et al.*, 2000). In both yeast and humans, the telomerase holoenzyme is a dimer, containing two active sites and two TER molecules per enzyme complex particle (Prescott and Blackburn, 1997), (Wenz *et al.*, 2001).

8. Telomere maintenance without telomerase

Some eukaryotic species have apparently completely lost the telomerase-mediated mode of telomeric DNA maintenance during evolution. In these organisms, telomeric DNA is not composed of tandem arrays of G-rich repeats and mechanisms other than telomerase are used to maintain a functional telomere. In *Drosophila melanogaster*, one retrotransposon is added onto the very termini of chromosomes by a variant retrotransposition mechanism; in the midge *Chrironomus tentans*, the onion *Allium cepa* and mosquito *Anopheles gambiae*, telomeres are maintained by recombination involving unequal homologous crossing over and/or gene conversion (reviewed in (McEachern *et al.*, 2000)).

Similar recombinogenic mechanisms are used when telomerase activity is depleted from organisms that generally maintain telomeres by telomerase. In telomerase deletion mutants from *S. cerevisiae*, *K. lactis* and *S. pombe*, a gradual telomere shortening is accompanied by a replicative senescence and a decrease in cell viability (Table. 4). Although eventually most cells die, a population of 'survivors' emerges that exhibits restored growth rates and that has overcome the normally fatal telomere shortening through circularization of their chromosomes (in *S. pombe*) (Nakamura *et al.*, 1998) and/or recombinational Rad52-dependent telomere elongation (in *S. pombe* (Nakamura *et al.*, 1998), *S. cerevisiae* (Lundblad and Blackburn, 1993), (Teng and Zakian, 1999), *K. lactis* (McEachern and Blackburn, 1996)). Likewise, in some cancers and immortalized human cell lines, telomeres are maintained despite the absence of telomerase. Such a telomerase-independent telomere maintenance, termed ALT (for alternative lengthening of telomeres) (Bryan *et al.*, 1997b), is capable of maintaining long and highly heterogeneous telomeres (Bryan *et al.*, 1995), (Kim *et al.*, 1994), (Rogan *et al.*, 1995) through a mechanism that involves recombination (Dunham *et al.*, 2000).

Organism	Telomerase component	Phenotype	Reference	Post-senescence Phenotype	Reference
S. cerevisiae	TLC1 (1300nt)	• Progressive telomere shorthening (4 bp/generation, ie ~1% / generation) • Gradual decrease in growth rate and viability	(Singer and Gottschling, 1994)	• Within ~100 generations, culture is overtaken by faster-growing cells: survivors • Global amplification and rearrangement of telomeric and subtelomeric regions (Rad52 dependent)	(Lundblad and Blackburn, 1993)
	Est2p	• Same as TLC1Δ	(Lendvay *et al.*, 1996)	• Same as TLC1Δ	(Lendvay *et al.*, 1996)
K. lactis	TER1 (1300nt)	• gradual loss of telomeric repeats (5 bp/generation; i.e. 0,5-1% / generation) • progressive decline of cell growth capability (growth senescence)	(McEachern and Blackburn, 1995)	• Survivors have lengthened telomeres • Recombination between short telomere tracts; amplification of subtelomeric repeats (Rad52 dependent)	(McEachern and Blackburn, 1996)
S. pombe	Trt1	• telomere shortening (2-4 bp/generation; i.e. ~1% / generation) • senescence	(Nakamura *et al.*, 1997)	• survivors after 120 divisions • most survivors have circular chromosomes, but some chromosomes maintain their telomeres presumably through recombination.	(Nakamura *et al.*, 1998)
Human	TERT (DN-hTERT)	• telomere shortening (150-250 bp/PD, i.e. ~3% / PD in 36M ovarian cancer cells; 60 bp /PD, i.e.0,5% / PD in A431 clones) • both types of cells cell undergo apoptosis in a telomere length dependent manner.	(Hahn *et al.*, 1999), (Zhang *et al.*, 1999)	• not observed in 36M ovarian cancer cells • in A431 cells, telomere shortening persisted for >109 doublings, until average TRF length approached ~4 kb (after this point, cells begin loosing DN-hTERT protein)	(Hahn *et al.*, 1999), (Zhang *et al.*, 1999)
	TR (451 nt) (RNAi)	• Loss of telomeric DNA (40bp / PD; i.e 0,5% / generation) • cell-death after 23 to 26 doublings	(Feng *et al.*, 1995),	NA	NA
Mouse	Terc (397nt)	• progressive telomere shortening with increasing mouse generations (5 kbp / organismal generation; 10-12% / organismal generation) • Minimal length for proper telomere function:1.5-3kb.	(Blasco *et al.*, 1997), (Niida *et al.*, 1998)	• Telomerase is the only efficient pathway for maintaining telomeres in the organism. • In some cells derived from $Terc^{-/-}$ mice, there's activation of ALT.	(Bryan *et al.*, 1995)
	TERT	• Cells from TERT-/- mice showed progressive telomere loss • On prolonged growth, mTert-deficient embryonic stem (ES) cells exhibit genomic instability, aneuploidy and telomeric fusions.	(Liu *et al.*, 2000)	NA	NA
A. thaliana (plant)	TERT (by homozygous trasnfer DNA)	• telomere shortening (500 bp/organismal generation; i.e. 12,5-25% / organismal generation) • survives up to 10 generations • late-generation mutants have an extended life-span and remain metabolically active.	(Fitzgerald *et al.*, 1999)	NA	NA

Table 4 Phenotypic consequences of the abolishment of telomerase activity in different organisms. To eliminate telomerase actvity, gene disruptions or gene replacements were undertaken in either telomerase reverse-transcriptase gene or RNA template gene. RNA antisense was used to analyse the role of hTR in HeLa cells. In all cases, the absence of telomerase leads to telomere shortening, progressive decline of cell growth and viability. In some organisms, a small population of cells is capable of recovering the "crisis" point and maintain telomeres through alternative mechanisms. NA: not available. PD: population doubling. DN: dominant negative.

9. Telomere length regulation

Although the length of individual telomeres is generally variable and derives from a balance between the addition of repeats by telomerase and the loss sequences because of the end-replication problem, the average number of repeats is typical of each species. The average size of telomeres is species-specific, varying from 350 bp *in S. cerevisiae* (Wang and Zakian, 1990) to 10-15 kb in humans (Harley *et al.*, 1990), (de Lange *et al.*, 1990) and 40-150 kb in mice (Kipling and Cooke, 1990). The relevance of maintaining telomeres within a defined length range is demonstrated by the deleterious consequences of most mutations that affect telomere elongation or shortening. A few exceptions exist in which the size range of telomeres is not fixed, but change according to environmental or developmental conditions, such as in *Trypanosoma brucei* and *Tetrahymena thermophila* (Bernards *et al.*, 1983), (Larson *et al.*, 1987).

The mechanisms and the factors involved in telomere length control are still not completely clarified. However, evidence points to a model in which the average telomere length results from an equilibrium between two states that allow access to telomerase or not. The current model suggests that double-stranded telomeric DNA binding-proteins nucleate a higher-order complex by recognising the telomere in a sequence-specific manner and by nucleating a tertiary complex through interactions involving other protein domains (Moretti *et al.*, 1994), (Liu *et al.*, 1994), (Krauskopf and Blackburn, 1996), (Konig *et al.*, 1996). These proteins are trans-acting factors that act similarly in all chromosome ends. In such a way, telomeres can rapidly switch between two states: "open" (accessible to telomerase) and "closed" (protected from telomerase activity). Rounds of incomplete replication and/or degradation of chromosome ends result in telomere shortening, and increase the probability of switiching to an open state. A shortened telomere temporarily opens and is acted on by telomerase. Once lengthened by telomerase, the telomere has an increased probability of switching back to the closed structural state. This opening-closing cycle keeps telomere length distributions confined within upper and lower limits (Blackburn, 2001).

10. Telomere loss and senescence

Telomerase is developmentally regulated in higher eukaryotes (Shay and Wright, 1996). In humans, telomerase activity is present in foetal tissues and, shortly after birth, becomes undetectable in most somatic tissues. Low levels of telomerase are found in proliferative cells of renewal tissues, such as the bone marrow progenitor cells, cord blood, blood mononuclear cells and intestinal mucosa (Marciniak and Guarente, 2001). High levels of telomerase are detected during all life span only in the germline.

Normal human somatic cells have a finite proliferative life span known as the Hayflick limit or cellular senescence (Hayflick and Moorhead, 1965). Without appropriate mechanisms to counteract the inadequate replication at the ends of linear chromosomes, telomeres shorten with successive rounds of DNA synthesis or every time a cell divides. The telomere hypothesis of ageing postulates that the progressive loss of telomeric DNA results in the eventual induction of the senescence program and ultimately signals cell cycle exit (Olovnikov, 1973). Telomeres have thus been called "mitotic-clocks", a mechanism the cell uses to determine its life span. Such considerations are a matter of importance not only for those interested in "ageing", but also in organ transplantation, as well as cloning of mammals.

Human somatic cells senesce in a two-stage manner, termed mortality stage 1 (M1) and mortality stage 2 (M2) (Wright *et al.*, 1989). Due to the end-replication problem, the telomere tract progressively shortens with continuous replication of normal cells. When a critical telomere length is reached, cells enter in replicative senescence, or M1 stage.

Transfection of normal somatic human cells with viral oncogenes that block p53 and pRb pathways allows cells to continue to proliferate for an extended period of time, during which they lose additional telomeric sequences (Greider, 1999), (Kiyono *et al.*, 1998) and accumulate chromosomal aberrations until eventually entering crisis (M2). Escape from crisis is rare and occurs with a frequency of approximately 10^{-7} (Wright *et al.*, 1989). In these immortal cells, telomere length is stable and generally telomerase is up-regulated. Sometimes, immortalisation also occurs by activation of alternative mechanisms of telomere maintenance (ALT) (Bryan *et al.*, 1997a) (blue line; Fig. 10).

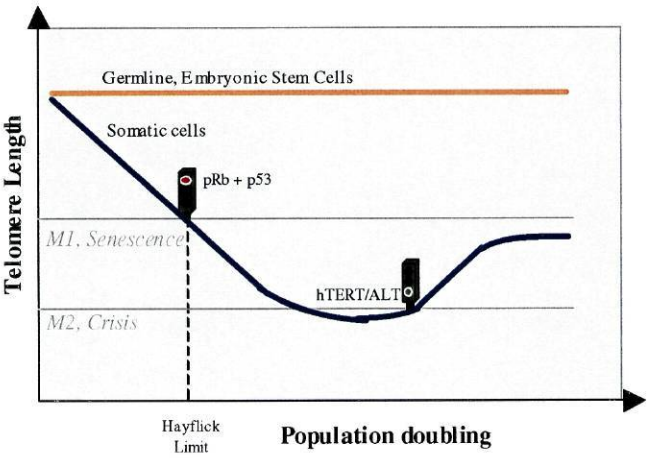


Figure 10. Dynamics of telomere length in human somatic and germline cells. Yellow line: because germline (reproductive) and embryonic stem cells express telomerase telomere length is constant. Blue line: normal human somatic cells express low or undetectable levels of telomerase activity. Telomeric sequences shorten progressively reaching replicative senescence (or Hayfflick limit, or M1 stage). Blocking p53 and pRb pathways allows cell division beyond M1 stage. Telomere tract continues to shorten. and eventually cells enter in crisis or M2. Most cells die at crisis. The rare cells that escape this fate generally express telomerase and have a stable telomere length. A minority of survivors activates alternative pathways (ALT) of maintaining telomeres (Figure based on (Harley, 1991)).

Although telomerase is expressed in 85-90% of tumour cell lines and tissues (White *et al.*, 2001), telomerase cannot be considered as an oncogene. First, hTERT and telomerase activity are expressed in normal immortal human embryonic cells (Amit *et al.*, 2000) and in germline cells (Kim *et al.*, 1994), (Wright *et al.*, 1996), both without signs of aberrant growth control (yellow line, Fig. 10). Secondly, pre-senescent cells in which expression of the TERT gene is forced on retained normal growth control and displayed no changes associated with the malignant transformation (Morales *et al.*, 1999), (Jiang *et al.*, 1999). These data indicates that telomerase is clearly not sufficient to overcome all of the barriers to the oncogenic state.

11. Telomerase Inhibition

The prevalence of telomerase expression in human cancers makes it an attractive candidate for new mechanism-based targets for cancer therapy (Kim *et al.*, 1994), (Counter *et al.*, 1994), (Damm *et al.*, 2001). Several strategies have been developed in order to inhibit telomerase activity and interfere with tumour development. The three potential targets are TERT, TR and telomeric DNA (Fig. 11) (reviewed in (Mergny *et al.*, 2002)).

Target	Type of drug	References
hTERT	Nucleoside analogues	(Gomez *et al.*, 1998)
	Isothiazolone derivatives (TMPI)	(Hayakawa, 1999)
	Rubromycins and analogues	(Ueno, 2000)
	Carboxylic amide derivatives (BIBR 1532)	(Damm *et al.*, 2001)
	Ribozymes	(Yokoyama and al., 1998)
hTR	Long antisense RNA	(Kondo *et al.*, 1998a)
	Short oligomers (PNA, N3'->P5' phosphoramidates)	(Feng *et al.*, 1995), (Shea-Herbert *et al.*, 2002)
	Ribozymes	(Kanazawa, 2000)
	RNA/DNA duplex binders	(Francis, 2001)
Telomeric DNA	Cisplatin and other antitumour agents	(Kondo *et al.*, 1998b), (Ishibashi and Lippard, 1998)
	G-quadruplexes ligands (eg. Acridines, ethidium)	(Mergny, 2001), (Riou *et al.*, 2002)

Figure 11. Telomerase inhibitors developed for cancer treatment in humans. There are three main categories of drugs. Some interfere with the catalytic component of telomerase (hTERT), others target the RNA molecule of telomerase (hTR). The third category does not target any component of the telomerase enzyme, but rather its substrate: the telomere itself. Nucleoside analogues (such as AZT) were first developed as anti-virals that interfered with HIV reverse-transcriptase. Ribozymes are small RNA molecules that possess specific endoribonuclease activity. The antisense approach uses RNA molecules or oligonucleotides that are complementary to part of hTR or to the mRNA of hTERT. Agents that bind to RNA/DNA duplex do not strictly target hTR, but its interaction with the substrate, the telomeric DNA. The folding of the telomeric 3'-ovehang in G-quartet inhibits telomerase activity *in vitro*. Thus, stabilisation of these structures can be considered an original strategy to achieve antitumour activity.

Two other elegant ways to achieve telomerase inhibition do not fall into the above mentioned classes. As telomerase is mainly expressed in tumour cells, the use of transcription regulatory sequences of telomerase (hTR and hTERT) to drive the synthesis of a suicide gene (such as Caspase 8) might lead to the selective killing of cancer cells (Plumb *et al.*, 2001). This is called **Suicide Therapy**. The other telomerase-based strategy to destroy tumour cells is **Immunotherapy**. Antigens from the catalytic component are presented by MHC-molecules at the surface of tumour cells (Vonderheide, 2002), allowing strategies of vaccination and generation of effective anti-tumour cytotoxic T lymphocyte (CTL) responses.

In single-celled organisms, telomeres need to be maintained above a threshold length, to assure the survival of the organism. If telomerase is essential for the maintenance of telomeres, drugs that interfere with telomerase might induce fatal shortening of telomeres leading to cell senescence and eventually cell death. Such strategy can be envisioned for single-cell pathogens, whose virulence depends on a fast proliferation. Thus telomeres can be considered as the 'Achilles Heel' of highly proliferating pathogens, such as protozoan pathogens. Bottius et al. have shown that reverse-transcriptase inhibitors such as dideoxy-GTP efficiently diminish *P. falciparum* telomerase activity *in vitro* (Bottius *et al.*, 1998), which suggests that Plasmodial telomerase can be used as a drug target.

12. TRAP: an in vitro assay used to measure telomerase activity

A telomere terminal transferase activity was first detected in Tetrahymena extracts by Greider and Blackburn (Greider and Blackburn, 1985). The *in vitro* test developed by these authors is nowadays the basis for the current method to measure telomerase activity, named TRAP (telomere repeat amplification protocol). This method has been successfully adapted to *P. falciparum* system and is schematically represented in Fig. 12. It consists of two steps. First, an oligonucleotide (Pf-I) that mimics a chromosome end is used as a substrate to which telomeric repeats are added *de novo* by telomerase, contained in a semi-purified *P. falciparum* protein extract. This **Elongation step** is not very sensitive. Thus, the products obtained, which may have a variable number of synthesised G-rich repeats, are PCR amplified in the **Amplification step.** The primers used for this reaction consist of Pf-I and Pf-CX, the latter representing an oligonucleotide that contains three telomeric repeats that hybridise with the

G-rich strand synthesised during the elongation step. The reaction is performed in the presence of a radio-labelled deoxynucleotide and after separation on a non-denaturing gel, products are detected by exposure to an auto-radiogram film. Given the size heterogeneity of the elongation products, a typical result of TRAP assay, consists of a ladder of bands, in which the smallest band corresponds to Pf-I + 1 telomeric repeat, the second band is Pf-I + 2 telomeric repeats, etc. The size difference between each band is therefore the size of a single telomeric repeat (6 nt in humans and 7 nt in *P. falciparum*, for example).

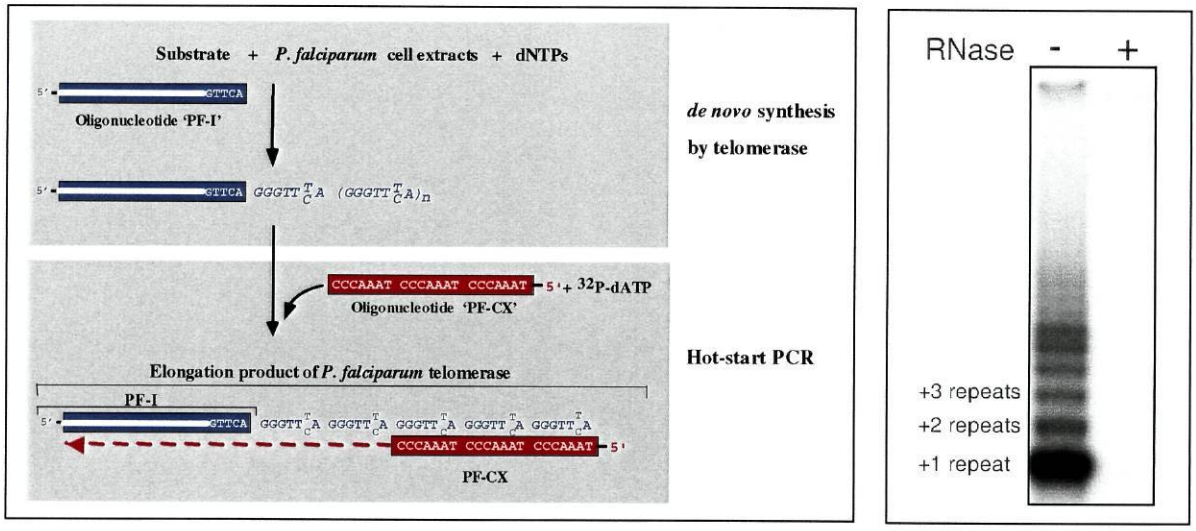


Figure 12. Telomere Repeat Amplification Protocol (TRAP) as used in *P. falciparum* to measure *de novo* synthesis of telomeric repeats *in vitro*. This reaction is most likely mediated by Plasmodial telomerase enzyme. (A) Diagram showing the two steps of TRAP: elongation by telomerase and amplification. (B) Typical result obtained on a TRAP assay: the size difference between bands of the ladder corresponds to the size of a telomereic unit (7 nt in this case). Telomerase activity is RNAse sensitive.

III. GENOME ORGANISATION IN PLASMODIA

The genome of Plasmodia comprises a ~30Mb nuclear genome and two other unrelated genomes: a mitochondrial genome (6 kb) and a plastid-originating genome (35 kb) (Feagin, 1994). The haploid nuclear genome is divided between 14 linear chromosomes, which range in size from 0,7 to 3,5 Mb, and harbour an estimated ~5000 genes (Reddy, 1995).

Plasmodial nuclear genomes are extremely AT-rich, varying around 70-82% (82% for *P. falciparum*), whereas for example human genome is 59% rich in AT (Lander *et al.*, 2001). The distribution of the base composition is not uniform in *P. falciparum*: genes have a lower AT-content (60-70%), which is still above average values in eukaryotes, whereas introns and DNA sequences flanking genes tend to be very AT-rich (>85%) (Fig. 13C).

1. Chromosome structure

A *P. falciparum* chromosome comprises two distinct compartments: a conserved central domain and a polymorphic terminal domain (Corcoran *et al.*, 1988), (Sinnis and Wellems, 1988). House-keeping genes map to the central chromosome regions, whereas some immunodominant antigens are located near the chromosome ends (Hernandez-Rivas *et al.*, 1997), (Thompson *et al.*, 1997) (Fig. 13A). Antigen genes are separated from the telomere repeats by an array of non-coding DNA elements that span between ~15-35 kb and consist in some cases of complex degenerated repeats (Oquendo *et al.*, 1986), (Vernick and McCutchan, 1988), (Dolan *et al.*, 1993), (Wasserman *et al.*, 1995) (Fig. 13B). Subtelomeric regions are unusually rich in GC, 30%, considering this is a non-coding region (Fig.13C). As mentioned above, arrays of repetitive elements were also described in *P. berghei* subtelomeric regions (Pace *et al.*, 1987).

All multigene families described in Plasmodia so far have at least some members at the chromosome ends: Py235 in *P.yoelii* (Khan *et al.*, 2001); *vir* in *P. vivax* (del Portillo *et al.*, 2001); *var* (Hernandez-Rivas *et al.*, 1996), *rif*, *Pf60.1*, *stevor* (Gardner *et al.*, 1998), (Bowman *et al.*, 1999) and *clag* (Manski-Nankervis *et al.*, 2000) in *P. falciparum*. Some gene-families contain 50 members or less (like *var, stevor* and *Py235*), while others are predicted to have between 600-1000 members (*vir*). The precise role of the majority of the gene families is not

yet clear. The best-studied gene-family is the *var* genes. These genes encode proteins that appear on the surface of the erythrocyte membrane (PfEMP1) and are involved with two phenomenon that are crucial for the parasite virulence: cytoadhesion and antigenic variation. The first allows the parasite to adhere to endothelial cells of the human host vessels and, in such a way, prevent being cleared by the spleen. The latter is necessary for immune evasion (Craig and Scherf, 2001).

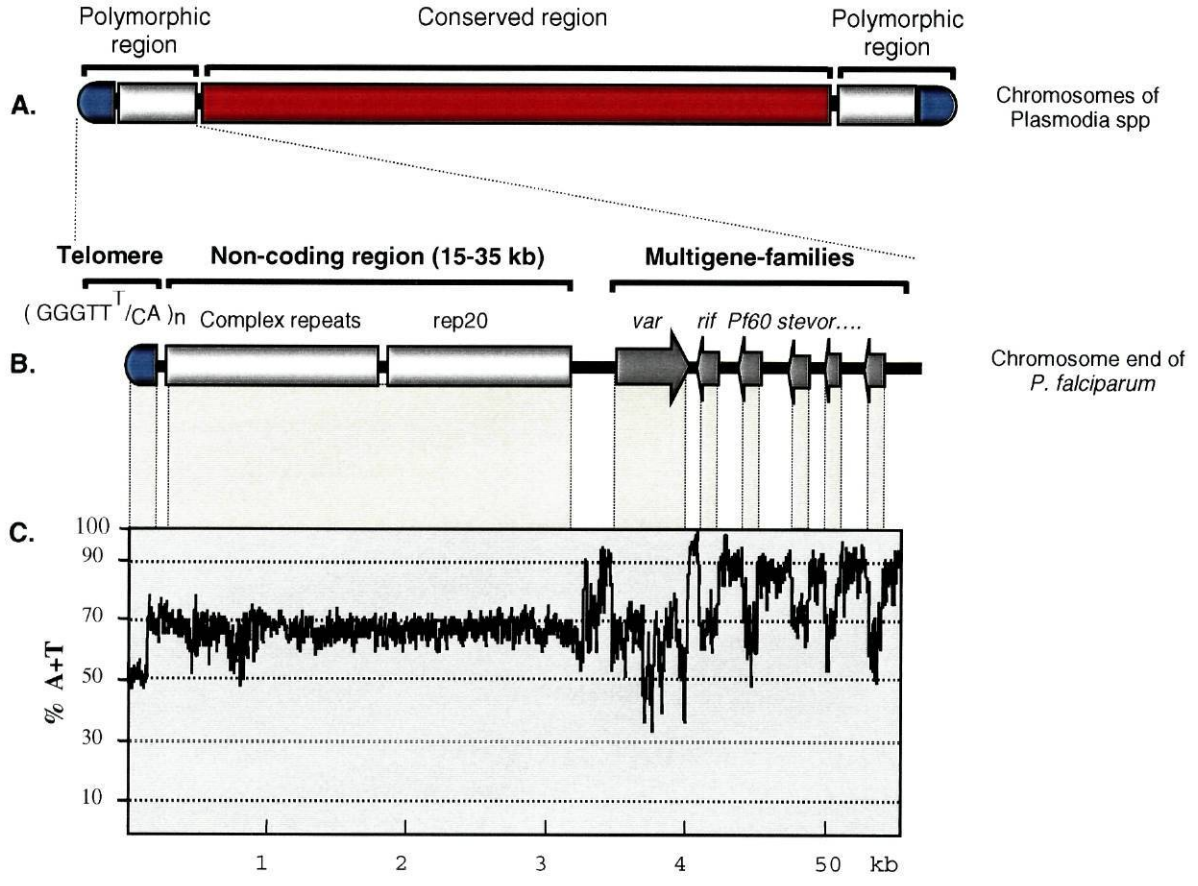


Figure 13. Structural organisation of chromosomes in Plasmodia. (A) Plasmodia chromosomes are compartmentalised in a polymorphic terminal region and a conserved central region (where house-keeping genes are located). (B) *P. falciparum* chromosome ends consist of a telomere at the terminus, a complex array of repeats (including Rep20, a common marker for mapping studies in *P. falciparum*) and several multigene families, important for the parasite virulence. (C) Subtelomeric regions display 70% content of A+T, a figure characteristic of coding-regions. Telomeres are only 50% rich in A+T. In average *P. falciparum* genome is 82% rich in A+T.

2. Chromosome polymorphism

Substantial genetic diversity exists between different *P. falciparum* isolates. Both antigen genes and individual parasite chromosomes display striking polymorphisms. This results from the phenomen of chromosome breakage and healing (see above, Fig. 8) or other genetic exchanges, such as translocations (Hernandez-Rivas *et al.*, 1996) and gene amplification (Foote *et al.*, 1989). Chromosomal polymorphisms can arise through meiotic recombination, as shown by repeated crossing-over between homologous chromosomes in all progeny clones of a laboratory cross (Dolan *et al.*, 1993).

3. Chromatin organisation

P. falciparum chromatin, like the majority of eukaryotes, is organised in fundamental units called nucleosomes. Cary et al. analysed the total nuclear chromatin and concluded that the mean nucleosomal size was approximately 180 bp (Cary *et al.*, 1994). Another study analysed the chromatin organisation only in subtelomeric regions and the authors observed a different nucleosomal size: 155 bp (Lanzer *et al.*, 1994a), (Lanzer *et al.*, 1994b). Several lines of evidence suggest that histone constitution and chromatin organisation of *Plasmodium* conform to that of other eukaryotes: the molecular cloning of several *P. falciparum* histone genes (Creedon *et al.*, 1992), (Bennet *et al.*, 1995), (Longhurst and Holder, 1997), the cloning of a nuclear factor of *P. berghei* probably involved in the dynamics of chromatin packaging (Birago *et al.*, 1996) and the biochemical evidence for a plasmodial histone deacetylase (Darkin-Rattray *et al.*, 1996).

4. Nuclear architecture in P. falciparum

Recent technological advances such as FISH (Fluorescent *In situ* Hybridisation) have given new insights into the three-dimensional organisation of the nucleus, in relatively small organisms such as yeast, Trypanosomes and *Plasmodium*. More than ten years ago, it was shown that *T. brucei* telomeres form clusters at the nuclear periphery (Chung *et al.*, 1990). Telomeres clusters adjacent to the nuclear envelope were also observed in *S. cerevisiae* (Klein *et al.*, 1992) and have been proposed to represent distinct nuclear compartment that may play important role in epigenetic gene regulation, DNA recombination and telomeric silencing (reviewed in (Pryde *et al.*, 1997), (Cockell and Gasser, 1999), (Gotta and Gasser, 1996)).

In yeast, a number of telomere associated proteins involved in the telomere length regulation, cluster formation, telomere anchoring to the periphery and gene silencing have been described and characterised (reviewed in (Tham and Zakian, 2000)). *P. falciparum* orthologues to most yeast telomere associated proteins have been identified (Scherf *et al.*, 2001), implying that yeast can serve as a model to investigate the role of biologically relevant aspects in *P. falciparum* such as the importance of virulence factor localisation at chromosome ends.

FISH analysis using a chromosome end specific fluorescent probe recently demonstrated that the 28 chromosome ends of *P. falciparum* are not randomly distributed in the nucleus, but are found at the nuclear periphery. Moreover, the telomeres form physical associations demonstrated by the observation that only 4 to 7 spots are detected in asexual and sexual blood stage parasites, suggesting that each cluster contains between 4 and 7 distinct chromosome ends (Freitas-Junior *et al.*, 2000) (Fig. 14).

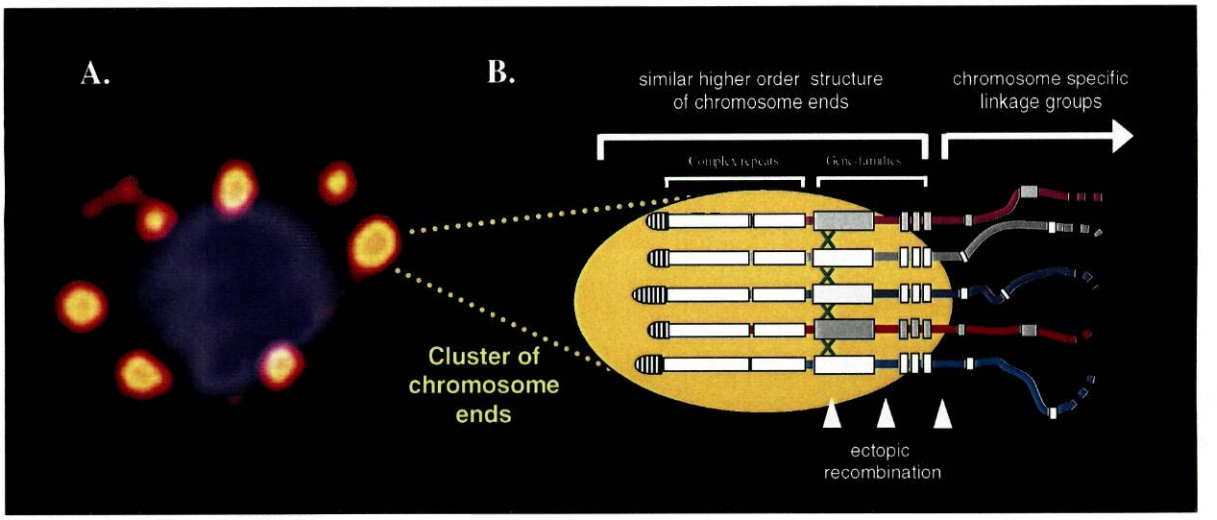


Figure 14. Organisation of chromosome ends in nucleus of *P. falciparum*. (A) FISH analysis with a telomere-specific DNA probe (rep20) showing that the chromosome termini form clusters at the nuclear periphery during trophozoite asexual blood stages (kindly provided by Freitas-Junior). (B) Schematic model of a telomere cluster: heterologous chromosomes are physically aligned due to the conserved higher-order structure of chromosome ends (complex repeats and multigene families: *var, rifins, Pf60.1, stevor*). Such organisation is supported by the observation of increased rates of recombination in gene families located in subtelomeres (green crosses represent ectopic recombination events detected among members of the *var* gene family). For a revised model refer to "Concluding Remarks" section of this thesis.

The molecular components involved in cluster formation are as yet unknown. The extent of chromosome end alignment is also unclear, however two lines of evidence support the idea that it spreads beyond the telomere repeats to a distance of 30 to 40 kb, including the subtelomeric region that encodes variant surface antigen families. DNA sequences in this region undergo ectopic (non-allelic) recombination at a much higher rate than expected for homologous recombination (Su *et al.*, 1999), (Freitas-Junior *et al.*, 2000). Moreover, preliminary chromosome painting studies revealed that a pair of subtelomeric probes distant of 60-80 kb co-localise in the same spot in the nucleus, whereas two chromosome internal probes equally distant, are clearly seen as two distinct signals (Freitas-Junior, L.H., unpublished data). We assume that the physical alignment of heterologous chromosome ends brings together homologous sequences from different chromosomes allowing efficient DNA recombination.

OBJECTIVES

Over the course of my thesis, I have investigated the telomere biology of malaria parasites, emphasizing two major objectives. Firstly, to obtain a clearer picture of the genomic environment of telomere-associated multigene families, which are essential for the parasite virulence. Secondly, to understand the dynamics of telomere maintenance in *P. falciparum* and to test the hypothesis that telomerase might be a valid target for anti-malarial drug treatment.

Specific aims:

- To examine how telomeric chromatin is organised in *P. falciparum.*
- To study telomere length regulation in *P. falciparum.*
- To identify the gene encoding the catalytic component of telomerase (TERT) in human and rodent malaria species.
- To obtain a general picture on how subtelomeric DNA is organised in the 14 chromosomes of *Plasmodium* nuclear genome, emphasizing the subtelomeric regions of *P. falciparum.*
- To understand the function of subtelomeric regions in *P. falciparum.*
- To identify proteins that bind to the subtelomeric regions of *P. falciparum.*

RESULTS

This chapter contains the results obtained during 3,5 years of experimental work and is organised into four sub-chapters. Sub-chapters I and II represent data that have been published in international journals and are presented here in their published format. Sub-chapters III and IV correspond to results that have not yet been published. For consistency of style, subchapters III and IV are presented in the format of journal articles. The order of the four subchapters corresponds to the chronological progress of the lab work.

Sub-chapters:

I. This sub-chapter contains the first paper, which was published in "*Molecular and Biochemical Parasitology*" journal (2000). Bio-informatic tools were utilised to analyse the DNA organisation of eight chromosome ends in *P. falciparum*. Micrococcal nuclease digestions studies were performed to investigate the nature of the chromatin organisation of telomeres.

II. This sub-chapter appeared in "*EMBO Journal*" (2002). In this work we measured the telomere length of several different Plasmodia species as well as several *P. falciparum* lab strains and investigated why some chromosome ends harbour longer telomeres than average. The results obtained permitted to assign distinct roles for telomeres and subtelomeric region in the spatial organisation of chromosomes in the nucleus.

III. Here, we characterised a putative Plasmodial telomerase reverse-transcriptase gene, *PfTERT*. *PfTERT* is predicted to encode an unusually large TERT. Antibodies raised against the recombinant PfTERT protein revealed a localisation pattern not previously described for telomerase from other organisms (manuscript in preparation).

IV. Using an approach as described in sub-chapter I, we analysed the chromosome end organisation of human and rodent malaria species, taking advantage of the greater amount of sequencing data available in 2002 as compared to 1999 (preliminary data).

RESULTS I

Genomic Organisation and Chromatin Structure of *Plasmodium falciparum* Chromosome Ends

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(* equal contribution to the project)

Short Communication

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MOLECULAR AND BIOCHEMICAL PARASITOLOGY

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Short communication

Genomic organisation and chromatin structure of *Plasmodium falciparum* chromosome ends

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1. Introduction

Telomeres play an essential role in a number of biological processes in eukaryotic cells. They ensure complete replication of chromosomes and are necessary for protection against degradation and fusion (reviewed in Ref. [1]). Chromosome ends of the protozoan malaria parasite *Plasmodium falciparum* consist of distinct structural regions: the telomere and the polymorphic subtelomeric region (reviewed in Ref. [2]). The telomeres are composed of degenerate G-rich repeats, in which GGGTT(T/C)A is the most frequent. Enzymes involved in telomere replication and chromosome length maintenance are of fundamental importance for organisms such as *Plasmodium* with a highly proliferative life cycle. One of these enzymes is the specialised reverse transcriptase, telomerase, which has recently been identified in *P. falciparum* cell extracts [3], defining a new target for the development of drugs that could induce parasite cell senescence. Genes coding for virulence factors are localised in the subtelomeric regions of *P. falciparum* chromosomes [4], in sites which are known to be highly recombinogenic [2]. In several organisms, telomere-associated regions function as a kind of adaptive domain, mediating rapid evolution of DNA sequences located in these regions [5,6]. A better definition of telomere organisation and telomere-associated sequences will contribute greatly to the understanding of the parasite's biology.

In this study, we present evidence for the packaging of *P. falciparum* telomeres in two distinct types of chromatin: a non-nucleosomal structure at the chromosome end and nucleosomal organisation further upstream. In addition, we present evidence that *P. falciparum* subtelomeric elements have evolved in a species-specific manner.

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2. Methods

We analysed DNA sequences from the eight distinct chromosome ends available on the International Malaria Genome Project Database (for further details see legend of Fig. 1). To search for repetitive elements, each chromosome end was analysed by DotPlot. In Fig. 1A, the result obtained for one end of chromosome 3 is presented, showing a mosaic of six blocks of repetitive sequences located between the telomere and the coding regions. The same organisation was observed for the seven other chromosome ends studied. All six elements are positioned in the same relative order while the size and DNA sequence of each block is polymorphic. We have called these telomere adjacent elements 'telomere associated repetitive elements' (TAREs). TARE-1 is found closest to the telomere. It consists of complex tandem repeats which have been previously described [7]. The size of this block is very polymorphic both within and between chromosomes ranging from 0.9 to 1.9 kb. TARE-2 is 1.6 kb long

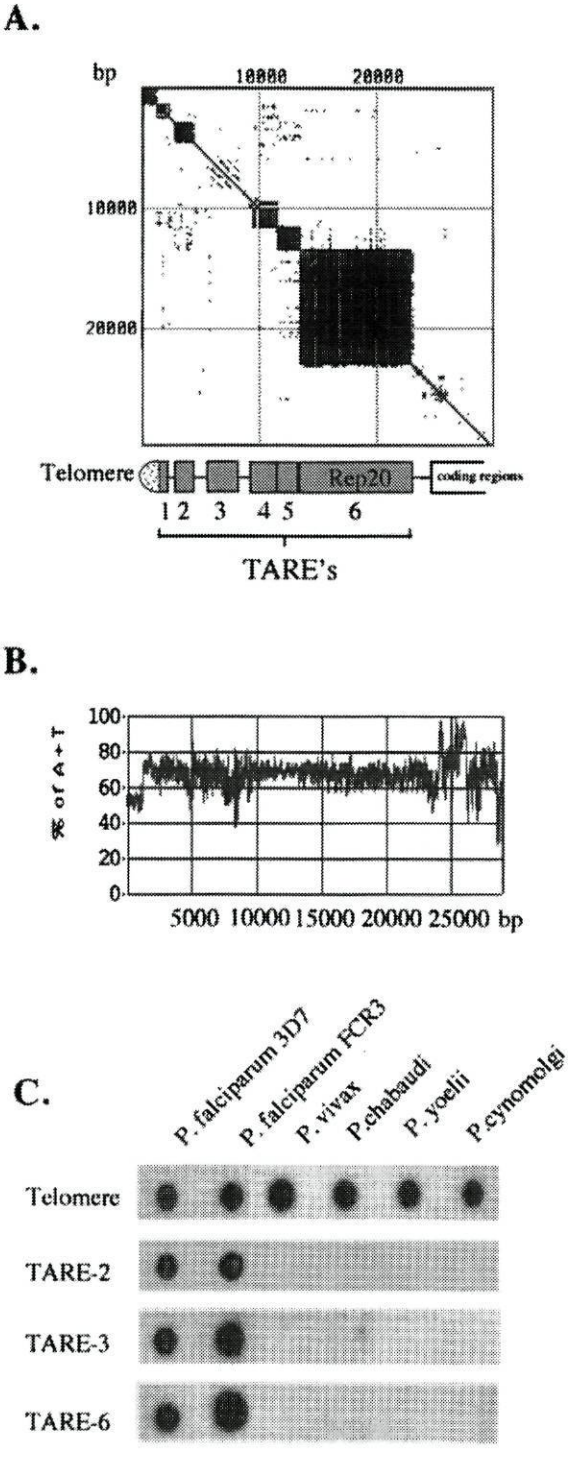


Fig. 1.

Fig. 1. Higher order organisation of chromosome ends of *P. falciparum*. A total of eight chromosome end contigs available on *P. falciparum* databases were analysed: one end of chromosome 1 (accession no. AL031747), the two extremities of chromosome 2 (accession nos. AE001364, AE001365 and AE001436, AE001435; [25]), the two extremities of chromosome 3 (accession nos. AL034560 and AL034559.3 ; [24]), one extremity of chromosome 11 (ftp://ftp.tigr.org/pub/data/p_falciparum/chr11/chr11_contigs.gz), one extremity of chromosome 12 (accession no. AC006278) and one extremity of chromosome 13 (accession no. AL096783). Each sequence was DotPlotted against itself to identify repetitive sequence blocks and against the other contigs to check the degree of homology and their relative position. For this purpose we used the Lasergene software, DNASTAR, with the following settings: 75% match and a 30-bp window size. Alignments of repeats and inter-block sequences were constructed using the CLUSTAL V algorithm from the same software. (A) DotPlot analysis of one end of chromosome 3 (AL034559.3). Each black square on the diagonal of the diagram represents a block of repetitive, tandemly repeated sequences, which we have called telomere associated repetitive elements (TAREs). These elements are schematically indicated under the graph, in a diagram representing the structure of the chromosome end. (B) Adenine and thymidine base content of the same chromosome end (same scale as in A). (C) Species-specificity of subtelomeric DNA elements. Dot-blot analysis of ~100 ng of genomic DNA from two different strains of *P. falciparum* (3D7 and FCR3) and from four other *Plasmodium* species (*Plasmodium vivax*, *Plasmodium chabaudi*, *Plasmodium yoelii* and *Plasmodium cynomolgi*). Each membrane was hybridised with one of the four different ^{32}P-labelled probes: *P. falciparum* telomere repeats, TARE-2, TARE-3 and TARE-6 (Rep20).

and consists of a 135-bp degenerate sequence repeated 12 times, interspersed by two distinct 21-bp sequences. TARE-3 is composed of three to four consecutive 0.7-kb elements, which have also been previously described [8,9]. The fourth repetitive element, TARE-4, is highly polymorphic in length, ranging from 0.7 to 2 kb. It is composed of highly degenerate, short repeats and an interspersed non-repetitive sequence of 230 bp. TARE-5 is located immediately adjacent to TARE-4. Its length varies from 1.4 to 2 kb and is composed of moderately degenerate tandem repeats of 12 bp (5′ ACTAACA(T/A)(C/G)A(T/C)(T/C)). TARE-6 corresponds to the well-known Rep20 element which contains a degenerate 21-bp sequence. [10]. DotPlot analysis revealed that the length of TARE-6 varies from 8.4 to up to 21 kb. The unique sequences found between the TAREs are highly homologous between different chromosomes, reaching up to 90% identity in one of the inter-block sequences. Analysis of the nucleotide composition revealed that the 20–40-kb non-coding, subtelomeric regions display a relatively low A + T content of ~ 70% compared to 82% in the internal chromosome regions (Fig. 1B). Similarly, long compositionally homogeneous DNA segments of differential AT/GC content (isochores) have been described in vertebrates [11]. While these isochores are dispersed among the chromosomes, the available data indicates that the *P. falciparum* shorter isochore-like regions seem to be limited to the chromosome ends. It is tempting to speculate that the subtelomeric elements have been generated from coding regions (A + T content ~ 70%).

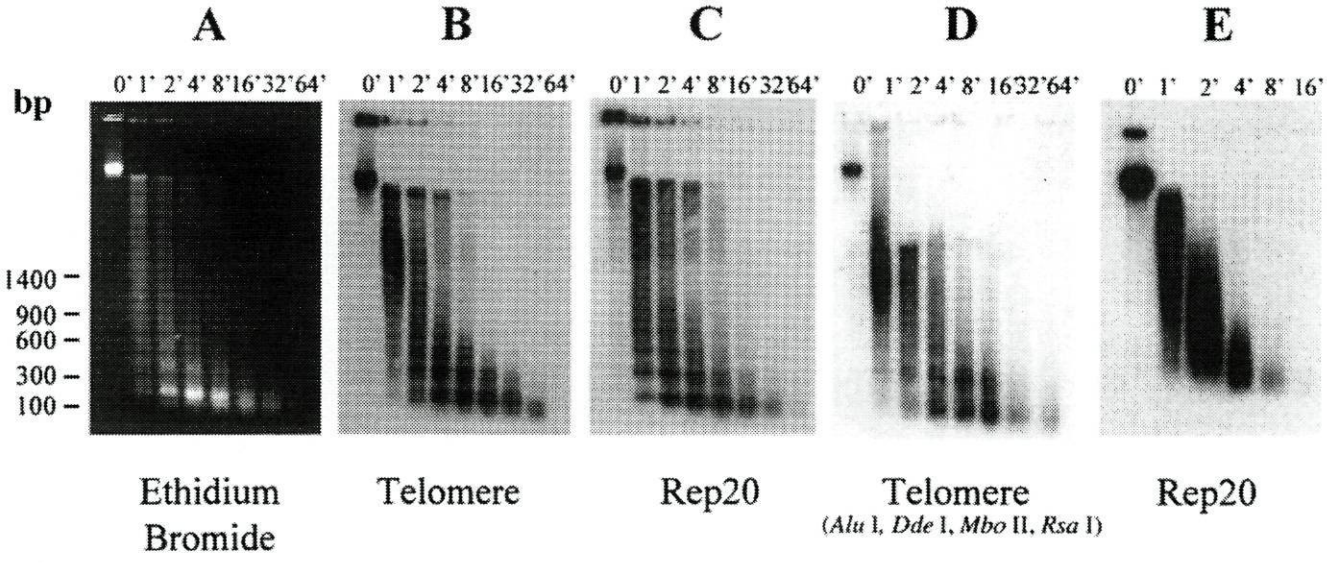
3. Conclusions

In conclusion, our analysis shows that all subtelomeric elements contribute to DNA sequence and size polymorphism at chromosome extremities, with the polymorphic TARE-6 (Rep20) element having a major impact on the size polymorphism of *P. falciparum* chromosomes. Our analysis demonstrates that the higher order subtelomeric organisation is strikingly well conserved, confirming and extending earlier models based on chromosome mapping studies [12–14]. This high homology might enhance ectopic recombination between extremities of the same and distinct chromosomes, which may have a direct impact on the generation of genetic diversity in antigen genes located just next to TARE-6 [4,15,16]. This idea is supported by a recent study in our laboratory showing recombination between two members of the *var* gene family located on different chromosome ends (Bottius et al., manuscript in preparation).

To test the species-specificity of the subtelomeric *P. falciparum* elements, we hybridised genomic DNA from five different malaria species with probes corresponding to the telomere, TARE-2, TARE-3 and TARE-6. No cross-hybridisation between the subtelomeric elements from *P. falciparum* and the DNA from the other species was detected at moderate wash conditions (2 × SSC, 0.1% SDS, 65°C), demonstrating that these elements are species-specific (Fig. 1C). In fact, telomeres were the only regions to cross-hybridise between *Plasmodium* species, suggesting that subtelomeric regions from different malaria species have undergone rapid evolution. This contrasts with internal chromosome regions which seem to be composed of relatively conserved linkage groups between malaria species [17,18].

It has been reported in a number of lower eukaryotes that telomeres have an unusual, non-nucleosomal chromatin structure. For instance in *Saccharomyces cerevisiae*, the entire telomere repeat region is packaged in a non-nucleosomal structure [19]. Telomeric DNA from these organisms is bound by proteins mediating specific functions associated with telomeres such as telomere length regulation [1]. We studied the chromatin organisation in the telomeres of *P. falciparum* by micrococcal nuclease (MNase) digestion of isolated parasite nuclei. MNase preferentially digests DNA in the linker region found between nucleosomes and partial nuclease digestion results in the formation of a characteristic nucleosomal ladder. Non-nucleosomal chromatin and naked DNA contain no nuclease hypersensitive sites and thus MNase digestion yields a smear-like pattern when hybridised with a telomeric probe [19]. In *P.*

falciparum the characteristic phasing of nucleosomes is observed by ethidium bromide staining of the partial digestion products (Fig. 2A). A periodicity of ~150 bp was observed between the bands, similar to that reported by other groups [20,21]. Hybridisation with the *P. falciparum* telomeric probe suggested that the telomeres are at least partially packaged in a nucleosomal-like structure. The banding pattern obtained by hybridisation with the telomeric probe differs from that obtained with the non-telomeric probe (Fig. 2B and C). The bands observed with the telomeric probe have a more diffuse appearance and the hybridisation signal is composed of fewer nucleosomal bands with a more intense background signal. To confirm that the hybridisation signal obtained was derived exclusively from telomeric sequences and not from molecules containing a portion of the adjacent subtelomeric region, we digested MNase products with frequently cutting restriction endonucleases. Telomere repeats are barren of almost all restriction sites and digesting genomic DNA with frequent cutters liberates *P. falciparum* telomeres leaving barely any non-



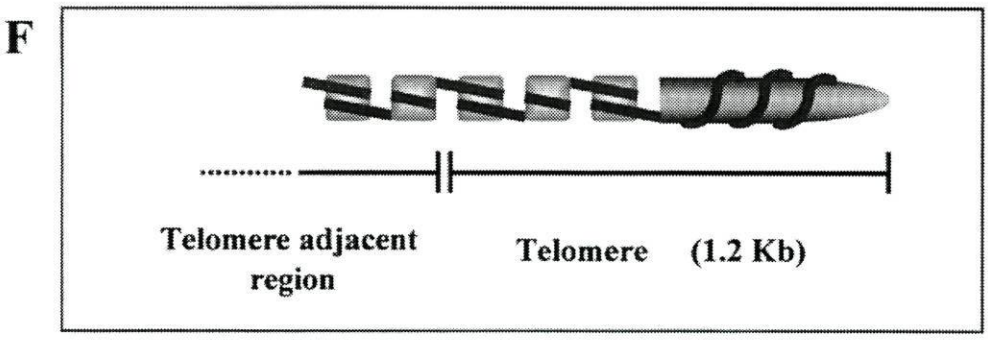


Fig. 2. Chromatin organisation in *P. falciparum* telomeric and subtelomeric regions. As described [20], 10^9 nuclei from asynchronous FCR3 strain parasites were isolated and digested for the times indicated (in minutes) with 5 U/ml of micrococcal nuclease. (A) Ethidium bromide stained gel; (B) hybridisation of MNase products with a telomeric probe; (C) MNase products hybridised with Rep20 (TARE-6) probe; (D) MNase products digested with four frequently-cutting restriction enzymes and hybridised with a telomeric probe; (E) negative control: micrococcal nuclease digestion of genomic DNA in the same conditions described for nuclei probed with Rep20; (F) model of chromatin organisation in *P. falciparum* telomeres. The model proposes two distinct types of chromatin: the inner region of the telomere organised in three to four consecutive nucleosomes and the chromosome terminus assembled in a non-nucleosomal structure, probably composed of distinct type(s) of telomere-specific binding proteins.

telomeric sequence. Fig. 2D shows that a maximum of three to four nucleosomes hybridised with the telomeric probe after restriction digestion of MNase products. This number would be sufficient to cover ~600–800-bp repeats leaving ~400–600 bp in a non-nucleosomal structure (the mean telomere length of the FCR3 strain is ~1.2 kb, data not shown). The hybridisation signal observed in Fig. 2D most likely results from the superposition of two distinct telomeric chromatin structures. Based on these data we propose that *P. falciparum* telomeres are composed of two types of chromatin: a nucleosomal region, and a non-nucleosomal, terminal, region (Fig. 2). A similar dimorphic chromatin structure has also been described in human and *Trypanosoma cruzi* telomeres [22,23]. In humans, the nucleosomal organisation of chromosomal DNA extends into the telomere repeat array and only a minor fraction of repeats at the very tip of the chromosome contains non-nucleosomal chromatin. In the future, it will be interesting to identify *P. falciparum* specific telomere binding proteins and to evaluate their role in chromosome stability, segregation, recombination and possibly on the expression of virulence factor genes located at chromosome ends.

Acknowledgements

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RESULTS II

A Central Role For *Plasmodium falciparum* Subtelomeric Regions in Spatial Positioning and Telomere Length Regulation

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A central role for *Plasmodium falciparum* subtelomeric regions in spatial positioning and telomere length regulation

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In the protozoan malaria parasite, *Plasmodium falciparum*, the telomere-associated sequences (TASs) of the 14 linear chromosomes display a similar higher order organization and form clusters of four to seven telomeres localized at the nuclear periphery. Experimental evidence has shown that the physical tethering of chromosome ends enhances the ectopic recombination between gene families involved in antigenic variation and parasite sequestration. Using FISH analysis, we observed that chromosome ends lacking the subtelomeric region are usually delocalized from telomere clusters, but still remain at the nuclear periphery. This indicates that subtelomeric DNA is necessary for cluster formation but is not essential for peripheral positioning. Intriguingly, these truncated chromosomes have unusually long telomeric tracts (up to three times longer than average length), showing that TASs play a role in telomere length regulation. On these chromosomes, the newly formed telomere frequently extends from truncated genes leading, in some cases, to the transcription of telomeric DNA. The implications of both subtelomeric gene expression and nuclear architecture in the virulence of this serious human pathogen are discussed.

Keywords: malaria/nuclear architecture/telomere-associated sequences/telomere clustering/telomere length

Introduction

Telomeres, the complex of repetitive DNA and associated proteins at chromosome ends, are essential for chromosome stability. They prevent chromosome ends from fusing and from being recognized as damaged DNA. The telomeres from several organisms have been shown to be located at the nuclear periphery. Transcriptional repression and heterochromatin formation are other processes intimately associated with telomeres and assigned to functional subdomains within the nucleus (reviewed in Greider, 1996; Cockell and Gasser, 1999). Telomeric DNA generally consists of tandemly repeated, short G-rich sequences and ends with a 3′ overhang, formed by the degradation of the ultimate primer used for synthesizing the lagging strand during DNA replication (reviewed in de Lange, 1995). It was recently observed that telomeres in mammalian cells, ciliates and trypanosomes end with large 'T loops' (for telomere loops), presumably formed by invasion of the 3′ telomeric overhang into the duplex telomeric repeat array. These structures are thought to protect chromosomal termini from degradation and recognition as broken ends (Griffith *et al.*, 1999; Murti and Prescott, 1999; Munoz-Jordan *et al.*, 2001).

Telomere length regulation involves a tight balance of competing forces: telomere shortening and telomere elongation (reviewed in McEachern *et al.*, 2000). In most organisms telomere length appears to fluctuate around a mean value, which is species specific. However, in certain cell types or organisms, this equilibrium can be unbalanced. For example, in human somatic cells continuous telomere shortening has been observed, whereas in African trypanosomes telomeres display an irregular pattern of growth and shortening (Bernards *et al.*, 1983; Pays *et al.*, 1983; Harley *et al.*, 1990; Hastie *et al.*, 1990). Telomere length is controlled by specific telomere-binding proteins. Several have been described: Rap1p, Cdc13p and Rif proteins in *Saccharomyces cerevisiae* (Berman *et al.*, 1986; Shore and Nasmyth, 1987; Nugent *et al.*, 1996; Virta-Pearlman *et al.*, 1996); Rap1p in *Kluyveromyces lactis* (Larson *et al.*, 1994); Taz1p and Pot1p in *Schizosaccharomyces pombe* (Cooper *et al.*, 1997; Baumann and Cech, 2001); TRF1 and TRF2 in humans (Chong *et al.*, 1995; Bilaud *et al.*, 1996; Broccoli *et al.*, 1997); and a TBP in the ciliate *Oxytricha nova* (Froelich-Ammon *et al.*, 1998). Mutations in these telomere-binding proteins alter the telomere length of each chromosome end.

The telomere adjacent regions are generally composed of a mosaic of non-coding repetitive elements, which seem to have evolved in a species-specific manner (reviewed in Pryde and Louis, 1997) and are considered to serve as molecular buffers of non-functional 'junk DNA' next to the telomere repeats (Wilkie *et al.*, 1991). Although their biological role remains ambiguous, specialized functions such as protecting subtelomeric genes from transcriptional silencing due to the telomere-position effect (TPE) may have evolved in eukaryotes (Fourel *et al.*, 1999; Pryde and Louis, 1999).

The protozoan parasite, *Plasmodium falciparum*, is responsible for the most fatal form of human malaria. The severity of malaria is correlated with the expression of virulence factor genes localized predominantly at chromosome extremities (reviewed in Craig and Scherf, 2001). The haploid nuclear genome of *P.falciparum* is extremely AT rich (82%) and consists of 14 linear chromosomes varying from 0.7 to 3.4 Mb. At each chromosome end,

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telomere repeats [GGGTT(T/C)A] are followed by a non-coding subtelomeric region of ~15–30 kb. This region is composed of six different telomere-associated repetitive elements (TAREs 1–6) and has a highly conserved organization (Figueiredo *et al.*, 2000). These elements are flanked by members of gene families coding for virulence factors, including *var* genes (Rubio *et al.*, 1996; Hernandez-Rivas *et al.*, 1997), which are responsible for antigenic variation and cytoadhesion. When cultivated *in vitro*, *P.falciparum* chromosomes spontaneously undergo breakage, occasionally leading to large terminal deletions. Broken chromosomes are frequently healed by the addition of new telomere repeats, most likely by a plasmodial telomerase (Bottius *et al.*, 1998). Recently, *P.falciparum* chromosome ends were shown to form clusters of four to seven telomeres at the nuclear periphery, facilitating ectopic recombination among heterologous subtelomeric chromosome regions, including genes coding for virulence factors (Freitas-Junior *et al.*, 2000).

In the work reported here, we studied *P.falciparum* mutant strains in which spontaneous terminal chromosome truncations occurred. We observed that the loss of the subtelomeric region changed the location of chromosome ends in the nucleus. Furthermore, we present evidence, for the first time, that telomere length on individual chromosome ends can vary drastically and is modulated *in cis* by the DNA sequences at the telomere junction.

Results

Telomere length varies strikingly among malaria species

The mean telomere length of five different species of *Plasmodium* was analysed using the 'telomere restriction fragment' (TRF) method (de Lange *et al.*, 1990). This technique takes advantage of the lack of restriction sites in the G-rich repetitive telomeric DNA as a means to liberate intact telomeres. Genomic DNA was digested with four frequently cutting restriction enzymes (*Alu*I, *Dde*I, *Mbo*II, *Rsa*I) and analysed by Southern blot using a *P.falciparum* telomere-specific probe (Figure 1A). The mean telomere length was determined using a PhosphorImager quantitative analyser (Figure 1B). Figure 1A shows the striking difference observed in the telomere length between the human malaria species *P.falciparum* and *Plasmodium vivax*, the rodent malarial species *Plasmodium yoelii* and *Plasmodium chabaudi* and the simian parasite *Plasmodium cynomolgi*. *P.vivax* exhibits the longest mean telomere length of ~6700 bp. *P.yoelii* and *P.cynomolgi* telomeres have similar mean lengths of 2000 and 2300 bp, respectively, while *P.chabaudi* has the shortest telomeric DNA tracts averaging 960 bp. Within *P.falciparum* strains, the mean telomere length variation is less striking, ranging from 850 to 1600 bp. The 'Banjul' strain displays an unusual hybridization pattern in which two different-sized peaks are observed: (i) a broad smear represents telomeric DNA similar to the mean length of the other strains and (ii) a sharper peak, corresponding to higher molecular weight telomeric molecules of ~3400 bp. Hence a large difference exists in the average telomere length among the different malarial species.

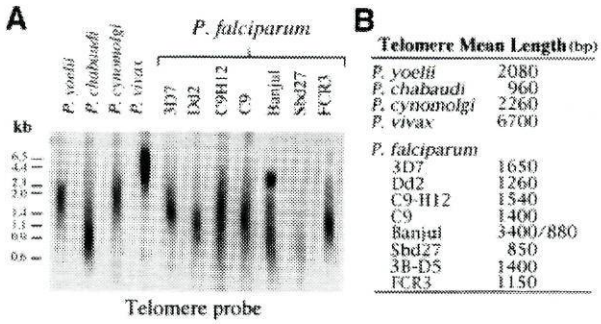


Fig. 1. Telomere length varies substantially among *Plasmodium* species, and to a lesser extent within the same species, as shown by TRF analysis. (**A**) One microgram of total genomic DNA was digested with four frequently cutting restriction enzymes: *Alu*I, *Dde*I, *Mbo*II, *Rsa*I. TRFs were identified using a *P.falciparum* telomere-specific probe, as telomere repeats in other *Plasmodium* species also consist of the same type of degenerate G-rich heptamer (Ponzi *et al.*, 1985; L.M.Figueiredo, unpublished data). The intensity of the signal in each lane was quantified by PhosphorImager analysis and the peak of highest intensity was taken as the mean value for telomere length. Based on the sequences available at the genome database, we calculate that the distance between the telomere and the first restriction site (among the sites of *Alu*I, *Dde*I, *Mbo*II, *Rsa*I) is ~30 bp in *P.falciparum* and *P.vivax*. (**B**) Summary of the average telomere lengths (in bp).

Large inter- and intra-chromosomal variation of telomere repeat length

The broad smears observed by TRF analysis in Figure 1 indicate the possibility of multiple size classes of telomeres in malarial parasites. Therefore, we examined the telomere length of individual chromosomes from three genetically different *P.falciparum* strains (FCR3, 3B-D5 and 3D7) using two-dimensional pulse-field gel electrophoresis (2D-PFGE). This type of analysis consists of separating the 14 *P.falciparum* chromosomes in a first dimension by PFGE followed by digestion with frequently cutting enzymes to liberate the telomere (as described above for TRF analysis). After separation in a second dimension, chromosome-specific telomere restriction fragments were visualized by Southern blot. A characteristic 'telomere fingerprint pattern' (TFP) was observed in the three parasite strains (Figure 2A), revealing in each case that the telomere length is heterogeneous among the 14 chromosomes of the parasite genome. Such fingerprints are stable in laboratory-cultured parasites for at least several months (data not shown). In the FCR3 strain, the majority of the chromosomes have a telomere length of ~1100 bp (see also Figure 1B), although several chromosomes harbour significantly longer telomeres. Specifically, chromosome 1 and some of the chromosomes in the compression zone (chromosomes 5–9) display an intermediate telomere length of 1900 bp. A few others in the compression zone are estimated to have telomeres of up to 3100 bp. Heterogeneity of telomere length also exists in the 3B-D5 strain, although it is less pronounced, ranging from 1400 to 2500 bp. In contrast, the TFP of the 3D7 strain shows that telomere length is much more homogeneous among the 14 chromosomes, ranging within a tighter interval from 1100 to 1600 bp. Moreover, comparison of the TFPs in Figure 2A reveals that the same chromosome can have telomeres of greater than the average length in one strain and the mean length in another strain.

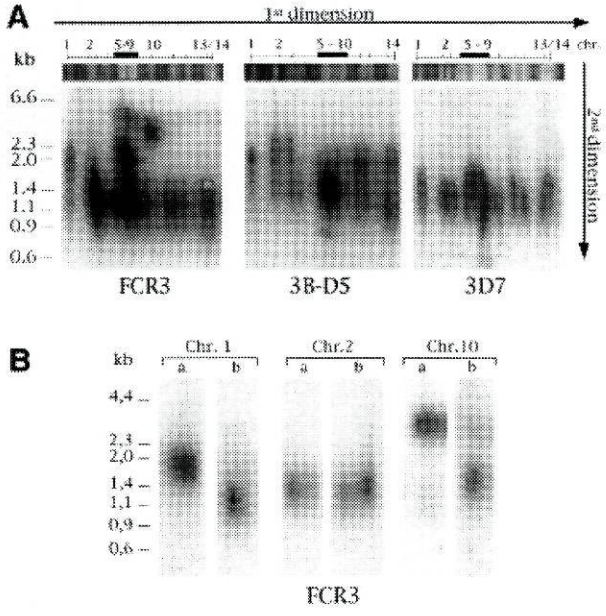


Fig. 2. (**A**) TFP is characteristic of each *P.falciparum* strain. Chromosomes of the genetically different parasite clones FCR3, 3B-D5 and 3D7 were separated by PFGE in a first dimension. Agarose strips containing the 14 chromosomes were next subjected to TRF analysis by digestion with four frequently cutting restriction enzymes (*Alu*I, *Dde*I, *Mbo*II, *Rsa*I), followed by conventional separation on an agarose gel for the second dimension. TRFs were identified using a telomere-specific probe. The observed compression zone is due to the size similarity of chromosomes 5–9/10. (**B**) Two telomeres from a single chromosome can have a different size, as measured by TRF analysis of both 'arms' of three different chromosomes from FCR3. Individual chromosomes (1, 2 and 10) were isolated by PFGE and digested overnight with enzymes that liberate large subtelomeric restriction fragments (*Sac*II for chromosome 1 and *Bss*HII for chromosomes 2 and 10). Restriction fragments were separated by PFGE, excised from the gel and subjected to TRF analysis as described in (A).

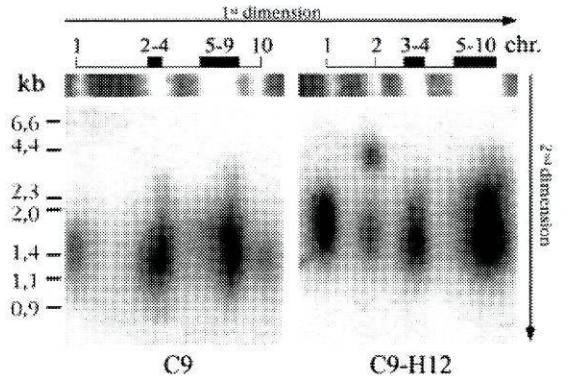


Fig. 3. Intact and truncated chromosome ends harbour telomeres of differing length. TFP of C9 and C9-H12 (a subclone of C9) are presented. Ethidium bromide-stained chromosomes 1–10 are shown. Chromosomes 2 and 10 in C9-H12 migrate more quickly than those of the parental strain C9 due to spontaneously generated large chromosomal truncations. The breakage sites in chromosomes 2 and 10 have been localized to *HRP-I* (this work) and *Pf11-1* (Scherf *et al.*, 1992), respectively.

The long smears observed for some chromosomes in the TFP indicated that there might be a difference between the lengths of the two telomeres on the same chromosome. To address this question, large subtelomeric restriction fragments from chromosomes 1, 2 and 10 of FCR3 were isolated by PFGE and analysed by TRF analysis (Figure 2B). The mean length of the chromosome 1 telomeres differs: 1800 versus 1150 bp. For chromosome 10, the difference in telomere length is more pronounced: 3600 and 1500 bp. However, in chromosome 2 both telomeres are of identical length (1400 bp). Together, these data show that the number of telomere repeats at a given chromosome end may vary dramatically both inter- and intra-chromosomally, demonstrating that regulation of telomere length can be chromosome end specific.

Evidence that DNA sequences at the telomere junction are involved in telomere length regulation

To investigate the molecular basis of the unusually long telomeres on specific chromosome ends, we used two *P.falciparum* clones with an identical genetic background (C9 and its subclone C9-H12), but which differ in their TFPs (Figure 3). Chromosomes 2 and 10 in C9-H12 migrate further than the corresponding chromosomes in C9, due to the fact that they contain large chromosome terminal deletions. Comparison of the TFPs for C9 and C9-H12 clearly shows that a new size population has arisen in C9-H12, indicating that one or both telomeres of chromosomes 2 and 10 have become much longer (the new mean lengths being 4400 and 2200 bp, respectively), whereas in C9 both telomeres of chromosomes 2 and 10 have the same average length. We cloned and sequenced the chromosome breakpoint on chromosome 2 of C9-H12, revealing that it is localized within the intron of a gene coding for the histidine-rich protein I (*HRP-I*). The *HRP-I* gene is normally located ~120 kb from the telomere, but in C9-H12 the entire 120 kb subtelomeric region has been deleted from chromosome 2 (corresponding to >10% of the chromosome), including all but the promotor and the first exon of *HRP-I*. This truncation was repaired by the addition of telomere repeats to the breakage site (schematically shown in Figure 4B). We investigated whether the longer telomere observed in chomosome 2 of C9-H12 is located at the truncated chromosome arm. Both terminal regions from *Bss*HII-digested chromosome 2 were isolated using PFGE. Hybridization analysis confirmed that the longer telomere (4400 bp) corresponds to the truncated arm of chromosome 2, while the intact subtelomeric arm of the same chromosome is only 1500 bp, corresponding to the average length for this strain (data not shown). The same type of analysis was performed for chromosome 2 of the C9 strain, which revealed that both intact subtelomeric arms harbour telomeres of 1400 bp, the average length for this strain (data not shown).

The presence of longer telomeres on truncated chromosomes was confirmed in all additional cases examined (see Table I for summary). A chromosome breakage and healing event had been reported previously for chromosome 10 of C9-H12, resulting in a large subtelomeric deletion of ~100 kb leaving a truncated *Pf11-1* gene adjacent to telomere repeats (Scherf *et al.*, 1992). Southern blot analysis of the chromosome breakpoint using the *Pf11-1* gene as a probe revealed that the newly formed telomere is 2200 bp long (data not shown), while the mean telomere length for this strain is 1540 bp. One of the

chromosome 1 arms (of the FCR3 strain), which has been described to have undergone chromosome breakage and healing at the *RESA* gene locus (Cappai *et al.*, 1989), has a longer than average telomere (1800 bp compared with strain mean average length of 1150 bp; see Figure 2B). In the same strain, a truncation in chromosome 10 has also been reported (Scherf *et al.*, 1992) and we again observed that one of the telomeres is longer (3600 bp). The longer telomere of chromosome 2 in the Dd2 strain is at a breakpoint also in the *HRP-I* gene (analysed in this work, data not shown). Thus, loss of the highly conserved TAS found at the telomere junction appears to lead to a local increase in telomere length. It appears that the sequences found *in cis* to the telomeric repeats are involved directly in the regulation of telomere length. Alternatively, the proximity of the transcription machinery to the telomeres in truncated chromosome ends might interfere with the telosome, a terminal non-nucleosomal structure described previously (Figueiredo *et al.*, 2000) that is thought to be involved in regulating telomere length.

Transcription of telomere repeats at a broken chromosome

To verify whether the proximity of genes close to the telomeres in truncated chromosomes has an effect on telomere length regulation, we first sought to demonstrate that intact chromosome ends are not transcribed and then examined the expression of truncated genes. In intact chromosome ends, a non-coding polymorphic region composed of several repetitive elements (TAREs 1–6) separates the telomeric repeats from the first genes (Figueiredo *et al.*, 2000). We designed a pair of oligonucleotides to amplify a 200 bp unique sequence generally found adjacent to *P.falciparum* telomeres (500–2000 bp). Using real-time RT–PCR, no products were amplified from cDNA, while a control gene generated a specific product (Figure 4A). We were also unable to detect transcripts using specific PCR primers of the TARE2 or TARE1 and the telomere region. Taken together, these results strongly suggest that intact chromosome ends are not transcribed.

To analyse the expression of genes that have been placed close to the telomere by chromosome deletion, we used RT–PCR to synthesize cDNA from asexual ring stage (the early form of the 48 h blood-stage cycle) RNA from C9-H12 and Dd2 clones using a telomere oligonucleotide as the primer for the reverse transcriptase reaction. A specific oligonucleotide matching the *HRP-I* exon 1 (110 bp from the breakpoint in C9-H12 clone, 290 bp from the breakpoint in Dd2) was used for the PCR. PCR products were transferred to a nylon membrane and

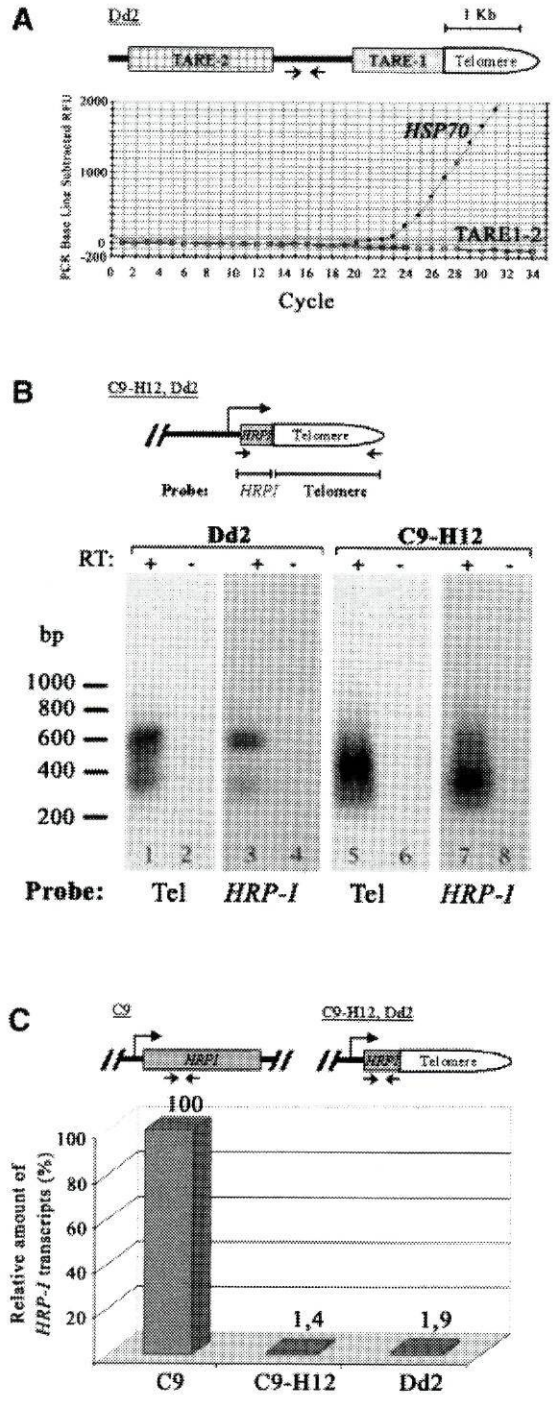


Fig. 4. Telomeric transcription status in intact versus truncated chromosomes. (**A**) Intact chromosomes ends are not transcribed. Real-time RT–PCR using a pair of oligonucleotides that match the non-repetitive region between TARE1 and TARE2 (see Materials and methods for sequences) of the Dd2 strain shows no amplification on hexamer-synthesized cDNA (open squares). However, a specific product was amplified using *HSP70*-specific oligonucleotides from the same cDNA (dots). (**B**) Truncated chromosomes are transcribed. Transcripts from genes that are positioned close to the telomeres can be detected by RT–PCR. Dd2 and C9-H12 cDNA was synthesized from blood-stage RNA using a telomere-specific oligonucleotide as primer for the reverse transcriptase enzyme reaction. Transcription at the breakage site was analysed by PCR using *HRP-I* gene-specific primers in combination with the telomere primer. Southern blot analysis of the RT–PCR products was performed using DNA probes specific to either *HRP-I* or the telomere, showing that transcription of truncated genes runs into telomeric repeats. (+) and (–) indicate the presence or absence of reverse transcriptase. (**C**) There is a decrease in the relative amount of transcripts when *HRP-I* is telomeric. cDNA from C9, C9-H12 and Dd2 strains was synthesized using an *HRP-I*-specific oligonucleotide. Quantitative real-time PCR of the *HRP-I* gene shows that when the gene is truncated (C9-H12 and Dd2), we can detect only 1–2% of the transcripts that exist in C9, where the gene is intact and >100 kb away from the telomere. RFU, reference fluorescence units; RT, reverse transcriptase.

Table I. Length and transcription of telomeres at truncated chromosomes

Chromosome	Locus of breakage	Strain	Telomere length of broken arm	Mean telomere length for strain	Length ratio	Telomere transcription
1	*RESA*	FCR3	1800 (–152)	1150	1.4	–
2	*HRP-I*	Dd2	2400 (–289)	1260	1.7	+
2	*HRP-I*	C9-H12	4400 (–127)	1540	2.8	+
10	*Pf11-1*	C9-H12	2200 (–86)	1540	1.4	–
10	ND	FCR3	3600 (ND)	1150	3.1	ND

The values obtained from TRF analysis correspond to the size of the telomere repeat tract and a short fragment between the first telomere repeat and the restriction site. In intact chromosome ends, this non-telomeric fragment is very small (~30 bp). In broken chromosome ends, the size of this fragment (shown in parentheses) was subtracted from telomere length obtained by TRF analysis in order to calculate the ratio between the telomere length of broken chromosomes and the average for the strain. Transcription through the telomeric tract next to the breakage site was detected by RT–PCR: + indicates presence of transcripts, – indicates absence of transcripts. ND, not determined.

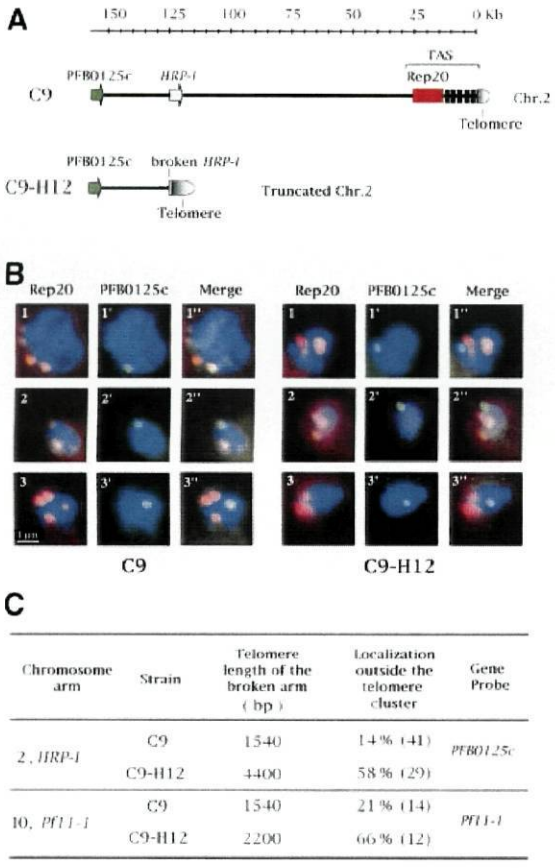
hybridized with a *HRP-I*-specific or a telomere-specific probe (Figure 4B). In both strains, amplification products were detected with a probe for both telomeres and *HRP-I* (lanes 1, 3, 5 and 7). Cloning and DNA sequence analysis of these products confirmed that they contained the truncated portion of the *HRP-I* gene and the adjacent telomeric repeats. No products were detected with any of the probes when RT–PCR was performed in the absence of reverse transcriptase (lanes 2, 4, 6, 8 and 10). Two other truncated chromosome ends were analysed: in chromosome 1 of the FCR3 clone, the breakage has removed the promoter of the *RESA* gene (Cappai *et al.*, 1989); in chromosome 10, of the C9-H12 clone, the truncated gene, *Pf11-1*, is not normally transcribed in the blood stages (Scherf *et al.*, 1992). In both cases, no transcription is expected to occur and indeed we were not able to amplify any transcripts from these truncated genes using RT–PCR (data not shown, but summarized in Table I).

To compare the relative expression of a gene when in its normal position in the chromosome and when it is telomeric, we quantified the relative transcription of *HRP-I* in C9, C9-H12 and Dd2 strains using real-time RT–PCR. When *HRP-I* is truncated and adjacent to the telomere (C9-H12 and Dd2), we detected only 1–2% of the transcripts compared with that of the same gene in the non-truncated chromosome (C9) (Figure 4C). In summary, no transcription was detected in intact chromosome ends, but telomeres can be transcribed if located next to an active promotor.

Distinct localization of truncated chromosomes at the nuclear periphery

Chromosome ends of *P.falciparum* form clusters of four to seven telomeres at the nuclear periphery (Freitas-Junior *et al.*, 2000). We addressed the question of whether the telomere repeats are sufficient to anchor the chromosomes at the periphery and to form clusters. We compared the nuclear positioning of chromosomes that were either intact at both ends or truncated at one end. Chromosomes 2 and 10 of parasites derived from the same genetic background (C9 and C9-H12) were used for the analysis. Both chromosomes are intact in C9. In C9-H12 however, chromosome 2 is truncated within the *HRP-I* locus and chromosome 10 is truncated within the *Pf11-1* locus. *HRP-I* is normally transcribed in asexual blood-stage parasites, while *Pf11-1* is not (sexual-stage-specific gene) (Scherf *et al.*, 1992). An example for chromosome 2 is shown in Figure 5. Two-colour fluorescence *in situ* hybridization (FISH) was performed on asexual blood-stage parasites of C9 and C9-H12 strains. *Rep20* (or TARE6), a large telomere adjacent repetitive element, was used as a marker for the positioning of the clusters in the nucleus (Freitas-Junior *et al.*, 2000). Hybridization with *PFB0125c*, a chromosome 2-specific marker located next to the *HRP-I* locus (Gardner *et al.*, 1998) showed one fluorescent spot located at the periphery of the nucleus in both C9 and C9-H12 strains (Figure 5B, panels 1′–3′). Merging *Rep20* and *PFB0125c* signals (panels 1″–3″), we observed that, while in C9 the two signals colocalize in most cells analysed (14% do not colocalize), in C9-H12 the two markers do not colocalize in 58% of the cells (Figure 5C). This result demonstrates that despite the presence of a telomere, localization outside telomere clusters is approximately four times higher for the truncated extremity of chromosome 2 (of C9-H12) than for its intact counterpart (of C9). The same tendency was observed in chromosome 10 using *Pf11-1* as a specific subtelomeric chromosome marker: the truncated chromosome end (C9-H12) localizes outside telomere clusters 66% of the time, i.e. three times more frequently than in the intact chromosome (C9) (21%). Thus, lack of a conventional subtelomeric region results in a chromosome end that is found more frequently outside telomere clusters. The question arises of whether the truncated chromosomes are still located at the nuclear periphery.

To address this question, we used a computer program to quantify the distance of telomeric probes from the centre of the nucleus and compared these results with the distance measured using a chromosome internal probe. Figure 6A shows the probes used for the FISH analysis, which are derived from *P.falciparum* chromosome 2, whose complete sequence has been determined recently (Gardner *et al.*, 1998). The results presented in Figure 6B and C show that the distribution of these probes in the nucleus is non-random. The truncated end of the chromosome, represented by the probe *PFB0125c*, is located in the periphery of the nucleus and has a similar hybridization pattern to the subtelomeric probe, *Rep20*. In contrast, the chromosome internal probe (*PFB0540w*) was located in the most central zone of the nucleus. A χ^2 analysis performed on the distributions proved that they are significantly different ($p < 0.01$) from each other.



Chromosome arm	Strain	Telomere length of the broken arm (bp)	Localization outside the telomere cluster	Gene Probe
2, *HRP-1*	C9	1540	14% (41)	*PFB0125c*
	C9-H12	4400	58% (29)	
10, *Pf11-1*	C9	1540	21% (14)	*Pf11-1*
	C9-H12	2200	66% (12)	

Fig. 5. Truncated chromosome ends localize preferentially outside telomere clusters. (**A**) Diagram showing the location of the FISH probes in intact (C9) and truncated (C9-H12) chromosome 2. (**B**) FISH analysis in C9 and C9-H12 asexual stages. FISH was performed as described in Materials and methods, using *Rep20* as marker for chromosome end clusters (in red, panels 1–3) and *PFB0125c* as a single copy gene marker present in both intact and truncated forms of chromosome 2 (in green, panels 1′–3′). DNA is counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (in blue). (**C**) Summary of the data obtained by TRF analysis and FISH for chromosomes 2 and 10 of C9 and C9-H12 strains. The number of nuclei analysed is given in parentheses.

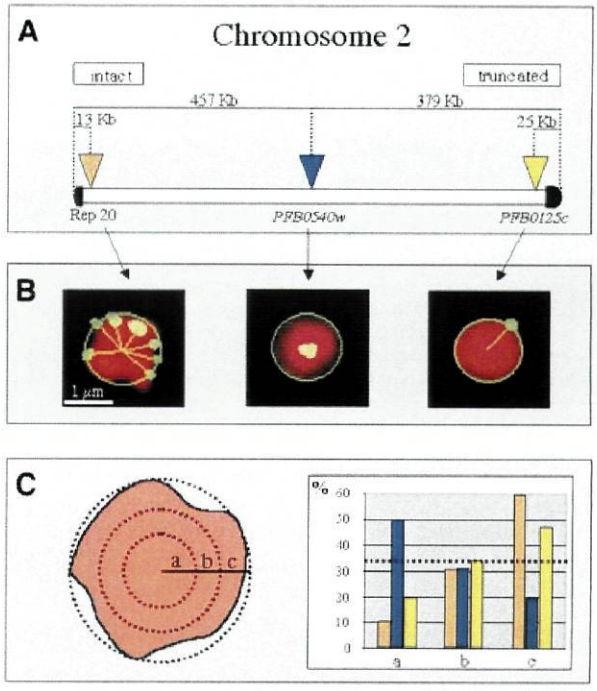


Fig. 6. Intact and truncated chromosome ends are preferentially located at the nuclear periphery. (**A**) The three probes, *Rep 20*, *PFB0540w* and *PFB0125c*, used to analyse *P.falciparum* chromosome 2 from the C9 H12 strain, are marked by arrows and the distance from each chromosome extremity is indicated. The truncated and intact extremities are also indicated. (**B**) FISH analysis was performed using each of the probes indicated in (A) individually. A computer graphic representation of the 2D FISH image is shown. A computer method was used to measure the distance of these signals from the centre of the nucleus. (**C**) The distance from the periphery of the nucleus was measured by dividing the nucleus into three zones, a = internal, b = intermediate and c = peripheral, as described in Materials and methods. The histogram on the right represents the percentage of signals found in each zone. The dashed line represents the theoretical values of a signal randomly distributed in the three zones mentioned above. The subtelomeric probe *Rep20* is represented in orange, the internal probe *PFB0540w* is represented in blue and the subtelomeric probe from the truncated end of the chromosome is represented in yellow.

Discussion

Chromosome-specific, cis-acting DNA elements can overrule regulation of telomere length

In this work we show that there are large inter- and intra-chromosomal length differences within the 28 telomeres of *P.falciparum*. Moreover, the same chromosome end that harbours a telomere of average size in one strain can be up to 3-fold longer in another. Our investigations pointed to spontaneously broken chromosomes, which have been healed by the addition of telomere repeats, as a primary event leading to longer telomeres. Once acquired, longer telomeres are stable within the population, resulting in a strain-specific TFP. However, each of the *de novo* formed telomeres displays a characteristic size. In the C9-H12 strain, for instance, the truncated arm of chromosome 2 has a 4400 bp telomere, while the chromosome 10 truncated arm harbours a smaller telomere of 2200 bp. We postulate that the sequence heterogeneity found in the DNA adjacent to the telomere accounts for the differences seen in telomere length at truncated chromosome ends (see Table I). This could also explain the telomere heterogeneity that has been described in human and murine cells (Zijlmans *et al.*, 1997; Martens *et al.*, 1998), as well as in the human protozoan pathogen *Trypanosoma cruzi* (Freitas-Junior *et al.*, 1999). How the genomic environment adjacent to telomeres is recognized by the machinery that regulates the telomere length remains unknown. One possibility is that specific proteins bind to the TAS and strengthen a telomere fold-back structure, in which the telomere end is protected. When the TAS is deleted, an unusual environment adjacent to the telomere might disturb the folding back and interfere with the machinery that controls telomere length.

Our findings indicate that the presence of conserved TASs on *P.falciparum* chromosomes helps to establish a specific and homogeneous telomere length. Consequently, the variation in the mean length observed between different *Plasmodium* species (960–6700 bp; see Figure 1) may result from the different TAS composition presented by each *Plasmodium* species. This idea is

supported by studies in yeast, which have shown that the telomere–non-telomere junction is important for the regulation of telomere length (Craven and Petes, 1999; Ray and Runge, 1999).

Transcription through telomere repeats at long repeat tracts

It was not possible to amplify specific transcripts from intact chromosome ends using pairs of oligonucleotides derived from the telomeres and adjacent sequences using either classical or real-time RT–PCR, demonstrating that intact chromosome ends are not transcribed. We tested the implications of the proximity of actively transcribed genes to a telomere by analysing the chromosome 2 *HRP-I* truncation in two genetically distinct strains of *P.falciparum*: Dd2 and C9-H12. Using RT–PCR, we were able to detect transcripts corresponding to the truncated *HRP-I* gene during blood stages in the two different strains. These transcripts also contained telomeric repeats, demonstrating that transcription continues into the telomere. Transcription of such truncated chromosomes might explain the signal obtained by Rudenko and Van der Ploeg (1989) when it was shown by nuclear run-on that telomere transcription occurs in *P.falciparum* and that it is α-amanitin sensitive. It has been shown in yeast that telomeric transcription results in a shortening of the telomere (Sandell *et al.*, 1994); however, in this study we observed that all *de novo* synthesized telomeres of truncated chromosomes display longer telomeres than intact chromosome ends, whether or not the truncated gene is transcribed. However, a modest increase in length exists for those truncated chromosomes that are transcribed.

Quantitative real-time PCR experiments revealed ~70 times less *HRP-I* transcripts when the gene is truncated and next to the telomere (in C9-H12 and Dd2) than when it is intact and internal (in C9). Such a difference may reflect the instability of the *HRP-I* transcript in C9-H12 and Dd2 due to the lack of a 3′ untranslated region in the mRNA, but it is possible that a telomere repression effect also accounts for this low transcript level. Telomere silencing has been reported in yeast (Gottschling *et al.*, 1990) and human cells (Baur *et al.*, 2001), for genes that are artificially positioned upstream of telomeres. Disrupting the tethering of telomeres in yeast derepresses telomeric silencing (Cockell and Gasser, 1999), indicating that the compartmentalization of telomeres at the nuclear periphery plays a role in gene regulation. It is conceivable that *P.falciparum* telomeres can also exist in a state that allows transcription and in a state that leads to repression of gene activity. Furthermore, it has been shown in another malaria species, *Plasmodium berghei*, that the stable insertion of a selectable drug marker adjacent to telomere sequences can be transcribed from the proximity of telomeres (van Dijk *et al.*, 1996; Pace *et al.*, 2000).

Implication of telomere-associated regions in cluster organization of chromosome ends

Clustering of *P.falciparum* telomeres at the periphery of the nucleus has been demonstrated recently in sexual and asexual stages of the parasitic life cycle. The conserved organization of the subtelomeric region is thought to be important for the physical alignment of heterologous chromosome ends (Freitas-Junior *et al.*, 2000). Telomere

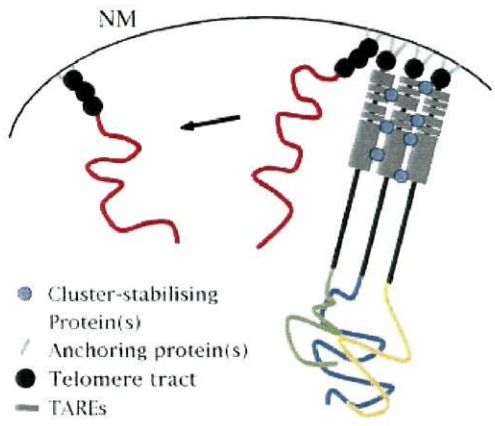


Fig. 7. Model for the spatial organization of chromosome ends in the nucleus of *P.falciparum* is shown. The model predicts a distinct role for telomeres and TASs in the nuclear architecture. We hypothesize that perinuclear localization of chromosome ends is mediated by putative 'anchoring proteins', which bind to the telomere and anchor it to nuclear membrane structural elements. As lack of TASs frequently delocalizes truncated chromosomes from clusters, we propose that TASs play an important role in the physical clustering of chromosome ends perhaps through interactions with specific cluster-stabilizing proteins, which could stabilize the association of chromosome ends. NM, nuclear membrane.

clusters probably account for the high level of genetic diversity observed in these chromosome compartments and generate a boundary for recombination between *var* genes found at telomeric and internal locations (Rubio *et al.*, 1996; Freitas-Junior *et al.*, 2000). Here, we have presented evidence that TASs are indeed important for the nuclear architecture by forming the genetic elements that mediate the clustering of chromosome ends. Analysis of two different chromosomes that have lost their entire TAS revealed that the truncated chromosome ends are frequently dissociated from a cluster (58 and 66%, respectively, for chromosomes 2 and 10). However, the same chromosome from the parental strain in which the subtelomeric elements are intact is found within clusters (only 14 and 21%, respectively, are not). No telomeric transcription is detected in the truncated chromosome 10, indicating that delocalization from clusters is due to the lack of the subtelomeric region rather than a result of telomeric transcription. A recent study has shown that a plasmid containing TARE6 (or *Rep20*), the major repetitive element on the subtelomeric region, colocalizes with telomere clusters in transfected *P.falciparum* (R.A.O'Donnell and B.Crabb, personal communication). Together, these results allow us to conclude that TAS is necessary and sufficient for telomere clustering.

Both intact and truncated chromosome ends localize at the perinuclear space, suggesting that the subtelomeric region is not required for anchoring chromosome ends to the nuclear periphery. We propose a model for the spatial organization of *P.falciparum* chromosome ends in which the telomeres and the conserved TASs have a distinct role in the chromosome architecture (Figure 7). The model predicts that the subtelomeric regions are involved in the formation and stability of telomere clusters. This could be mediated by specific TAS-binding proteins, 'cluster-

stabilizing proteins', that would cross-link the different ends. Telomere repeat-specific proteins may mediate localization at the periphery, as has been shown in yeast, where telomeres are tethered to the nuclear membrane by interactions with a number of proteins (Laroche *et al.*, 1998; Galy *et al.*, 2000). At this point, we cannot rule out the possibility that the organization of the cluster contains inherent properties necessary for telomere length regulation. Consequently, the dissociation of a telomere from a cluster might also cause the observed increase in telomere length.

In *P.falciparum* blood-stage parasites, the resolution of FISH is ~10 kb, meaning that two individual signals are visualized for probes with a 10 kb distance from each other (data not shown). It is therefore surprising that a probe localized ~100 kb from one of the extremities of chromosome 2 colocalizes with telomere clusters. Nevertheless, similar results have been observed earlier in FISH studies of three different chromosomes ends (10, 11 and 13; Freitas-Junior *et al.*, 2000) using chromosome-specific probes ~100 kb from the closest *Rep20* element. In contrast, a combination of two internal probes from chromosome 2 separated by 150 kb gave an almost negligible colocalization index (8.7%, background presumably due to random colocalization). To explain such results, we hypothesize that *P.falciparum* subtelomeric chromosome regions are more compact than chromosome internal regions. This could be explained by the telomere folding back over the subtelomeric regions, as has been observed in yeast (de Bruin *et al.*, 2001). A large number of homologues to yeast proteins involved in nuclear architecture of the telomere, such as Sir proteins, the Ku and the Mlp complexes, have been found in the *P.falciparum* genome database (Scherf *et al.*, 2001), suggesting that yeast can serve as a model to study the telomere biology of this parasite.

In conclusion, our observations provide an original concept giving new insights into the complex processes of control of telomere length and nuclear architecture. Moreover, this work opens new avenues into the understanding of the recombination processes that occur between heterologous chromosomes, as well as the epigenetic processes that control the transcription of subtelomeric gene families involved in antigenic variation in malaria.

Materials and methods

Strains and culture conditions

Plasmodium falciparum intra-erythrocytic stages were cultivated as described (Trager and Jensen, 1976). Genetically distinct strains from different geographical areas used in this study were: 3D7 (Walliker *et al.*, 1987), FCR3 (Scherf *et al.*, 1988), Dd2 and 3B-D5 (Wellems *et al.*, 1990), C9 and C9-H12 (Scherf *et al.*, 1992), Banjul and Sbd27 (Kahane *et al.*, 1987). C9-H12 is a subclone from the C9 strain generated by selection for a knob$^-$ phenotype (generally associated with a spontaneous *HRP-I* deletion in chromosome 2). Genomic DNA from *P.cynomolgi* and *P.vivax* was kindly provided by P.H.David and *P.yoelii* and *P.chabaudi* by D.Mattei.

TRF analysis

Genomic DNA (500 ng) was digested overnight with 5 U of four frequently cutting enzymes (*Alu*I, *Dde*I, *Mbo*II and *Rsa*I), separated on a 1% agarose gel and subjected to Southern blot analysis. The membrane was hybridized with an α-^{32}P-labelled DNA probe specific for *Plasmodium* telomeres (Bottius *et al.*, 1998) and signals were quantified by PhosphorImager (Molecular Dynamics). Peaks were used to calculate the mean telomere length.

Telomere fingerprint pattern

A TFP was obtained by 2D-PFGE analysis. Individual chromosomes were fractionated in the first dimension using a Pulsaphor/Gene Navigator PFGE apparatus (Pharmacia), subsequently digested with *Alu*I, *Dde*I, *Mbo*II, *Rsa*I and separated in the second dimension as described (Hernandez-Rivas and Scherf, 1997). Transfer and hybridization with a telomere-specific probe were as described below.

Southern blotting

DNA products resolved on agarose gels were transferred to Hybond N$^+$ membrane (Amersham) as recommended by the manufacturer. Probes were labelled with [α-^{32}P]dATP by random priming. *HRP-I* and *Pf11-1* probes were as described previously (Scherf and Mattei, 1992; Scherf *et al.*, 1992). The telomere-specific probe consisted of 20 *P.falciparum* telomere repeats (Bottius *et al.*, 1998). The *Rep20* probe was provided by G.Langsley (Patarapotikul and Langsley, 1988). The probe for the *PFB0125c* gene (Gardner *et al.*, 1998) was obtained by PCR amplification (5′-GGAGAAAAATGCAGATGTGG-3′ and 5′-GCCTCATATA-AGGTTTCATATC-3′) and cloning into pCR2.1 vector. Blots were washed twice for 20 min in 2× SSC 0.1% SDS, and once for 30 min in 0.2× SSC 0.1% SDS, at 65°C.

RT–PCR

Ring-stage total RNA was isolated from infected erythrocytes using the TRIzol method (as described in Kyes *et al.* (2000). One microgram of total RNA was used for the first-strand cDNA synthesis. A telomere antisense oligonucleotide [5′-GGCGCG (T G/A AACCC)$_3$-3′] was used as a specific primer to initiate the first strand cDNA synthesis using the M-MLVL Superscript II reverse transcriptase. The reaction was performed as suggested by the supplier (Gibco-BRL). PCR was carried out for 35 cycles as follows: 95°C for 10 s, 50°C for 30 s, 72°C for 1 min, followed by a final extension period of 5 min at 72°C. The primers used were the antisense telomere oligonucleotide and the specific primer 5′-CCGGGATCCATGAAAAGTTTTAAGAACAA-3′ for the *HRP-I* gene from chromosome 2. RT–PCR products were separated on a 2% agarose gel and analysed by Southern blot. The products were purified from agarose (Qiagen Gel Extraction Kit), cloned in pCR2.1 vector (TOPO TA cloning kit; Invitrogen) and sequenced.

For quantitative real-time PCR amplified products were designed to be <200 bp in size for optimal RT–PCR measurements. For the *HRP-I* gene, the 5′-TTATTAGAGCACTTCAAAACCC oligonucleotide was used to synthesize the cDNA and then paired with oligonucleotide 5′-ATACTTTGAGGAGAAAGAAGGC to PCR amplify exon 1 of the *HRP-I* gene. In order to amplify putative transcripts close to the telomeres of intact chromosome ends, cDNA was synthesized from random hexamers and two oligonucleotides that match the non-repetitive region between TARE1 and TARE2 were used. The TARE1–2A primer is 5′-AGAAAACATAACATCATAAGTCC-3′ and the TARE1–2B primer is 5′-TAATAAAGTACTCGGTTG GGC-3′. The *P.falciparum* calmodulin and HSP70 housekeeping genes were used as standard controls. PCR was performed on an iCycler apparatus (Bio-Rad) using the SYBR green detection system (Perkin-Elmer). In three independent experiments (in which each reaction was carried out in triplicate), the following PCR programme was used: 10 min at 95°C; 35 cycles of 15 s at 95°C/30 s at 55°C/30 s at 60°C. The iCycler apparatus measured the fluorescence of each sample in every cycle during the elongation step.

FISH

FISH analysis of asexual blood-stage parasites (trophozoites) was performed as described (Freitas-Junior *et al.*, 2000). The FISH data presented here are two-dimensional images of three-dimensional parasite nuclei. Thus, accidental overlap of the hybridization signals cannot be ruled out. In these experiments colocalization is defined as total or partial overlap of the hybridization signal.

Image analysis

The spatial localization of telomeres in the nuclei was assessed by a computerized method that automatically counts the number of telomeres (Olivo-Marin, 2002) and measures their distance from the centre of the nucleus, using a representation corresponding to an orthogonal projection of the three-dimensional sphere into a two-dimensional circle. The program computes the normalized distance $\Delta_i = d_i/R$ of each telomere i to the centre of its corresponding nucleus, where the radius of nucleus R is determined by thresholding the DAPI image and d is the distance

between the probe and centre of the nucleus. The values obtained for Δ were accumulated from >100 cells and grouped into three concentric circles of radii 0.487 (internal zone a), 0.72 (intermediate zone b) and 1 (peripheral zone c). Signals found outside the DAPI staining, but in close vicinity (0.2 of radial distance) were considered to be part of the peripheral zone. These radial values define the partition of a unitary sphere into three zones of equal volume (33.3% each). Statistical significance was assessed by a χ^2 test, confirming that the distribution for each probe is significantly non-random (p <0.05 for the three probes).

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RESULTS III

Plasmodium Telomerase Reverse-Transcriptase Is Unusually Large and Localises in a Discrete Subnuclear Compartment

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Abstract

Telomerase activity has been previously reported in the human malaria parasite, *Plasmodium* falciparum. Here, we have identified the gene that encodes the catalytic component of this enzyme: *P. falciparum* telomerase reverse-transcriptase (*PfTERT*). It encodes a very basic protein that contains nearly all the expected motifs described in previous TERTs: the RT motifs at the C-terminus side of the protein (1, 2, A through E motifs) and four telomerase specific motifs at the N-terminus (T2, QFP and T motifs). In contrast to other described TERTs, in which the average molecular weight is 120 kDa, PfTERT is much larger. It contains around 2500 amino acids and western blotting analysis confirmed that its apparent molecular weight is larger than 200 kDa. Indirect fluorescence antibody staining of asexual blood stage parasites revealed that PfTERT is not detectable in early ring forms (G1-like phase); however in parasites that have begun DNA synthesis, PfTERT forms a single discrete spot at the nuclear periphery. As successive rounds of nuclear division take place, a multinuclear parasite is formed, but again never more than one spot per nucleus is observed. Telomerase has been proposed to be a good drug target because its activity is likely required in the highly proliferating blood stage of *P. falciparum*. Indeed, telomerase activity can be efficiently inhibited by reverse-transcriptase inhibitors *in vitro*. Our studies will now allow structural modeling studies of the human and *P. falciparum* TERT proteins, eventually aiding in the design of drugs that would specifically inhibit *P. falciparum* TERT. In this study, we have also identified putative TERT genes from three rodent malaria species, which will facilitate the design of pre-clinical trials.

Introduction

Human malaria is re-emerging as the world's most lethal infection, affecting 300 million people and killing 1-2 million people every year. It is caused by an obligate intracellular (unicellular) protozoan of the genus Plasmodia, *Plasmodium falciparum* being the most virulent species. Resistance to existent antimalarials is a major problem in most tropical areas, making the need for new therapeutic drugs (Ridley, 2002) especially urgent. In the human host, *P. falciparum* undergoes exclusively asexual reproduction (in the hepatocytes or erythrocytes) and it is characterised by an extremely high proliferating capacity (reviewed in (Knell, 1991)). Proteins that are essential for the parasite replication can thus be envisioned as potential drug targets, since blocking the proliferation of the parasites would be a major step in controlling the disease.

In the majority of eukaryotes, telomeres consist of tandem arrays of short repeats, maintained by an RNA-dependent DNA polymerase called telomerase. The absence of this enzyme leads to a fatal shortening of telomeres, resulting in growth arrest and eventually senescence (Lingner et al., 1997), (Nakamura et al., 1998), (Blasco et al., 1997), although studies in yeast and human cells have shown that alternative mechanisms for maintaining chromosome integrity can be employed (reviewed in (Lundblad, 2002), (Henson et al., 2002)). The essential core components of telomerase enzyme consist of a catalytic protein component and an RNA molecule that serves as an internal template from which telomeric repeats are added to the 3'-end of the telomere G-rich strand. Other

proteins associate with telomerase, several of which are required for its action *in vivo* (Evans and Lundblad, 2000). The protein component was first purified from *Euplotes aediculatus* (Lingner et al., 1997). By sequence homology, TERTs have been identified in other yeast (Meyerson et al., 1997), (Counter et al., 1997), (Nakamura et al., 1997), (Metz et al., 2001), ciliates (Bryan et al., 1998), (Collins and Gandhi, 1998), mouse (Greenberg et al., 1998), plants (Oguchi et al., 1999), *Giardia lamblia* and *Caenorhabditis elegans* (Malik et al., 2000) and *Xenopus laevis* (Kuramoto et al., 2001). Almost all TERTs contain telomerase-specific motifs in the N-terminus (T2, CP, QFP and T) and reverse-transcriptase specific motifs in the C-terminus (1, 2, A-E) (see Fig. 1) (Lingner et al., 1997) (Friedman and Cech, 1999), (Xia et al., 2000), (Miller et al., 2000), (Malik et al., 2000), (Friedman and Cech, 1999). While the RT domain of TERT is essential for catalytic activity, the N-terminus is implicated in efficient binding of the RNA molecule, definition of the 5' RNA template boundary, functional multimerisation with other TERT and interaction with associated proteins (reviewed in (Liu, 2002)). Telomerase is developmentally regulated in higher eukaryotes (Shay and Wright, 1996). In humans, telomerase activity is present in fetal tissues and, shortly after birth; it is undetectable in most somatic tissues. Low levels of telomerase are found in bone marrow progenitor cells, cord blood, blood mononuclear cells and intestinal mucosa (Marciniak and Guarente, 2001). In contrast, in 85-90% of tumours, telomerase is up-regulated (Kim et al., 1994), (Counter et al., 1994), which makes it an attractive candidate for new mechanism-based targets for cancer therapy (Damm et al., 2001).

The haploid nuclear genome of *P. falciparum* contains around 30Mb, organised in 14 linear chromosomes (reviewed in (Hoffman et al., 2002)). The ends of the chromosomes consist of a tandem array of a degenerate G-rich heptamer, GGGTT T/C A being the most frequent (Vernick and McCutchan, 1988). The mean telomere length is ~1,2 kb and it is maintained at a constant size during blood-stage proliferation, as long as no spontaneous breakages occur in internal regions of the chromosomes (Figueiredo et al., 2002). Chromatin studies have shown that telomeres are partially organised in a non-nucleosomal structure (Figueiredo et al., 2000), suggesting that specific proteins constitute telomeric chromatin. In yeast and humans, several proteins have been shown to interact directly or indirectly with telomeric DNA (for review see (McEachern et al., 2000)). In *P. falciparum* genome database, putative telomeric-specific proteins have been found and are currently being characterised (Scherf et al., 2001). Telomerase activity has been detected in semi-purified nuclear extracts of *P. falciparum* asexual bloodstages (Bottius et al., 1998). This same study also showed that telomerase can use as its substrate oligonucleotides that mimic both telomeric sequences and chromosome breakpoints, suggesting that *P. falciparum* telomerase contributes to chromosome maintenance and to *de novo* telomere formation on broken chromosomes.

Plasmodial telomerase is likely to be necessary to maintain a constant telomere length during blood stages. Given the highly proliferative capacity of the parasite in this stage of the life cycle, one would predict that drugs that interfere with telomere maintenance would induce fatal shortening of telomeres, leading to the death of the parasite. Moreover, because *P. falciparum* telomeres are much shorter than human telomeres (10-15 kb), the inhibitory effects that a telomerase-directed drug might potentially have on human telomerase would be proportionally less important. In the present work, we

report the identification and characterisation of a putative TERT from the human malaria parasite *P. falciparum* (PfTERT). We also identified partial TERTs from three murine Plasmodial species, opening new possibilities for testing telomerase-specific drugs in malaria mouse model.

Materials & Methods

Genome searches and sequence alignments

Genes coding for putative telomerase reverse-transcriptases in *Plasmodium* species were obtained from both NCBI Custom Blast in Malaria Genetics & Genomics (http://www.ncbi.nlm.nih.gov/Malaria/*Plasmodium*blcus.html) and PlasmoDB (www.plasmodb.org), using TBLASTN. To identify the first Plasmodial TERT gene (PfTERT), Motif T and other reverse-transcriptase motifs were used as a query. For *P. yoelii*, *P. berghei* and *P. chabaudi* TERT genes, the entire PfTERT protein was used as a query. Multiple sequence alignments were constructed using CLUSTALW algorithm with gap opening and extension penalties of 20 and the Blosum series of similarity matrices.

RT-PCR

Total RNA was extracted from *in vitro* cultures of *P. falciparum* asexual blood stages using the TRIzol method (GIBCO) as described in (Kyes et al., 2000). 1 µg of total RNA was used for cDNA synthesis, after confirming the absence of DNA contaminants by PCR. Random hexameres or *PfTERT* gene specific oligonucleotides were used as primers to initiate the first strand cDNA synthesis by the M-MLVL Superscript II Reverse Transcriptase. The reaction was performed as suggested by the supplier (GIBCO-BRL). The pairs of primers used to amplify PfTERT gene were: TEL 7 (5'ATGAGAAG TTCCAGAGAAGTGTG3') and TEL8 (5'TCTGTGTAATTAG TACAGCTTG3') for F1, TEL11 (5'GA GTCGCAG AATTATACCATC3') and TEL2 (5'GTA CATAATCCATTTTGGGT C3') for F2, TEL1 (5'TCTAACCAAATCTGAGCAA ACC3') and TEL4 (5'CAT AACAGTTTG AGAAATCTCC3') for F3, TEL3 (5'TG CCAGAAATTGTCAAGC AACG3') and TEL6 (5'GGATGAATT AATATTAC TTCCCC3') for F4 and TEL5 (5'GGCTTACCACAAGGTTTTAGC3') and TEL12 (5'CTATGTTTGG ACA CCAAGCC3') for F5. RT-PCR products were separated on a 1% agarose gel, purified from agarose (QIAGEN Gel Extraction Kit), cloned in pCR2.1 vector (Invitrogen, TOPO TA cloning kit) and sequenced.

Pulse-field gel electrophoresis

Individual chromosomes were fractionated in the first dimension using a Pulsaphor/Gene Navigator PFGE apparatus (Pharmacia) as described (Hernandez-Rivas and Scherf, 1997). The gel was then exposed for 5 min to 254 nm UV-light to allow an efficient transfer of large DNA fragments. Resolved chromosomes were transferred to positively charged membrane (Q-BIOgene) as recommended by the manufacturer in 0.4N NaOH. A PfTERT gene-specific fragment of 1520 bp *was* labelled with [α-32P]dATP by random priming (AMERSHAM). After overnight hybridisation at 65°C in 7% SDS, 1% BSA, 0.5M Phosphate buffer pH7.2, blots were washed four times for 15 min in 1x SSC 0.1% SDS, at 65°C and exposed to a KODAK film.

Production of antibodies anti-PfTERT

A 660 bp fragment containing Motifs 1, 2 was amplified from gDNA using the following oligonucleotides: TELX1 (5'CG

CGGATCCCATATGAGAATGGCAAA AGAAG 3') and TELX2 (5'CCGGA ATTCTTGCTTGACAATTTCTGGCAC 3'), which contain a *Bam*H I and an *Eco*R I restriction site respectively. The PCR amplified product was gel purified (QIAGEN gel extraction kit), digested with *Bam*H I and *Eco*R I and cloned into pGEX-B vector (Pharmacia). Vector construction was confirmed by sequencing. Recombinant protein was expressed in DH5a *E. coli* bacterial strain, purified with Glutathione-Sepharose and used to immunise mice (C129 strain). A 15 amino acid peptide from PfTERT C-terminus (position: 2476-2487) was coupled to Keyhole Limpet Hemocyanin (KLH) carrier protein and used to immunise rabbits.

Western blotting analysis

Total extracts containing proteins from asexual blood stage parasites were resolved on a 7.5% SDS-PAGE. Western blotting analysis was performed following a standard protocol. Proteins were transferred to a Hybond nitrocellulose membrane (Amersham) for 2 hr at 150 mA. After Ponceau staining, strips were cut containing ~$3x10^7$ parasite equivalent proteins. Strips were pre-incubated overnight with PBS, 5% non-fat milk, 0.05% Tween20 at room temperature. As primary antibody we used the following: non-purified sera from mice immunised against a GST-PfTERT recombinant protein, non-purified sera from pre-immunised mice, or monoclonal mouse antibody anti-PfHSP70. Anti-mouse IgG (H+L) alkaline-phosphatase conjugate (PROMEGA) was used as secondary antibody.

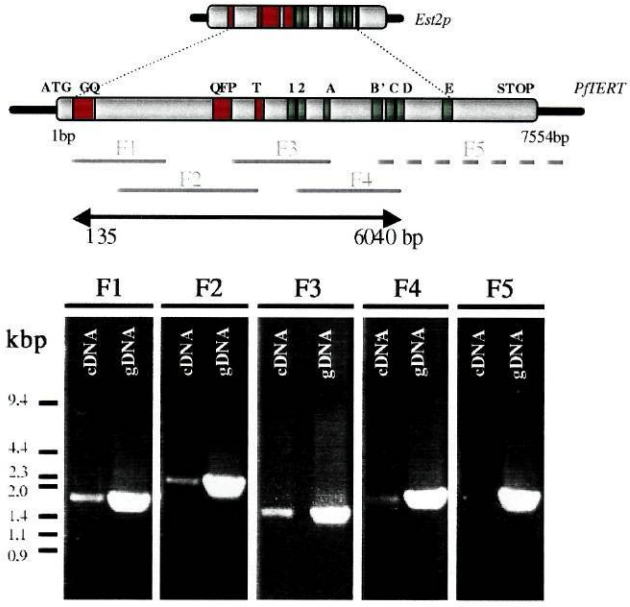


Fig. 1. *PfTERT* candidate gene. (A) Schematic diagram comparing the size of the yeast *Est2p* and *PfTERT* open reading frames. *Est2p* does not harbour any introns. The 5'-end region of the *PfTERT* gene encodes several motifs that are specific to telomerases and which are not found in other reverse-transcriptases (boxes in red): T2, QFP and T. The remainder of the gene encodes motifs that are characteristic of reverse-transcriptases: 1, 2, A through E (boxes in green). (B) Agarose gel showing the RT-PCR fragments covering almost completely the PfTERT gene (F1-F5), performed in parallel on cDNA and gDNA. cDNA was previously analysed to have no DNA contaminants (data not shown).

Primary and secondary antibodies were incubated with strips for 1 hr at room-temperature in PBS, 5% non-fat milk, 0.05% Tween20. Intermediate and final washes were made in the same buffer three times 5 min and twice 10 min. The specific proteins were detected using the NBT/BCIP system from PROMEGA.

Immunofluorescence microscopy

Slides containing mono-layers of air-dried parasite-infected erythrocytes were prepared as described in (Schlichtherle et al., 2000). Incubation with primary antibody (non-purified mouse sera) was performed for 1 hr. After washing twice

for 10 min with PBS 0.1% BSA, slides were incubated with anti-mouse Ig-G FITC conjugate (SIGMA) for 30 min. Incubations with antibodies were performed at room temperature, in a humid dark chamber. Slides were washed thoroughly, mounted in propidium iodide and anti-fadeand covered with a coverslip.

Results

Identification of P. falciparum TERT gene

Two previous observations led us to search for a telomerase reverse-transcriptase (*TERT*) gene in *P. falciparum*. First, telomeric DNA is composed of canonical G-rich repeats, suggesting that they are maintained by telomerase. Second, telomerase activity has previously been detected in semi-purified nuclear extracts from *P. falciparum*. Attempts to PCR amplify the *PfTERT* gene based on consensus motifs were not successful and only after advances in the sequencing project of malaria genomes, were we able to identify a candidate TERT gene. The *P. falciparum* genome database was searched using as a query the conserved motifs described previously: the T motif and the seven reverse-transcriptase motifs (Nakamura et al., 1997). Two overlapping contigs displayed a high score. Their alignment produced a sequence of ~10 kb, containing a large open reading-frame of 7554 bp. The translation of this sequence, starting at the first methionine, results in a predicted protein of 2518 amino acids, that we named PfTERT (recently catalogued with accession number: chr13_400065.gen2 in PlasmoDB database and as AX112155 in GenBank).

PfTERT: gene characterisation

Analysis of the genomic sequence containing the 7554 bp ORF coding for PfTERT did not reveal any convincing matches to intron consensus sequences. To confirm the absence of introns, we performed an RT-PCR analysis. We amplified fragments of 500-2000 bp from gDNA and cDNA and ran out the resulting products in parallel on an agarose gel to check for differences in size. Fig.1 shows the results obtained for the largest fragments amplified. In reactions 1-4, cDNA and gDNA amplification products have the same length, which strongly suggests the absence of introns in the ORF sequence from 135 to 6040bp. In reaction 5, we were not able to amplify any product from the cDNA, suggesting that the oligonucleotide used as the 3'-end did not hybridise to the expected 3'-UTR. Other pairs of oligonucleotides will have to be designed to confirm the absence of introns in this region of the gene.

The initial contigs obtained from the genome database originated from two distinct chromosomes: 13 and 14, raising the possibility of the existence of two copies of TERT gene in the *P. falciparum* nuclear genome. To investigate the chromosomal location of PfTERT, we prepared a probe corresponding to F3 fragment in Fig. 1.

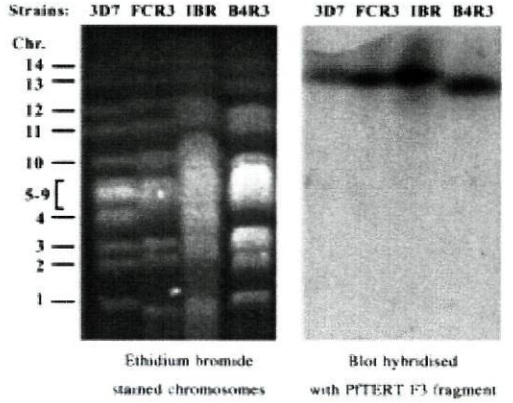


Fig. 2. *PfTERT* gene locus is on chromosome 13 in four different strains of *P. falciparum*. The left panel is a picture of an agarose gel of *P. falciparum* chromosomes after separation by pulse field gel electrophoresis and staining with ethidium bromide. The right panel represents the blot obtained after gel transfer and hybridisation to a radioactively labelled *PfTERT* F3 fragment (see diagram Fig. 1).

A.



B.

	Tt	Ot	Ea	Sc	Sp	Ca	At	Mm	Xl	Hs	Gl
% of overall identity to PfTERT	12,3	12,5	11,9	11,4	13,3	12,2	11,0	9,6	10,4	7,4	10,4
% of Motif T identity to PfTERT	24,5	24,5	32,7	22,4	28,6	12,2	18,4	24,5	22,4	22,4	-

Fig. 3. Comparison of the consensus motifs of PfTERT with telomerase reverse-transcriptases identified til present: *Tetrahymena thermophila* (TtTERT), *Oxytricha trifallax* (OtTERT), *Euplotes aediculatus* (Ea_p123), *Saccharomyces cerevisiae* (Sc_Est2p), *Schizosaccharomyces pombe* (Sp_Trt1p), *Candida albicans* (CaTERT), *Arabidopsis thaliana* (AtTERT), *Mus musculus* (mouse) (mTERT), *Xenopus laevis* (XlTERT), *Homo sapiens* (human) (hTERT) and *Giardia lamblia* (GlTERT) (A) Multiple sequence alignment of conserved amino acid motifs. The top lines represent an identity consensus sequence (when 8 out of 12 sequences are identical) and a consensus showing a conservation on the group of amino acid (when 9 out of 12 sequences belong to the same group) respectively; a= acidic (DE), b=basic (HKR), f=hydrophobic (AFILMPVW) and p=polar (CGNQSTY). On the bottom of motifs, squares represent telomerase-specific amino acids, not present in viral reverse-transcriptases; circles represent amino acids essential for reverse transcriptase activity. Motif spacing and distance to the protein ends (in amino acids) are shown. Motif CP and motif T have not been found in *G. lamblia*. Abbreviations for amino acids are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp and Y, Tyr. (B) Amino acid sequence identity between *P. falciparum* TERT and previously described TERTs. In the first line, amino acid composition of entire sequences was compared; in the second line, only sequences of Motif T (aligned as shown in (A)) were compared.

The 14 chromosomes of different laboratory strains of *P. falciparum* were firstly resolved by pulse-field gel electrophoresis and then transferred to a membrane, which was probed with F3 fragment. As shown in Fig. 2 (right panel), only one band is observed for each strain, corresponding to chromosome 13. Thus, *PfTERT* gene is located in a single chromosome.

Features of P. falciparum TERT protein

PfTERT contains nearly all the canonical motifs of other telomerase reverse-transcriptases. In the N-terminus half of PfTERT, we found two previously described motifs: T2 and QFP (Xia et al., 2000), (Miller et al., 2000). No motif CP was found. Motif T is roughly in the middle of the protein and motifs 1, 2 and A to E are located towards the C-terminus (Fig.3A). Amino acids known to be invariant within specific motifs were found in PfTERT (shown with a square in Fig.3A): Arg in Motif 1, an aromatic residue (Phe or Tyr) following the two critical Asp residues in Motif C and the Trp-X-Gly-X-Ser/Leu in motif E. PfTERT also contains amino acids identified to be critical for the reverse-transcriptase activity (Lingner et al., 1997), (Weinrich et al., 1997) (shown with a circle in Fig.3A). Like other TERTs identified previously, PfTERT is a highly-basic protein with an isoelectric point (pI) of 10.0 (Bryan et al., 1998).

PfTERT has several peculiar features, uncommon to other TERTs. The most striking difference is its predicted molecular weight: ~280 kDa. This is more than two times the molecular weight of a well-characterized TERT: the 103 kDa telomerase from *S. cerevisiae* (diagram Fig. 1). The slight differences in length observed previously among TERTs was mainly due to the size of the N-terminus half and, partially due to the distance between Motifs A and B' (Bryan et al., 1998). The difference in size of the Plasmodial TERT also lies in the N-terminus half of the protein, but the difference is more drastic (e.g. from the first methionine to Motif T, there are 1046 amino acids in PfTERT, and 461 amino acids in TtTERT). But, motif spacing is also generally larger in *P. falciparum* TERT: the distance between Motif T and Motif 1 is 310 amino acids in *P.*

falciparum while in other TERTs, this distance varies from 2-10 amino acids. Only motifs 1 and 2 show a conserved distance between them of only 1 amino acid (Fig.3A). Consequently, the overall percentage of amino acid identity between PfTERT sequence and the other TERTs is relatively low (Fig.3B): *S. pombe* TERT is the sequence with highest identity, while human TERT is just 7.4% identical. Given that Motif T is specific to telomerases and conserved among all TERTs, except for *G. lamblia*, we examined the extent of homology between Motif T of all identified TERTs and compared it with PfTERT (Fig.3B). The percentages of identity are, as expected, higher than when we compared the entire protein sequence, varying around 22-25%. Higher homology is observed with *E. aediculatus* (32.7%) and *S. pombe* (28.6%) sequences, while the lowest score is with *A. thaliana* (18.4%) and *C. albicans* (12.2%).

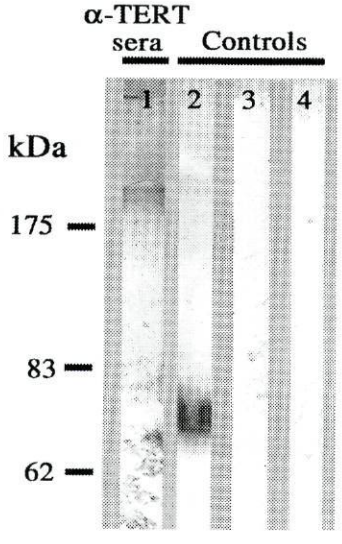


Fig. 4. High-molecular weight protein recognised by sera from PfTERT-GST immunised mice. A total protein extract from asynchronous blood stage parasites was resolved on a 7,5% acrylamide / bis-acrylamide gel, transferred to a Nylon membrane and incubated with the following primary antibodies: sera from mice immunised with PfTERT-GST fusion protein (strip 1); anti-PfHSP70 mouse monoclonal antibody (strip 2); pre-immune sera (strip 3) and just PBS/BSA (strip 4).

Expression of PfTERT

Telomerase activity has been detected in asexual blood stages of *P. falciparum* life cycle, a stage characterised by high levels of proliferation. By Western Blotting analysis, a large protein of apparent molecular weight > 200 kDa was recognised by mouse antibodies anti-PfTERT-GST fusion protein (strip 1, Fig. 4). This is consistent with our predictions of PfTERT being ~280 kDa. The second strip was incubated with an anti-PfHSP70 monoclonal antibody, as a positive control of the Western Blotting. Pre-immune mouse sera did not recognise any proteins in *P. falciparum* extracts (strip 3). Secondary-antibody (goat anti-mouse) did not cross-react with *P. falciparum* extracts either (strip 4).

Localisation of PfTERT in the cell

Based on an algorithm that predicts sorting signals and localization sites coded in protein sequences (PSORTII) (http://psort.nibb.ac.jp/psort; Kenta Nakai; Human Genome Center, Tokyo, Japan) and considering that the nuclear localisation signals (NLS) are conserved among eukaryotes (including *P. falciparum*), PfTERT contains several NLS-like motifs. In fact, the algorithm used by this program, the k-nearest neighbours classifier (k-NN) predicts that PfTERT has 78,3% probability of being nuclear (for *Tetrahymena* TERT, for example, the probability was 52,2%). Whether, these NLS-like sequences can work as functional NLSs would need to be confirmed.

Human TERT localises mainly in the nucleus, although some cytoplasmic staining can be observed in certain stages of the cell cycle (Seimiya et al., 2000). Immunofluorescence assays were undertaken on air-dried asynchronous parasites from a blood stage culture

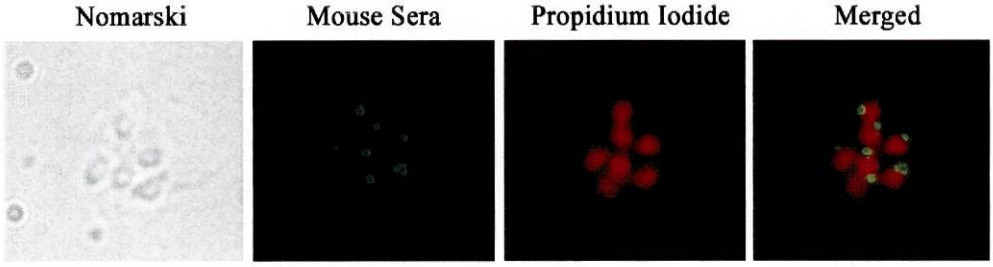


Fig. 5. Late-forms of asexual blood stages of *P. falciparum* are labelled by sera from GST-PfTERT immunised mice. A schizont is shown containing probably eight individual merozoites. In the Nomarski phase-contrast figure, individual forms (the merozoites) can be distinguished and are surrounded by a membrane (the erythrocyte membrane). GST-PfTERT mouse sera (shown in green) recognises each merozoite and not the erythrocyte. In each merozoite, the labelling is more concentrated in one spot, which apparently co-localises with the nuclear DNA, stained in red by the propidium iodide.

Immunolocalisation studies with mouse sera anti-PfTERT-GST, resulted in a characteristic pattern. In general, no staining was observed in early blood stages (ring: 0-18hr). In trophozoites (18-38hr) and schizonts (38-48hr), there seems to be both a cytoplasmic and a nuclear staining. The intensity of the nuclear signal increases in schizogony, where we can count one dot per nuclei of each merozoite (Fig. 5). Rabbit sera produced against a PfTERT peptide generates the same localisation pattern (data not shown).

Identification of other Plasmodial TERTs

Although *P. falciparum* was the first malaria species whose genome began to be sequenced, other malaria species are now being sequenced too: *Plasmodium* species that cause malaria in rodent animals (*P. berghei*, *P. yoelii*, *P. chabaudi*), in monkeys (*P. knowlesi*) and humans (*P. vivax*). Using PfTERT protein as a query, we searched the other *Plasmodium* species genome databases in order to find candidate *TERT* genes. Not all genome projects are equally advanced, thus good hits were obtained only in *P. yoelii* ($E=10^{-154}$), *P. chabaudi* ($E=10^{-21}$) and *P. berghei* ($E=10^{-22}$). In *P. yoelii*, an ORF predicts a very basic protein with 2189 amino acids and contains Motifs T2, QFP, T, 1, 2 and A-E. Thus we called it PyTERT. Alignment with PfTERT, suggests that the N-terminus of this protein is not included in the contig (Fig. 6A). *P. chabaudi* hit refers to a sequence with just 159 amino acids, spanning the region of motifs B', C and D (Fig. 6A). At the time we did these searches, the available sequencing data of *P. berghei* genome consisted of an EST mungbean library. Thus the hit we obtained referred to a 600 bp sequence that coded for a protein highly homologous to PfTERT N-terminus. We decided to extend the sequence of the *PbTERT* putative gene, by designing oligonucleotides that matched conserved regions between *PfTERT*, *PyTERT* and *PcTERT* genes. We were able to PCR amplify a 3942 bp sequence that span Motifs T2, QFP, T, 1 and 2 (Fig. 6A).

Although, we lack the complete sequences of TERT genes from *P. yoelii*, *P. chabaudi* and *P. berghei*, we carried out a multiple alignment of the four Plasmodial TERT proteins (Fig. 6B). We observe that the most divergent regions are mainly at the N and C-terminus of the proteins. The internal regions covering from Motif QFP to Motif A and from Motif B to D are highly conserved.

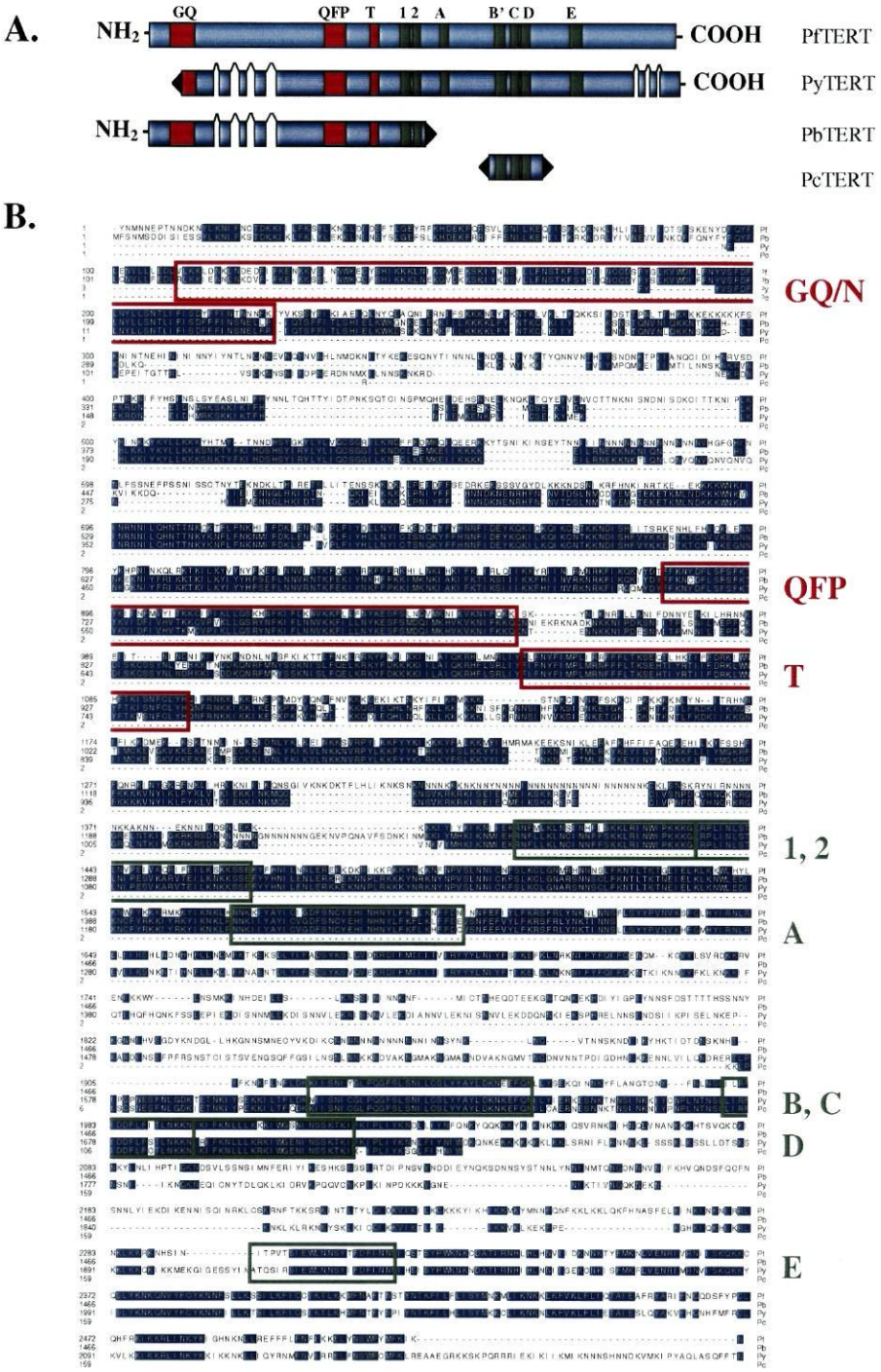


Fig. 6. The primary structure of Plasmodial TERT proteins is conserved. (A) Diagram showing the four putative TERT proteins, found in the malaria genome databases. Red boxes represent telomerase reverse-transcriptase specific motifs (T2, QF and T); green boxes are motifs common to other reverse-transcriptases (1, 2, A-E). The only gene that is completely sequenced is the *PfTERT* gene. In PyTERT protein is missing the N-terminus, in PbTERT is missing the C-terminus and in PcTERT only a 160 amino acid region was found. Note that PyTERT and PbTERT lack some amino acid sequences present in PfTERT. (B) Multiple sequence alignment of plasmodial TERTs. Motifs are shown in the same colour-code. Abbreviations for amino acids are as indicated in legend of Fig.1.

PfTERT contains insertions of amino acids that are absent from PbTERT and PyTERT. One of these regions is very peculiar: it consists of a stretch around 30 Arginines (N), interspaced by a few Lysines (K), Isoleucines (I) and Tyrosines (Y). The overall percentage of amino acid identity among Plasmodial TERT sequences is high. PyTERT and PbTERT are highly homologous (72,6% identity), while PfTERT shares 41,9% identical amino acids with PbTERT and 38,7% with PyTERT.

Discussion

Plasmodial chromosomes are capped by canonical telomeres, i.e. tandem arrays of G-rich degenerate heptameres (Vernick and McCutchan, 1988). It has been shown for other eukaryotes that such a telomere structure is maintained by a specific enzyme: telomerase. It has been previously shown that *P. falciparum* semi-purified nuclear extracts (Bottius et al., 1998), are capable of *de novo* telomere repeat synthesis, suggesting the presence of a telomerase enzyme in this human malaria parasite. In the present work, we identified and characterised a gene in *Plasmodium falciparum* that codes for the catalytic protein component of telomerase, named here PfTERT. It contains structural features identical to TERTs described in other organisms: reverse-transcriptase motifs, telomerase-specific motifs, molecular mass > 100 kDa and pI=10. We also found homologous genes in other Plasmodial species: *PyTERT*, *PbTERT* and *PcTERT* genes.

The TERT gene found in the genome database of *P. falciparum* is present on chromosome 13 and it contains an open reading-frame of 7554 bp. Although, no introns were predicted by the Gene Finder sequence analyser software, we started an RT-PCR analysis to confirm the absence of introns. PCR fragments varying from 300 to 2400 bp did not reveal differences in size between amplification products from cDNA and gDNA, suggesting that there are no introns on the region from +135 to 6040 bp. However, we cannot exclude the possibility that a small intron might exist due to the low resolution of agarose gel electrophoresis.

To obtain antibodies against the endogenous PfTERT, we produced a GST recombinant fusion protein that contained a 220 amino acid peptide spanning the reverse-transcriptase Motifs 1 and 2. The purified recombinant protein was used to immunise eight mice and sera was tested by Western blot and immunofluorescence microscopy. Mice sera recognised a band at high molecular weight, compatible with the predicted molecular weight for PfTERT of ~280 kDa. Immunofluorescence assays on air-dried slides of asexual blood stage parasites, produced a distinct nuclear pattern. This staining consists of a single dot, which appears to be close to the periphery of the nucleus. Some diffuse cytoplasmic labelling could also be observed. The nuclear signal was not detected in young forms of the parasite, but only in trophozoite stages through schizogony (a stage with more than one nucleus per parasite cell) and in newly formed merozoites. Rabbit antibodies against a C-terminal PfTERT peptide yielded a similar punctuated nuclear pattern. The reason why Plasmodial TERT localises in a discrete perinuclear spot remains mysterious, given the expected role of this enzyme at all chromosome ends during S phase. Very little is known about the localisation of telomerase in the cells of other organisms, and how it evolves with the progression of the cell-cycle. For hypotrichous ciliates, Fang and Cech

showed that telomerase RNA generally concentrates in several discrete foci of the nucleus that are devoid of DNA. During S phase, telomerase RNA is enriched in the replication band, a structure that contains the DNA replication machinery, suggesting that a sub-fraction of telomerase is coordinated with semiconservative DNA replication (Fang and Cech, 1995). More recently, it was observed that human TERT exhibits a subtle punctuate accumulation in the nucleus, which is dependent on the interaction of TERT with 14-3-3 family of proteins (Seimiya et al., 2000).

The cell-cycle progression in *P. falciparum* is not yet completely understood (Arnot and Gull, 1998). DNA synthesis begins in relatively small trophozoites, but nuclear subdivision, which leads to the formation of multinucleate cells, occurs only during schizogony. Not all nuclei progress through the cell cycle in a co-ordinated manner (Read et al., 1993), which means that a variable number of rounds of DNA synthesis and mitosis occur within each schizont. Whether or not any gap phases (G) exist between each round of DNA synthesis and mitosis phase is unknown. Finally, the schizont composed of 8-30 nuclei undergoes segmentation, which culminates with the formation of individual merozoites. Our observations with the anti-PfTERT sera seem to indicate that Plasmodial telomerase is not detectable in ring forms, but when the synthesis of DNA begins, a single discrete signal appears at the nuclear periphery. Given that multiple rounds of DNA synthesis occur before schizont segmentation, it is not surprising to detect the presence of telomerase throughout trophozoite and schizont stages. What is unexpected is the localisation of PfTERT in a discrete peripheral region of the nucleus. We need to investigate whether ths compartment corresponds to telomerase place of action, or if it is just a resevoir for the catalytic component of telomerase. The presence of a distinct intense signal after mitosis has been completed and merozoites have completely formed remains a mystery. More thorough studies will be necessary to correlate the localisation pattern with the cell cycle and timing of telomere replication.

In this work, we have identified by homology to PfTERT other putative telomerase reverse-transcriptases from the genome databases of *P. yoelii*, *P. berghei* and *P. chabaudi*. We do not retrieve the entire sequences for any of these three rodent malaria species. Nevertheless, motifs characteristic of telomerase and revere-transcriptases were found, as well as a few amino acids known to be specific to TERTs (such as a conserved Arg in motif 1). *PyTERT* gene, which we estimate is lacking around 300 bp at its 5' end including the ATG start codon, encodes a predicted protein of high molecular weight (~ 240 kDa) and pI 10.0, and contains general features of TERT proteins. TERTs among the *Plasmodia* genus are highly conserved. The sequences from rodent malarias share more than 70% amino acid identity and a lower homology to PfTERT, which is consistent with previous phylogenic analysis based on comparison of the small-subunit ribosomal RNA gene sequences (Waters et al., 1991). It is worth noting that the divergence of sequences between the human and rodent TERTs is not only due to amino acid substitutions, but also to stretches of amino acids present in *P. falciparum* TERT that are absent in other species. We do not know if these peptide "insertions" play important roles, but given that they are scattered throughout the PfTERT sequence, it is unlikely that they define a novel protein domain.

The identification of rodent TERTs is extremely important for pre-clinical trials of drugs designed to block TERT activity.

In fact, it has already been shown that *P. falciparum* telomerase activity can be efficiently inhibited *in vitro* by reverse-transcriptase drugs, such as nucleoside analogues (Bottius et al., 1998). These same drugs are currently being tested on *in vitro* cultures and preliminary data indicate that some are lethal to *P. falciparum* parasites (data not shown). If telomerase is validated as a good drug-target in culture, these drugs will subsequently have to be tested on mice and monkeys. For this purpose, characterisation of rodent TERTs will be essential.

Attempts to obtain *PfTERT* knock-out parasites by gene-interruption with a vector construction containing a positive selective marker (DHFR) and *PfTERT* sequence derived from the 5'-end of this gene has not been successful in our hands. In other organisms, in which telomerase knock-outs were generated, authors have observed a progressive shortening of telomere length and a decline of cell growth capability. In yeast, post-senescence survivors appear after more than 100 generations. In these telomerase knock-outs, telomeres are maintained in a telomerase-independent manner and cells grow normally (Lundblad and Blackburn, 1993), (Singer and Gottschling, 1994), (Lendvay et al., 1996), (Nakamura et al., 1998). In *P. falciparum*, time of selection for drug resistance takes several weeks. After this time, we did not detect any parasites containing the telomerase-gene disrupted, which could suggest that telomerase is essential for the parasite viability. But we cannot exclude the possibility that the gene-disruption failed due to a problem in the targeting of plasmid to this particular chromosome locus. The identification of Plasmodial telomerase gene opens new avenues that might lead to new intervention strategies against this important human pathogen responsible for 1-2 million deaths every year.

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RESULTS IV

Organisation of Subtelomeres in Human and Rodent Malaria Species

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(Preliminary data)

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The majority of eukaryotic chromosome ends comprise a specialised nucleoprotein structure called the telomere and an adjacent region called the subtelomere, or Telomere Associated Sequences (TAS). TAS are extremely polymorphic in size and are usually composed of a mosaic of repetitive blocks. Each block is usually highly variable in location and copy number. Despite such variability, the overall DNA organisation of subtelomeric region is generally conserved within a species.

The role of subtelomeric regions in different organisms is still a matter of debate. It was hypothesised that subtelomeres act as a buffer in telomere silencing events (Levis *et al.*, 1985), (Gottschling *et al.*, 1990), (Nimmo *et al.*, 1994). In yeast, some genetic elements of subtelomeres are implicated in the heterochromatin-like structure presented by chromosome ends (Pryde and Louis, 1999). The high degree of homology between subtelomeric regions of different chromosomes was shown to be important in the maintenance of telomeres, when telomerase is disrupted in yeast (Lundblad and Szostak, 1989), (Teng and Zakian, 1999) and humans (Dunham *et al.*, 2000). Recent studies in *Plasmodium falciparum* support a role for the subtelomeric region in cluster arrangement of chromosome ends (Figueiredo *et al.*, 2002), (O'Donnell *et al.*, 2002).

Data released from Plasmodial genome sequencing projects has revealed that the different Plasmodia species display a high degree of sequence conservation and gene synteny at internal domains of the chromosomes (Carlton *et al.*, 1998). However, cross-hybridisation studies indicated that subtelomeres present each a species-specific organisation. Previous studies have revealed how TAS are organised of in the majority of chromosomes in *P. falciparum* (Figueiredo *et al.*, 2000) and *P. berghei* (Pace *et al.*, 1987), (Dore *et al.*, 1990). The organisation of TAS in *P. falciparum* is highly conserved among the eight chromosome ends analysed. Upstream of *P. falciparum* telomeres, lies a non-coding region of 20-40kb composed of a mosaic of six different polymorphic repetitive elements (called Telomere-Associated Repetitive Elements: TAREs1-6). As observed in yeast and humans (Mefford *et al.*, 2002), in the vicinity of *P. falciparum* subtelomeric repeats, one can find members of multigene-families. The first gene upstream from TARE6 (or rep20) is a member of the *var* gene family, which code for virulence factors (Rubio *et al.*, 1996), (Hernandez-Rivas *et al.*, 1997). The *var* gene is followed by other multigene-families such as *rif, stevor* and *Pf60.1* (Gardner *et al.*, 1998), (Bowman *et al.*, 1999) (Fig. 1 for review).

In *P. berghei* subtelomeric regions consist of a tandem array of a 2.3 kb unit, clustered exclusively in a subtelomeric position of several chromosomes, but not all (Dore *et al.*, 1990). The 2.3kb unit includes ~160bp stretch of telomere-related sequences, which appear to be important for recombinational events that contribute to chromosome-size polymorphism (Pace *et al.*, 1990). No gene-families have been described so far adjacent to these subtelomeric repeats (Fig. 1 for review).

In this work, we took advantage of the progress of malaria sequencing projects, to determine and compare the DNA organisation of TAS in different Plasmodia species.

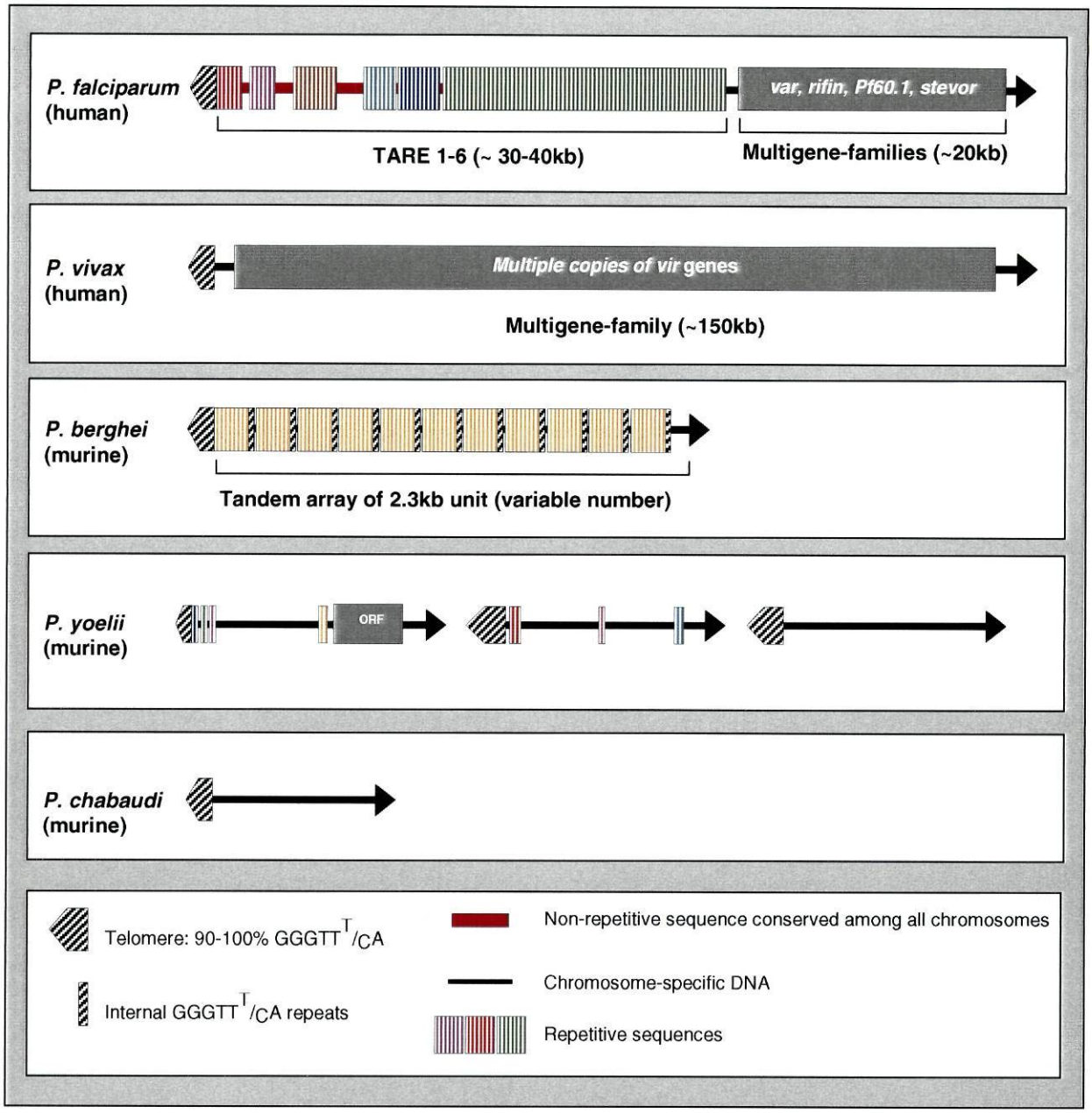


Figure 1. Model showing the organisation of subtelomeres in several *Plasmodium* species. The *Plasmodium* haploid nuclear genome is organised in 14 linear chromosomes. Each is composed of an internal region, where housekeeping genes are located, and a chromosome end. Adjacent to *P. falciparum* telomeres, there is a non-coding region that contains six TAREs, always positioned in the same order but of variable length. Upstream from TARE6 there is a locus for several gene-families that encode important virulence factors, such as the *var, rifin, Pf60.1* and *stevor* genes. In *P. vivax*, another human malaria parasite, only a YAC clone contains terminal telomeric repeats. In this sequence, the subtelomeric region does not contain any repetitive motifs. Instead, members of a large gene-family, *vir*, locate at just ~1 kb from telomeric repeats. The majority of TAS from *P. berghei* consist of a tandem array of 2.3 kb unit, in which 160 bp are telomeric repeats. No gene-families have been described at *P. berghei* subtelomeric locations. *P. yoelii* subtelomeres are highly divergent from chromosome to chromosome. Some TAS contain short repetitive motifs, other TAS present other motifs. But the majority of *P. yoelii* TAS analysed do not present any repetitive motifs. Several subtelomeric ORFsin *P. yoelii* belong to a novel putative gene family. Finally in *P. chabaudi*, the five contigs analysed did not reveal the presence of any repetitive motifs.

P. falciparum subtelomeres

The DNA organisation of *P. falciparum* subtelomeric regions in six TAREs has been proposed based on the analysis of eight subtelomeric contigs (Figueiredo *et al.*, 2000). Given the progress of the *P. falciparum* sequencing project, we were now able to study ten more subtelomeric contigs. We have now covered the subtelomeric regions from the following chromosomes: 1-4, 9-12 and 14 (both arms), 5 and 13 (only one arm). All TAS display the general organisation described previously: six different repetitive motifs, always in the same order. Only two exceptions were found; arm "b" of chromosome 1 lacks TARE1-3 and arm "a" of chromosome 9 lacks TARE1.

	Chromosome Arm "a"			Chromosome Arm "b"		
Chr.	TARE1-5 (kb)	TARE -6 (kb)	TAREs missing ?	TARE 1-5 (kb)	TARE -6 (kb)	TAREs Missing ?
1	10.8	15.7	NO	10.5	20	1, 2, 3
2	11,0	10	NO	11,8	8,4	NO
3	12,1	9,8	NO	10,4	21	NO
4	10	16	NO	10.5	6	NO
5	12	6	NO	NA	NA	NA
BLOB	NA	NA	NA	NA	NA	NA
9	10	6	1	10	25	NO
10	10	14	NO	10	> 7.6	NO
11	10	23	NO	11.5	8.7	NO
12	10	Inc.	Inc.	Inc.	9	Inc.
13	11	6.5	NO	NA	NA	NA
14	11	6.5	NO	10	Inc.	Inc.-

Table 1. Variability in the size of *P. falciparum* subtelomeric regions. The domain spanning TARE1-5 is less polymorphic in size (~10 kb) than TARE6. Alone, TARE6, or rep20, varies from 6 to 23 kb. In all subtelomeric contigs, TARE1-6 are present in the same order. Two contigs lack some TAREs: arm "b" of chromosome 1 and arm "a" of chromosome 9. Inc.: "incomplete" sequencing, i.e. there are no contigs spanning the entire subtelomeric region. NA: no available contigs.

These shorter TAS might be the result of breakage and healing events, as observed in strains that are cultivated for some time in culture (Scherf and Mattei, 1992). Overall, the subtelomeric region covering TAREs1-5 varies from 10-12 kb. TARE6 (rep20) alone is much more polymorphic in size, varying from 6 to 23 kb (Table 1).

P. vivax subtelomeres

At present, *Plasmodium vivax* genome database contains a single hit for telomeric DNA: a YAC clone constructed by del Portillo *et al.* (del Portillo *et al.*, 2001). In contrast to *P. falciparum* and *P. berghei*, no repetitive blocks exist in this *P. vivax* subtelomere (Fig.1). Instead, at ~1kb from telomere repeats there is an ordered array of members of the *vir* gene-family. They are present at about 600-1000 copies per haploid genome and encode proteins that are immunovariant in natural infections.

P. yoelii subtelomeres

Analysis of the DNA sequence data available in the *P. yoelii* genome database suggests that subtelomere organisation is distinct among heterologous chromosomes. For example, some chromosome ends present short repetitive blocks (<1kb), but the type and number of these is highly variable. Some subtelomeres do not present any repetitive motifs (Fig. 2). In some of the chromosome ends analysed, we found novel putative genes, members of a large gene-family (at least 50 distinct hits in the *P. yoelii* database) coding for putative small size proteins (22-35kDa). Alignment of some of the members of this family reveals a great conservation of the N and C-termini (Fig. 3). This adds another subtelomeric gene-family to the one described recently, the Py235 gene-family (Khan *et al.*, 2001).

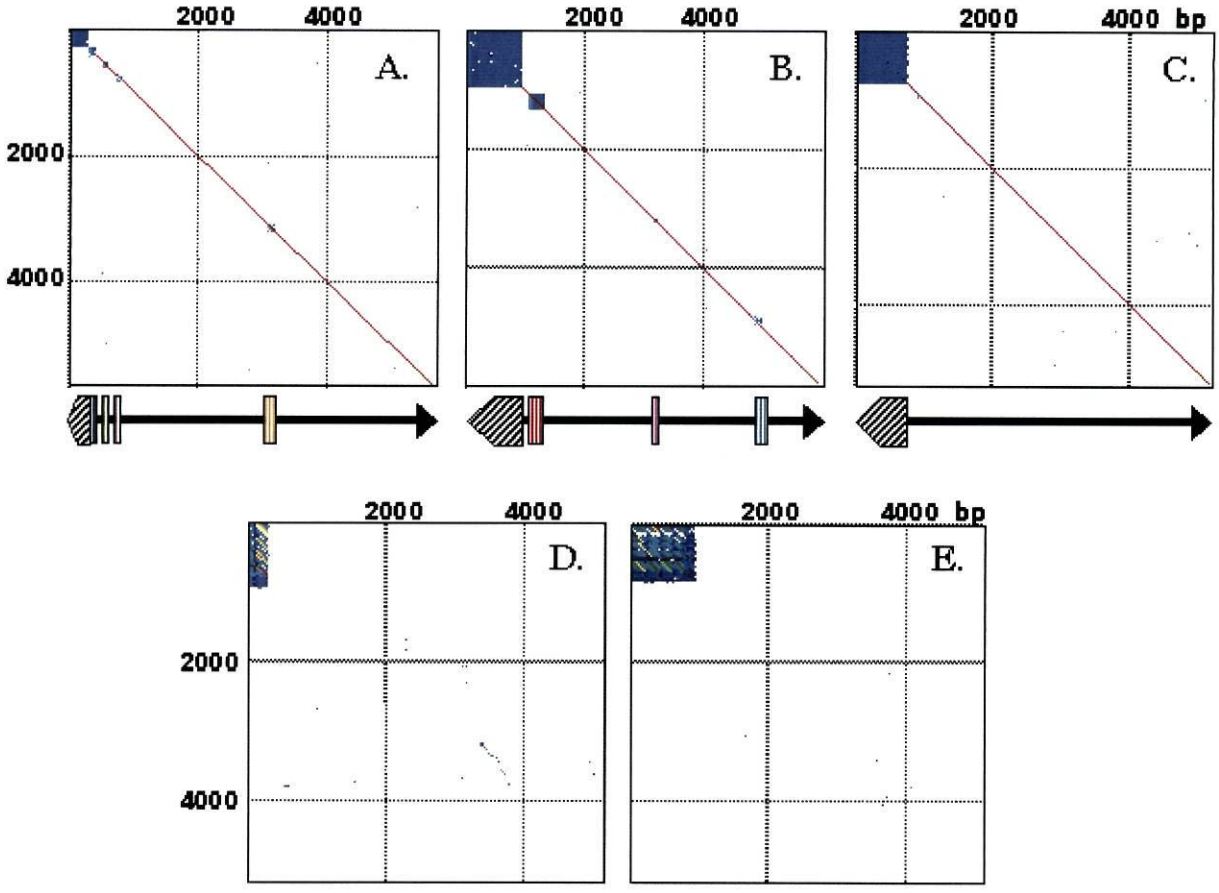


Figure 2. Example of Dot-Plot analysis of *P. yoelii* subtelomeric contigs. Genome database of *Plasmodium* species were interrogated though PlasmoDB (www.plasmodb) or NCBI-CustomBlast (http://www.ncbi.nlm.nih.gov/Malaria/plasmodiumblcus.html) websites using as a query telomeric repeats. Only contigs containing more than ten terminal telomeric repeats followed by a non-telomeric sequence larger than 4 kb were considered subtelomeric-originating sequences. Using the Lasergene software, DNASTAR, with the following settings: 75% match and a 30-bp window size, each sequence was Dot-Plotted against itself to identify repetitive sequence blocks and against the other contigs to check the degree of homology and their relative position. Each blue square on the diagonal of the diagram represents a block of repetitive, tandemly repeated sequences. The red diagonal means identical sequences. Plots A, B, and C are self-plots of three different subtelomeric contigs (called A, B and C respectively). Short repetitive elements can be observed in A and B but not in C. D is a plot of contig A against B. The only common element between the two sequences are the telomeric repeats. Plot of B against C, is shown in E. There are no unique subtelomeric elements conserved among the two subtelomeres.

P. chabaudi subtelomeres

At this stage, *P. chabaudi* genome project is not as advanced as the other rodent malaria species. Thus, only 3 hits were found containing telomere repeats and their size was not larger then 5kb. Sequence analysis did not reveal any open-reading frames, nor repetitive elements upstream (up to 5 kb) from telomere repeats (Fig. 2).

In the two rodent species *Plasmodium yoelii* and *Plasmodium chabaudi*, like in *P. falciparum*, telomeric repeats are degenerate. Telomeric DNA is composed approx. of 90% GGGTT(T/C)A repeats (the remaining consist of other short G-rich repeats).

Although considerable progress has been made in this last two years, the complete genomes of Plasmodium species are still not available. Therefore, we cannot exclude the possibility that some not yet sequenced subtelomeres present a different organisation from the pattern we described in this work. Nevertheless, the comparative analysis of the DNA organisation of Plasmodial subtelomeric regions allow us to take a number of important conclusions: 1. Each *Plasmodium* species analysed (except for *P. vivax*) contains distinct non-coding sequence elements adjacent to the same type of telomere repeats. 2. In *P. falciparum*, a conserved higher order structure is found on virtually all chromosome ends. This contrasts with *P. yoelii*, which displays distinct elements adjacent to the telomeres of different chromosomes. 3. Chromosome ends harbour generally highly diverse gene-families.

Plasmodial subtelomeres follow therefore the general organisation of other eukaryotic subtelomeres, given the presence of variable size repetitive motifs. *P. falciparum* TAS show however unique features. The DNA organisation in six distinct repetitive elements (TARE1-6) seems to be a trait of all chromosome ends. The same motifs are always positioned in the same order. Moreover, the inter-motif sequences are almost identical among heterologous chromosomes (Figueiredo *et al.*, 2000). As far as we know, such a strict conserved higher-order structure of the subtelomeric regions has never been observed in other organisms, suggesting that *P. falciparum* TAS have acquired crucial functions. We have previously shown that TAS are implicated in clustering of chromosome ends (Figueiredo *et al.*, 2002), which facilitates enhanced ectopic recombination among subtelomeric gene families (Freitas-Junior *et al.*, 2000). Clustering of chromosome ends might also have a role in epigenetic regulation of the expression of those genes. In yeast, Telomere Positiong-Effect (TPE) is responsible for the silencing of genes placed at subtelomeric locations. This effect is mediated through a heterochomatin-like structure that begins at the telomeres and is extended over the subtelomeric region. In *P. falciparum*, we can envision that the epigenetic control of subtelomeric gene-families may be mediated by a TPE-like mechanism.

Acknowledgments

We thank J. Lowell for helpful comments and discussion. This work was supported by an EC grant QLK-CT-2000-00109, a grant VIH-PAL from the French Ministry and by the fellowship from Fundação para a Ciência e a Tecnologia, PRAXIS XXI/BD/16020/98, Portugal (L.M.F.). *P. vivax* YAC data was obtained from the Sequencing Group at the Sanger Institute and can be retrieved from ftp://ftp.sanger.ac.uk/pub/pathogens/vivax. *P. berghei* sequence data was obtained from the University of Florida, Malaria Genome Tag Sequencing Project (Carlton and Dame, 2000). Data produced by the *P. chabaudi* Partial Genome Shotgun Sequencing Group at the Sanger Institute can be obtained from ftp://ftp.sanger.ac.uk/pub/pathogens/chabaudi. Preliminary sequence and/or preliminary annotated sequence data from the *P. yoelii* genome was obtained from The Institute for Genomic Research website (www.tigr.org). We wish to thank the scientists and funding agencies comprising the international Malaria Genome Project for making sequence data from the genome of *P. falciparum* (3D7) public prior to publication of the completed sequence. The Sanger Centre (UK) provided sequence for chromosomes 1, 3-9, & 13, with financial support from the Wellcome

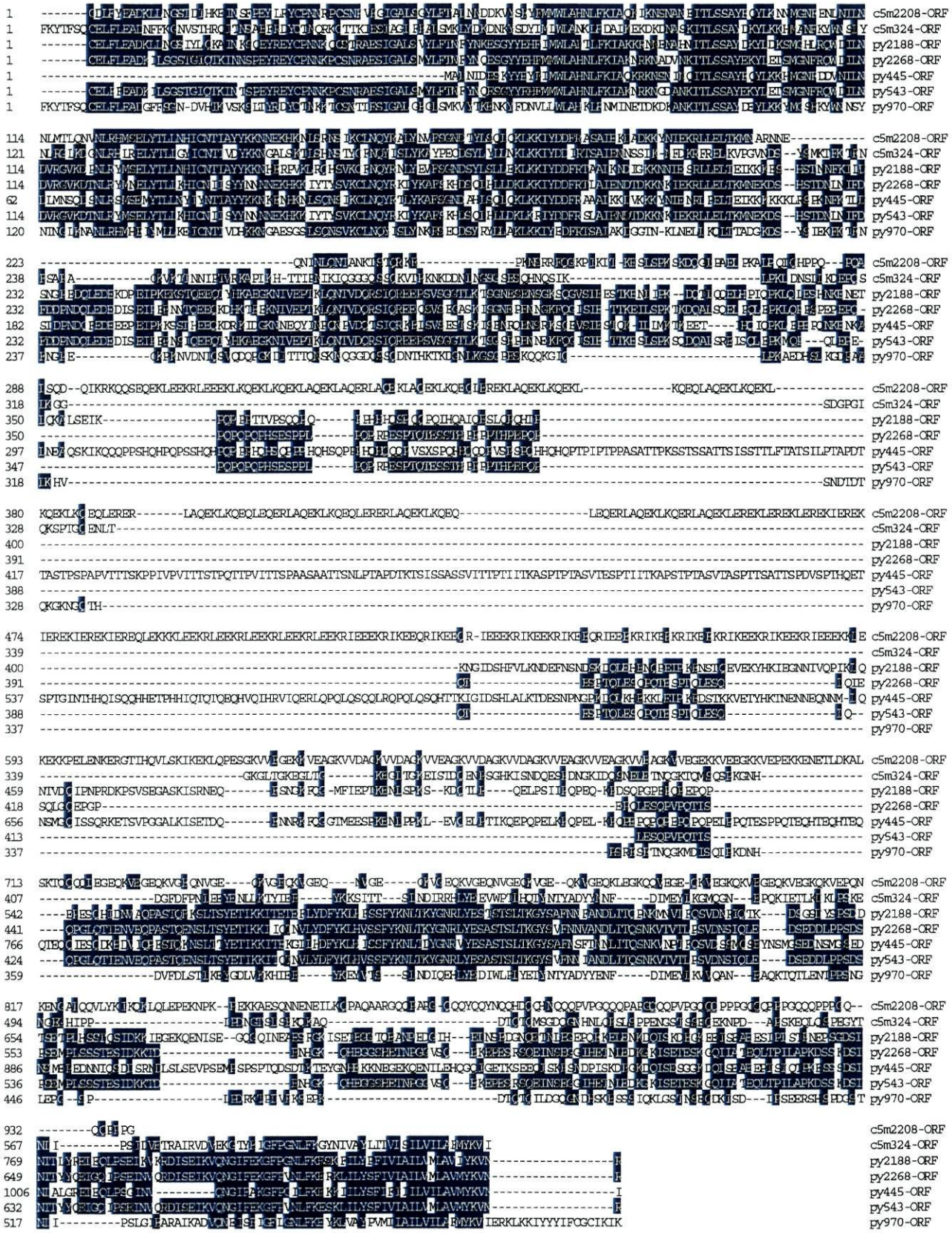


Figure 3. Alignment of seven subtelomeric members of a newly identified *P. yoelii* gene-family. It is at the N and C-termini that the highest amino acid identity is detected. The sequences were aligned with the CLUSTALW algorithm, with the following parameters: Gap Penalty 10 and Gap Length Penalty 10.

Trust. A consortium composed of The Institute for Genome Research, along with the Naval Medical Research Center (USA), sequenced chromosomes 2, 10, 11 & 14, with support from NIAID/NIH, the Burroughs Wellcome Fund, and the Department of Defense. The Stanford Genome Technology Center (USA) sequenced chromosome 12, with support from the Burroughs Wellcome Fund.

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REVIEW ARTICLES

In this chapter, I have included two review articles written by the Scherf laboratory and in which I participated.

The first work was published on Current Opinion in Microbiology (2001) and summarises our understanding of telomeres from malaria species, with an emphasis placed on *P. falciparum*.

The second work is a chapter of the book "Genomes and the Molecular Cell Biology of Malaria Parasites" (2002). Here, we describe the conserved higher-order structure of chromosome ends and their positioning in the nuclear space, focusing on the relationship between subtelomeric regions and the multigene families that are contained within these regions.

REVISION WORK I

Plasmodium Telomeres: a Pathogen's Perspective

Artur Scherf, Luísa M Figueiredo, Lúcio H Freitas-Junior

Review

in

Current Opinion in Microbiology 4: 409-414 (2001)

Plasmodium telomeres: a pathogen's perspective

Artur Scherf*, Luisa M Figueiredo and Lúcio H Freitas-Junior

New data on the organization of plasmodial telomeres has recently become available. Telomeres form clusters of four to seven heterologous chromosome ends at the nuclear periphery in asexual and sexual parasite stages. This subnuclear compartment promotes gene conversion between members of subtelomeric virulence factor genes in heterologous chromosomes resulting in diversity of antigenic and adhesive phenotypes. This has important implications for parasite survival.

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Abbreviations

FISH	fluorescent *in situ* hybridization
t-loop	telomere loop
TARE	telomere-associated repetitive element
TAS	telomere-associated sequence

Introduction

Linear eukaryotic chromosomes present three problems that do not exist in circular genomes. First, chromosome extremities need to be protected from degradation. Second, they have to avoid end-to-end fusion by DNA repair mechanisms. Third, they have to compensate for the gradual loss of DNA. How do eukaryotic organisms solve these problems? The answer lies in a specialized structure, found at the physical termini of chromosomes, called the telomere. For the vast majority of eukaryotes, telomeres consist of G-rich repetitive DNA and specific associated proteins. A variety of other functions have been assigned to telomeres, such as transcriptional silencing [1], chromosome positioning in the nucleus [2] and homologous and ectopic (non-allelic) recombination during meiosis (for reviews, see [3,4]).

Human malaria is re-emerging as the world's most lethal infection, affecting 300 million people and killing 2–3 million people every year. The disease is caused by the protozoan pathogen *Plasmodium*, of which *Plasmodium falciparum* is the most virulent. In this review, we summarize present knowledge of *P. falciparum* telomere biology and describe relevant aspects associated with chromosome extremities.

Plasmodium chromosome extremities: not just the end

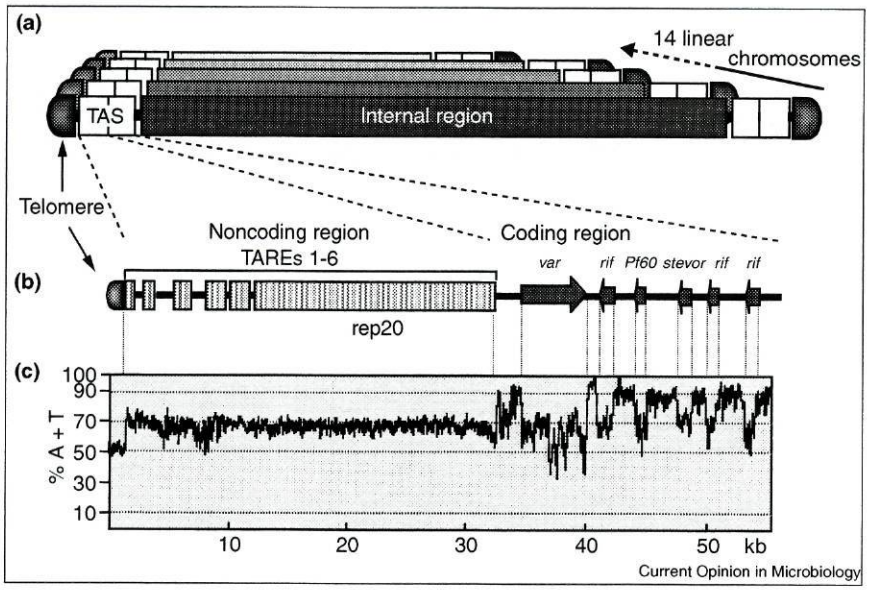
The extremely AT-rich nuclear genome of *P. falciparum* (~80% AT) is organized into 14 linear chromosomes. Telomeric DNA has been cloned from several malaria parasites and found to consist of degenerate canonical G-rich tandem repeats, with GGGTT(T/C)A being the most frequent motif [5–7]. The mean length of the telomeric array shows significant interspecies variation (for example, 1.2 kb for *P. falciparum* and 6.7 kb for *Plasmodium vivax*), but is relatively conserved between *P. falciparum* strains (LM Figueiredo, LH Freitas-Junior, E Bottius, A Scherf, unpublished data).

The terminal tract of *P. falciparum* telomeric DNA is assembled into a non-nucleosomal chromatin structure, called the telosome [8], as has been described in *Saccharomyces cerevisiae*, human and *Trypanosoma cruzi* telomeres [9,10,11••]. Proteins of the telosome complex mediate specific properties associated with telomeres. For example, a telomere structure has been reported for humans, ciliates and African trypanosomes, and found to form a terminal telomere loop (t-loop) at the chromosome end [12••,13,14]. T-loops are thought to be formed by invasion of the 3′ telomeric overhang into the duplex telomeric repeat array. It has been proposed that this structure protects chromosomal termini from degradation and recognition as broken ends. On the other hand, the short telomere tracts of yeast (around 0.3 kb) seem to fold back and interact with an adjacent region via specific protein–protein interactions, establishing a heterochromatin-like structure that prevents telomere elongation and reversibly represses transcription of subtelomeric genes [15,16••]. The formation of such telomere loops in *P. falciparum* could contribute to the epigenetic regulation of adjacent genes.

Most models for telomere length regulation take into account mechanisms that act *in trans* on each telomere via specific telomere-binding proteins (for a review, see [17•]). In a recent study, it was shown that *P. falciparum* chromosome ends that have lost the telomere-associated sequences (TASs) harbour longer telomeres (an increase in length of up to threefold), suggesting that telomere maintenance mechanisms in *P. falciparum* are sensitive to the genomic environment upstream of the telomere. This is the first evidence to suggest that telomere length regulation depends not only on *trans*-acting factors, but also on the genomic environment adjacent to the telomere (LM Figueiredo, LH Freitas-Junior, E Bottius, A Scherf, unpublished data).

Telomere repeat similarity has been shown in several malaria species by DNA cross-hybridization [18]. Additionally, internal chromosome regions appear to display a high degree of gene synteny in plasmodial species [19]. This is in striking contrast to the TASs, which are highly species-specific in their organisation. *P. falciparum* TASs consist of both a coding and a noncoding region (Figure 1a,b). The noncoding regions are adjacent to the telomere repeats and are composed of a mosaic of six different polymorphic repetitive elements called telomere-associated repetitive

Figure 1



A model of *P. falciparum* chromosome organisation. **(a)** The *P. falciparum* haploid nuclear genome is organized into 14 linear chromosomes. Each chromosome is composed of an internal region, in which housekeeping genes are located, and a chromosome end, displaying a higher-order DNA organisation common to all chromosomes. **(b)** The terminus of the left arm of chromosome 3 is schematically represented as an example of a chromosome end organisation. Just upstream of telomeres, there is a highly polymorphic TAS, composed of two zones: a noncoding and a coding region. The noncoding region contains six TAREs, always positioned in the same order but of variable length. TARE-6 (rep20) is composed of a repetitive unit of 20 bp. It spans a region of variable length (8–20 kb), being responsible for most of the length polymorphism observed at the noncoding region of TAS. The coding region is the locus of several gene families encoding important virulence factors, such as the *var* and *rifin* genes. **(c)** AT-content of a chromosome end. Telomeres and noncoding TASs are unusually rich in GC. The diagrams and graph are drawn to scale, except for the telomeres in (a).

elements (TAREs 1–6), which are always found at the chromosome ends in the same order and span 20–40 kb [8]. TARE-6, which is also known as rep20, is responsible for most of the chromosome-end size polymorphism. However, large subtelomeric deletions, which occur relatively frequently in laboratory-cultured strains and have been observed in clinical isolates, generate viable truncated chromosomes with no TAREs [20]. Surprisingly, noncoding TASs are as AT-rich as coding regions (70% AT) (Figure 1c), instead of as rich as noncoding regions (>90% AT). The noncoding region of the TAS may have evolved from coding regions, as has been proposed for isochores in vertebrates [21]. We cannot, however, discard the possibility that the maintenance of such a relatively G-rich noncoding region results from functional constraints, as is probably the case for telomere repeat sequences.

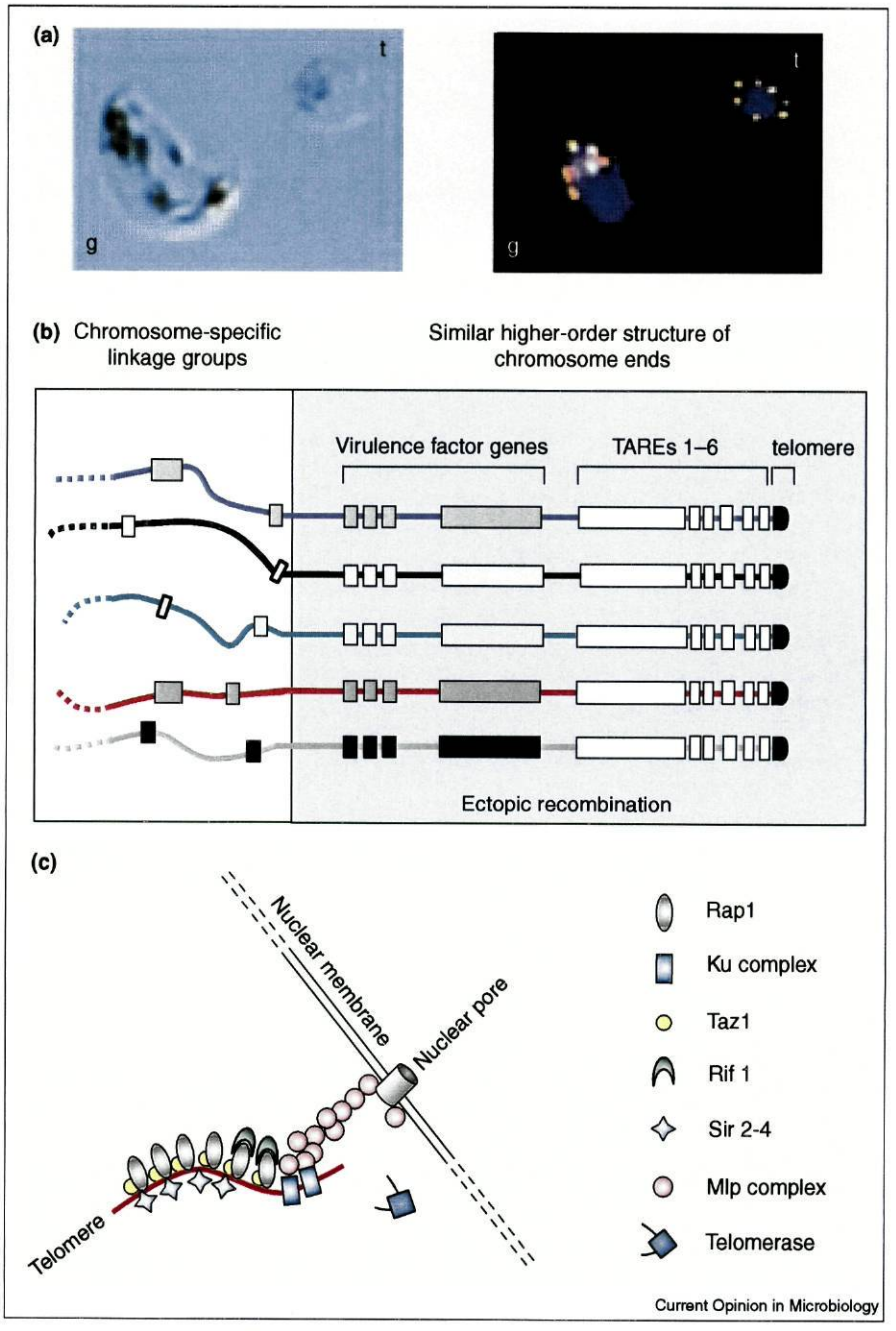
The species-specificity of the subtelomeric compartment is not limited to the noncoding region of the TAS. Systematic sequence analysis of several *P. falciparum* chromosomes has revealed that gene families coding for species-specific virulence factors involved in antigenic variation (*var* and *rifin* gene families) and cytoadhesion (*var* gene family) are found adjacent to the noncoding region [22,23]. Undoubtedly, a number of genes implicated in parasite–host-specific molecular interactions have evolved in this chromosome compartment .

Telomeres – are they the 'Achilles heel' of *Plasmodium*?

In single-celled organisms, telomere length needs to be maintained within a minimal size range to ensure the survival of the cell. However, because of the incomplete replication of linear chromosomes, there is a net loss of telomeric DNA with each successive cell division. To compensate for this loss, new telomeric repeats are added onto chromosome ends in a reaction catalysed by a specialized reverse transcriptase called telomerase. Telomerase is a holoenzyme, in which the catalytic core is composed of a protein, called TERT, and an RNA component. Telomerase RNA serves as the template for the addition of telomeric repeats. Interaction with other proteins may be important for the regulation of telomerase activity (for review, see [17•]).

In the absence of telomerase activity, telomeres shrink with each replication cycle and the erosion is so extensive (approximately 50 to 200 bp per replication in human somatic cells) that, with time, the telomere capping function becomes severely compromised, leading to growth arrest and, eventually, senescence [24]. The mean telomere length of *P. falciparum* is maintained at a constant average size during blood-stage proliferation, and telomerase activity has been detected in protein extracts of cells at this stage [25•]. Telomerase activity has also been identified in three kinetoplastid species: *Trypanosoma brucei*, *Leishmania major* and *Leishmania tarentolae* [26]. Drugs that interfere with telomere maintenance may induce fatal shortening of telomeres, leading to the death of the parasite. Thus, telomeres can be considered as the 'Achilles heel' of highly proliferating protozoan pathogens. However, in the absence of telomerase, it is possible that parasites may make use of alternative mechanisms to maintain a functional telomere. Recombination-mediated telomere maintenance has been described in yeast [27–29••], and chromosome

Figure 2



Nuclear architecture of *P. falciparum* chromosome ends. **(a)** FISH analysis of a telomere-specific DNA probe (rep20) showing that the chromosome termini form clusters at the nuclear periphery during trophozoite asexual blood stages (t), and form a bouquet-like structure at the pre-meiotic gametocytes sexual blood stage (g). The left-hand panel shows an image obtained by Nomarski interference contrast of free parasites. The right-hand panel shows parasite nuclei stained with DAPI (in blue) and telomere clusters (in yellow). **(b)** A schematic model of a *P. falciparum* telomere cluster is shown to illustrate the physical alignment of heterologous subtelomeric regions in which increased rates of recombination occur. **(c)** Hypothetical model of telomere-associated proteins in *P. falciparum*. Orthologues to *S. cerevisiae* (Rap1, Ku complex, Rif1, Sir 2-4, Mlp complex and telomerase) and *S. pombe* (Taz1) telomere-associated proteins detected in the *P. falciparum* genome database are shown. Potential protein–telomere or protein–protein interactions are shown in analogy to data established for yeast [32], (for details, see also: http://www.proteome.com).

circularization has been observed in *S. pombe* [29]. A candidate for the *P. falciparum* telomerase catalytic subunit has been identified in the genome database. Knockout experiments are in process to verify that this is an essential gene for the survival of the parasite and that it can be considered as a new target for the development of antiparasite therapies (LM Figueiredo, A Scherf, unpublished data). Telomerase has also been considered an attractive target for cancer therapeutics, as more than 90% of tumors show telomerase activity. Inhibition of telomerase activity in human cancer cells arrests their growth *in vitro* and *in vivo* [24].

The role of telomeres in the nuclear architecture

Recent technological advances such as FISH (fluorescent *in situ* hybridization) have provided new insights into the three-dimensional chromatin organization of the nucleus, even in relatively small organisms such as yeast and *Plasmodium*. Telomeres have been shown to form clusters and to anchor chromosomes to the nuclear periphery, thus forming distinct nuclear compartments in yeast. These nuclear subdomains are thought to have an important role in epigenetic gene regulation, DNA recombination and telomeric silencing (reviewed in [30,31,2]). The telomere position effect (TPE) brings about a heritable yet variegated

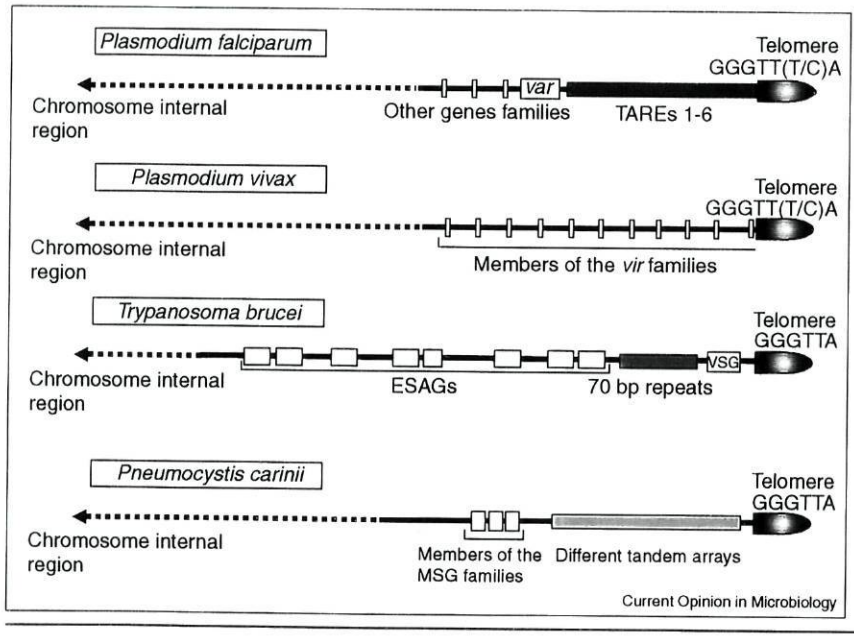
repression of genes placed near telomere repeats [1]. This effect has been associated with chromatin organization, as proteins essential for telomeric silencing are found to be concentrated at the telomeres and/or subtelomeric regions. In yeast, a number of telomere-associated proteins involved in telomere-length regulation, cluster formation, telomere anchoring to the periphery and gene silencing have been described and characterized [32]. *P. falciparum* orthologues to most yeast telomere-associated proteins have been identified (LH Freitas-Junior, A Scherf, unpublished data; for more details, see Figure 2c), implying that yeast can serve as a model for investigating the role of biologically relevant aspects in *P. falciparum*, such as the importance of virulence factor localization at chromosome ends.

FISH analysis using a chromosome-end-specific fluorescent probe recently demonstrated that the 28 chromosome ends of *P. falciparum* are not randomly distributed in the nucleus. The telomeres form physical associations demonstrated by four to seven spots at the nuclear periphery of asexual and sexual blood stage parasites, suggesting that each cluster contains between four and seven distinct chromosome ends (Figure 2a) [33]. The molecular components involved in cluster formation are as yet unknown. The extent of chromosome-end alignment is also unclear. However, several lines of evidence support the idea that it spreads beyond the telomere repeats by a distance of 30 to 40 kb, including the subtelomeric region that encodes variant-surface-antigen families. DNA sequences in this region undergo ectopic (non-allelic) recombination at a much higher rate than expected for homologous recombination [34]. We assume that the physical alignment of heterologous chromosome ends brings together homologous sequences from different chromosomes allowing efficient DNA recombination (Figure 2b). Also, it was recently shown that the *P. falciparum* subtelomeric region is crucial for the maintenance of chromosome termini in clusters (LM Figueiredo, LH Freitas-Junior, E Bottins, A Scherf, unpublished data). In parasites that have lost the subtelomeric domain because of spontaneous chromosome breakage, the large majority of chromosome ends are not associated with clusters. We speculate that specific TAS-binding proteins exist that crosslink chromosome termini and stabilize the cluster. Thus, deletion of chromosomal TAS would explain the observed dissociation of truncated chromosome termini from the cluster. We predict that chromosome ends not associated with clusters have a significantly lower frequency of ectopic recombination between subtelomeric gene families than do normal chromosome ends. This hypothesis is supported by recent reports showing that mutant fission yeast with defective telomere cluster formation have reduced meiotic recombination between chromosome termini [35], and ectopic recombination is no longer restricted to telomere-associated regions [36••].

The subtelomeric region and antigenic variation

What are the biological implications of the *P. falciparum* nuclear organization on genes located close to telomeres? It is now well established that plasmodial chromosome ends form a highly dynamic chromosome compartment and most of the chromosome polymorphism is due to DNA rearrangements occurring in the subtelomeric region. The high recombination frequencies observed in subtelomeric regions seem to create an environment that allows the expansion and diversification of gene families located at chromosome ends [37]. Thus, telomeres provide an ideal setting for genes involved in mechanisms such as antigenic variation. In *P. falciparum*, antigenic variation and cytoadhesion

Figure 3



Model showing the subtelomeric organisation in several human pathogens. The gene families that express variant cell-surface molecules on *T. brucei* [39] and *P. carnii* [43], or on the surface of infected erythrocytes in *P. falciparum* [22,23] and *P. vivax* [7] are indicated. The *P. vivax* chromosome end model has been obtained from the sequence analysis of a telomeric yeast artificial chromosome (YAC) clone. ESAG, expression-site-associated gene; MSG, major surface glycoprotein; VSG, variant surface glycoprotein.

are mediated by the *var* gene family, which consists of approximately 50 members per haploid genome. Most *var* genes are localized in the subtelomeric region of the chromosomes (Figure 1b) and expression of these genes occurs in a mutually exclusive manner. Programmed DNA rearrangement is not necessary to switch expression from one variant to another.

In a recent study, it was shown that chimeric *var* forms were created by gene conversion involving two *var* members located at different chromosome ends [33]. Thus, telomere clusters appear to provide a platform for ectopic recombination between virulence factors from different chromosomes. The continuous generation of variant-surface-antigen diversity is particularly important for *P. falciparum* [33,38] and for other pathogens whose survival in the vertebrate host depends on antigenic variation. This is demonstrated by the localization of variant-surface-molecule genes at subtelomeric chromosome regions in numerous human pathogens (Figure 3). *P. falciparum* contains a relatively small set of ~50 *var* genes, compared to other organisms that express variant surface antigens. African trypanosomes, for example contain around 1000 VSG genes [39] and *P. vivax* has between 600 and 1000 *vir* genes [7]. Nevertheless, *P. falciparum* populations harbor many *var* forms, whose diversity and continual renewal support the success of this species against host immunity. We speculate that parasite isolates carrying different *var* repertoires are more likely to initiate new infections than isolates that have previously infected the same human host.

Telomeric location of variant gene families may provide a special chromatin environment for reversible, mutually exclusive gene regulation. The mechanisms that control the regulation of *var* and *rifin* gene expression in *P. falciparum* are still unresolved, but many lines of evidence point to epigenetic factors being critically involved in the transcription of a single *var* gene family member at a time [40,41]. In analogy to the yeast system, we suggest that *P. falciparum* telomeres could mediate reversible silencing of *var* genes. The fact that *P. falciparum* telomeres closely resemble yeast telomere organization should facilitate the identification of the molecules that contribute to the control of epigenetic *var* gene transcription. However, further studies are needed to find a concept explaining how one subtelomeric *var* gene can be transcribed while the remaining members are repressed.

Conclusions

For many years, telomere biology was of interest to only a handful of specialists. Recently, the subject has gained a much broader interest, partly because of the fact that telomeres play a central role in cell senescence, and thus provide a new target for therapy development not only against human cancer but also against protozoan pathogens.

The recent discovery of telomere clustering at the nuclear periphery and its role in ectopic recombination suggests that *P. falciparum* subtelomeric regions define a specific subcompartment able to generate continual diversity in virulence factor genes. Other protozoan pathogens may follow the same blueprint. Another important feature of the telomere is its potential involvement in epigenetic gene regulation. The recent principles established for the telomere position effect in yeast [42•] may also be applicable to transcriptional control mechanisms of antigenic variation in *Plasmodium*, which remains, at the moment, enigmatic.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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REVISION WORK II

Chromosome Structure and Dynamics of *Plasmodium* Subtelomeres

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Book Chapter

in

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Abstract

Substantial gene synteny has been observed between malaria species. However, new data on the organization of plasmodial subtelomeres has recently become available and demonstrates that chromosome ends show, unlike the central region, a very dynamic evolution of its DNA sequence. Sequences of the subtelomere elements vary greatly among malaria species. Duplications among subtelomeres have created large families of expressed genes often encoding variable surface antigens. Fluorescence in situ hybridisation (FISH) combined with three-dimensional microscopy has demonstrated that chromosome ends in Plasmodium are not randomly arranged in the nucleus. Telomeres form clusters of 4 to 7 heterologous chromosome ends and are associated with the nuclear periphery. The physical alignment of subtelomeres promotes frequent recombination between members of telomere-associated virulence factor genes in heterologous chromosomes. This has important implications for the parasite survival and its adaptation to environmental stress.

Introduction

Chromosome mapping studies and DNA sequence analysis has revealed that specific linkage groups are conserved between malaria species (Carlton *et al.*, 1998), (Tchavtchitch *et al.*, 2001). However this conserved location of homologous genes and non-coding DNA sequence elements appears to be confined to the central chromosome part, whereas the subtelomeres consist of sequences that are often not found in other malaria species (Pace *et al.*, 1987), (Dore *et al.*, 1990), (Figueiredo *et al.*, 2000). Here we summarise our knowledge of a dynamic chromosome compartment, the subtelomeres of *Plasmodium* and discuss relevant aspects for the biology of plasmodial virulence factor genes. We also highlight the impact of novel findings on the nuclear organisation of chromosome extremities for DNA recombination and the regulation of transcription of telomere-associated genes.

Functional consequences of nuclear organisation

The far-ends of eukaryotic chromosomes: structure and function

Systematic genome analysis has provided precious information on the sequence and localisation of genes, which has improved our understanding of basic biological processes and has revealed that a significant part of a genome consists of domains devoid of genes. The extremities of chromosomes are one of these domains. Chromosome ends comprise a specialised nucleoprotein structure called the telomere and the adjacent region is called subtelomere, or Telomere Associated Sequence (TAS). For the vast majority of eukaryotes, telomeres consist of a tandem array of short G-rich repeats and specific associated proteins. They are responsible for protecting chromosome

extremities from degradation, prevent end-to-end fusion by DNA repair mechanisms and solve the 'end replication problem' (the gradual loss of DNA at the chromosome end with each replication cycle) (for review see (McEachern *et al.*, 2000)). Telomeres are also implicated in transcriptional silencing (Gottschling *et al.*, 1990), chromosome positioning in the nucleus (Gotta *et al.*, 1996), as well as homologous and ectopic recombination during meiosis (for reviews see (Cooper, 2000) and (Ishikawa and Naito, 1999)).

Subtelomeric regions appear to be species-specific, extremely polymorphic in size and usually are composed of a mosaic of repetitive blocks. Each block is usually highly variable in location and copy number. In subtelomeres, it is common to find members of gene families, such as the human olfactory receptor gene family (Trask *et al.*, 1998). The role of subtelomeric regions in different organisms is still a matter of debate. It was hypothesised that subtelomeres act as a buffer in telomere silencing events (Levis *et al.*, 1985); (Gottschling *et al.*, 1990); (Nimmo *et al.*, 1994). More recently, it was shown that the high degree of homology between subtelomeric regions is important in the maintenance of telomeres, when telomerase is disrupted in yeast (Lundblad and Szostak, 1989), (Teng and Zakian, 1999) and humans (Dunham *et al.*, 2000). Recent studies in *Plasmodium falciparum* support a role for the subtelomeric region in cluster arrangement of chromosome ends (Figueiredo *et al.*, 2002), (O'Donnell *et al.*, 2002).

Plasmodial chromosome ends: a specific compartment for multigene families

All Plasmodial species display a canonical telomere structure: G-rich repeats in tandem arrays. Telomeric DNA was first cloned from *Plasmodium berghei* (Ponzi *et al.*, 1985) and later from *P. falciparum* (Vernick and McCutchan, 1988). It was shown to be a degenerate heptamere, in which GGGTT(T/C)A are the two type of repeats in *P. berghei* and the most frequent in ones observed in *P. falciparum* (including some more degenerate type of telomere repeats). Sequencing data from *Plasmodium yoelii, Plasmodium chabaudi* and *Plasmodium knowlesi* showed that telomeric DNA is composed approx. of 90% GGGTT(T/C)A repeats (approx. of 10% consists of degenerate G-rich repeats), which confirmed previous cross-hybridisation results obtained with *P. berghei* telomeric DNA (Dore *et al.*, 1986).

Data released from Plasmodial genome sequencing projects has revealed that the different Plasmodia species display a high degree of sequence conservation and gene synteny (Carlton *et al.*, 1998). Subtelomeres, however, diverge dramatically. Since the beginning of the Plasmodial spp genome project, our group has undertaken a broad approach, looking at the DNA composition of all available sequenced chromosome ends with the help of bioinformatic tools. In *P. falciparum* we observed a unique conservation of the higher-order structure of subtelomeres (Figueiredo *et al.*, 2000). Upstream of all sequenced *P. falciparum* telomeres (18 analysed till present), lies a non-coding region of 15-30 kb composed of a mosaic of six different polymorphic repetitive elements (Telomere-Associated Repetitive Elements: TAREs1-6) (see Fig. 1). In *P. falciparum*, four

subtelomere blocks of complex repetitive DNA had previously been described (Oquendo *et al.*, 1986), (Vernick and McCutchan, 1988), (Dolan *et al.*, 1993), (Patarapotikul and Langsley, 1988). The six elements are present in all intact chromosomes and are always positioned in the same order, in contrast to previously described subtelomeres from other organisms that display higher variability (reviewed in (Scherf *et al.*, 2001), (Mefford and Trask, 2002). The AT content of this region is ~70%, which is unusually high for a non-coding region (90% AT), suggesting that subtelomeres might originate from coding regions. Towards the centromere, TAREs are flanked by members of the *var* gene family coding for virulence factors (Rubio *et al.*, 1996), (Hernandez-Rivas *et al.*, 1997). All *var* genes located next to the TARE6 repeated element are transcribed toward the centromere. The *var* gene is followed by other multigene families such as *rif, stevor* and *Pf60.1* (Gardner *et al.*, 1998), (Bowman *et al.*, 1999). Their precise role is described elsewhere in this book.

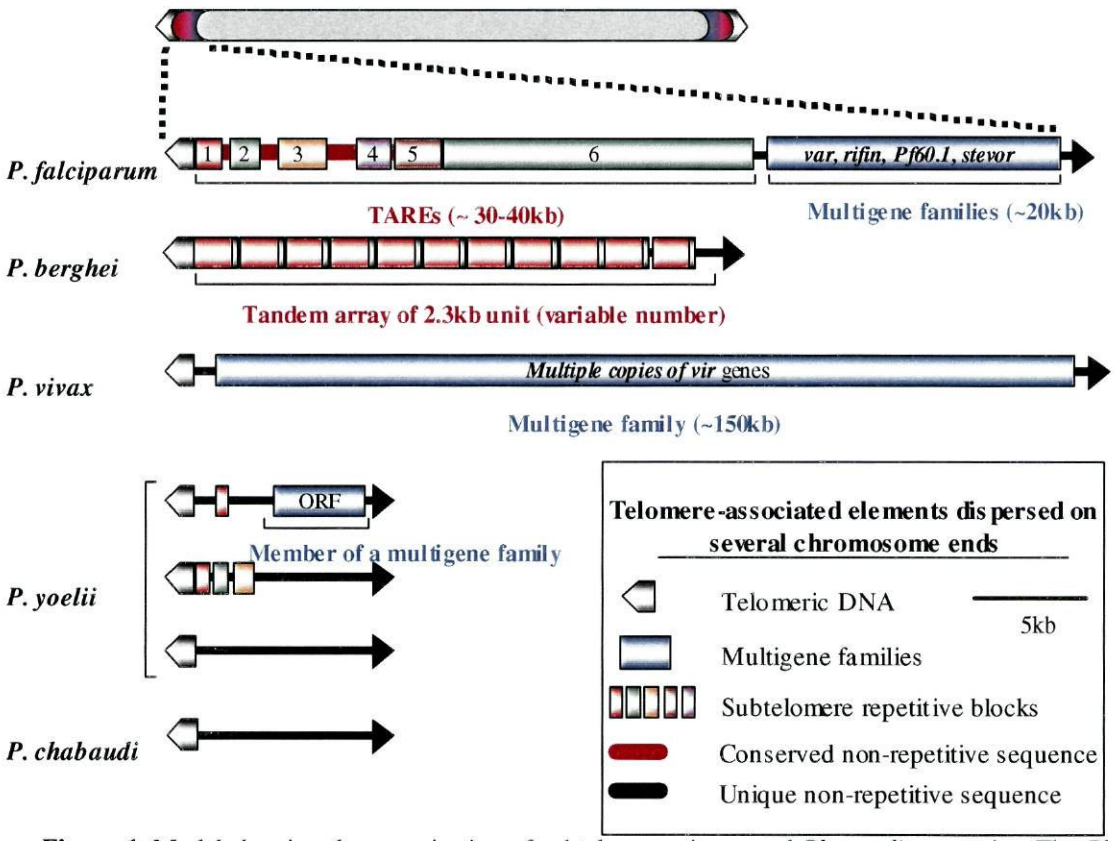


Figure 1. Model showing the organisation of subtelomeres in several *Plasmodium* species. The *Plasmodium* haploid nuclear genome is organised in 14 linear chromosomes. Each is composed of an internal region, where housekeeping genes are located, and a chromosome end. The terminus of the left arm of a chromosome is schematically represented as an example of a chromosome end organisation. Adjacent to *P. falciparum* telomeres, there is a highly polymorphic TAS, composed of two zones: a non-coding and a coding region. The non-coding region contains six TAREs, always positioned in the same order but of variable length. The adjacent coding region is the locus of several gene-families encoding important virulence factors, such as the *var* and *rifin* genes. The subtelomere organisation of four distinct *Plasmodium* species, (whose genome sequences are in the process of being determined) is also shown. See text for details.

The analysis of *P. falciparum* chromosome mutants revealed that telomere repeats and TARE's do have distinct functions. Telomeres are absolutely essential for chromosomal function, whereas TARE's can be deleted without any direct effect on the viability of the parasite during mitotic and meiotic divisions. The application of FISH analysis revealed novel functions for these genetic elements. Chromosome ends still are able to attach to the nuclear periphery in the absence of TARE's. However, these mutant chromosome ends tend to delocalise from the clusters suggesting strongly their involvement in cluster formation and/or maintenance ((Figueiredo *et al.*, 2002); see Fig. 2A and B).

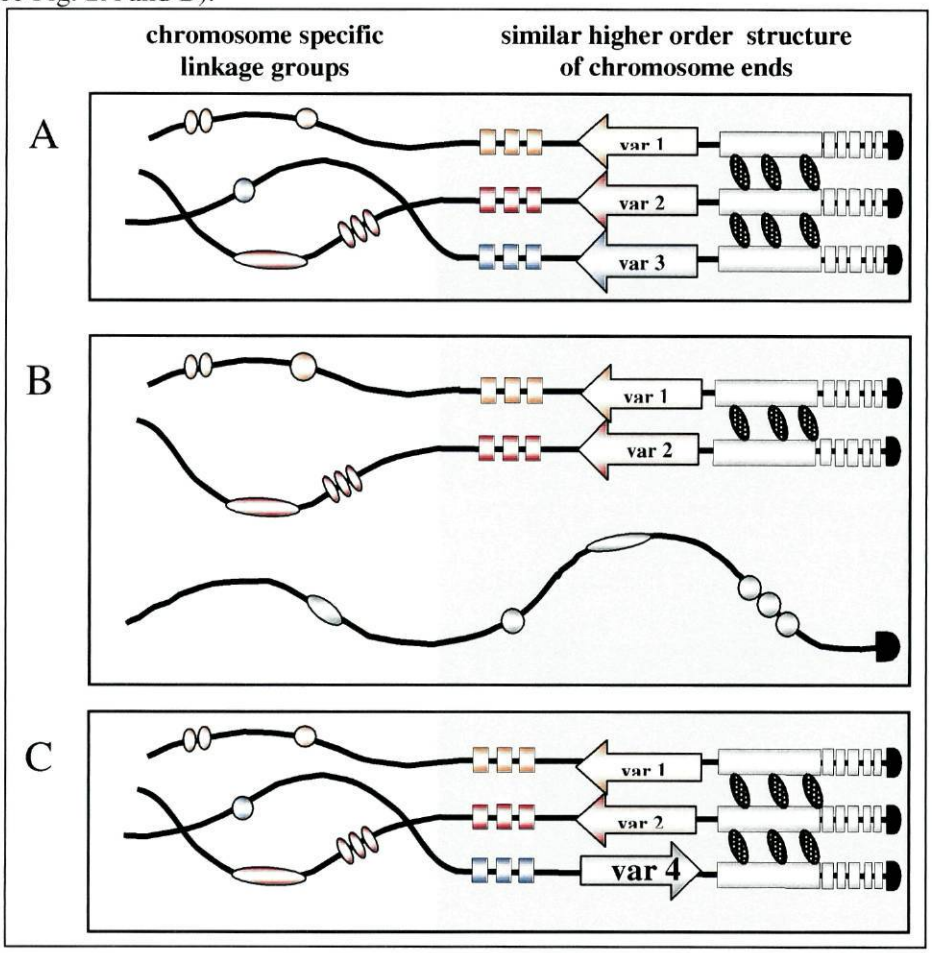


Figure 2. Chromosome clusters provide a nuclear environment for frequent recombination events. A similar subtelomere organisation is conserved among *P. falciparum* chromosome ends. A. The physical alignment of chromosome ends is shown where increased rates of recombination occur between homologous loci of heterologous chromosomes (ectopic recombination). *Var* genes adjacent to TAREs are generally transcribed towards the centromere. TARE6 specific DNA binding proteins that might cross-link chromosome ends are indicated. B. The deletion of the TARE is associated with the delocalisation of this chromosome end from a cluster. C. An unusual orientation of a *var* gene within a chromosome end has been observed (Vázquez-Macías *et al.*, 2002). Ectopic recombination between opposite orientated *var* genes predicts that ectopic recombination events would lead to dicentric chromosomes (lethal event).

P. berghei subtelomeric regions were the first ones to be described: they consist of a tandem array of a 2.3 kb unit, clustered exclusively in a subtelomeric position on several chromosomes (Pace *et al.*, 1987), (Dore *et al.*, 1990). The 2.3 kb unit includes ~160 bp stretch of telomere-related sequences, which appear to be important for recombinational events that contribute to chromosome-size polymorphism (Pace *et al.*, 1990). No gene families have been described so far adjacent to the 2.3 kb unit.

At present, *Plasmodium vivax* genome database contains a single hit for telomeric DNA: a YAC clone constructed by del Portillo *et al.* (del Portillo *et al.*, 2001). In contrast to *P. falciparum* and *P. berghei*, no large repetitive blocks exist in this *P. vivax* subtelomere. Instead, at ~1 kb from telomere repeats there is an ordered array of members of the *vir* gene-family. They are present at about 600-1000 copies per haploid genome and encode proteins that are immunovariant in natural infections, suggesting a role in the establishment of a chronic infection through antigenic variation.

Analysis of the DNA sequence data available in the *P. yoelii* genome database (February 2002), suggests that subtelomere organisation is distinct among heterologous chromosomes. For example, some chromosome ends present short repetitive blocks (<1 kb), but the type and number of these is highly variable. Another chromosome end carries a novel putative gene sequence, which is a member of a large gene-family (at least 50 distinct hits in the *P. yoelii* database) coding for putative proteins of small size (22-35 kDa). Alignment of some of the members of this family reveals a great conservation of the N and C-termini. This adds another subtelomeric gene family to the one described recently, the Py235 gene-family (Khan *et al.*, 2001).

At this stage, the *P. chabaudi* genome project is not as advanced as the other rodent malaria species. Thus, only 3 hits were found containing telomere repeats and their size was not larger then 5 kb. Sequence analysis did not reveal any open-reading frames, nor repetitive elements upstream (up to 5 kb) from telomere repeats.

The restricted sequence data available for the subtelomeres of the rodent malaria species do allow drawing a number of important conclusions: 1. Each *Plasmodium* species analysed (except for *P. vivax*) contains distinct non-coding sequence elements adjacent to the same type of telomere repeats. In *P. falciparum*, a similar higher order structure is found on virtually all chromosome ends. This contrasts with *P. yoelii*, which displays distinct elements adjacent to the telomeres. 2. Chromosome ends harbour generally highly diverse gene families.

Non-Random nuclear organisation of chromosomes

A prevailing view is that chromosomes are like spaghetti randomly floating around in the nucleoplasm. However, more and more data from different organisms point to non-random organisation of chromosomes in the nucleus (Marshall, 2002). We demonstrated, using FISH technology, that the 28 distinct chromosomes ends in *P. falciparum* are located at the nuclear periphery of asexual and sexual blood stage parasites. Several chromosome ends are physically grouped together and the number of clusters per haploid nuclei varies between 4 and 7. This observation implies that each cluster consists of 4 to 7 non-homologous chromosome ends (Fig. 3A).

It has been a mystery as to how the heterologous subtelomeres are physically aligned within a cluster and what provides the anchor for telomeres to the nuclear envelope. Two independent studies point to the subtelomeres as a critical DNA region involved in clustering. One study showed that the deletion of the entire subtelomeric region delocalises the truncated chromosome end from the cluster (Figueiredo *et al.*, 2002) (see Fig. 2B). However, this mutation apparently does not change the anchoring to the nuclear periphery. A second study demonstrated that a plasmid carrying a subtelomeric sequence called rep20 (TARE6) co-localises preferentially to the cluster in *P. falciparum* blood stage parasites, whereas a control plasmid showed a random distribution in the nucleus (O'Donnell *et al.*, 2002). These results suggest a function for the subtelomeric element rep20 in keeping together chromosome ends. How this is promoted is still unknown. Nonetheless, preliminary data from our laboratory indicate that specific proteins bind to rep20 repeats, raising the possibility that these molecules are involved in cross-linking *P. falciparum* chromosome ends (LM Figueiredo and A Scherf, data not shown).

Is clustering of *P. falciparum* chromosome ends random or are the same ends always involved in cluster formation? In order to address this question, the ends of three distinct chromosomes were analysed by multicolour FISH to determine the frequency of paired heterologuous telomeres. Promiscuous cluster formation was observed for the three chromosome ends studied (Freitas-Junior *et al.*, 2000). This observation implies that telomere clustering in *P. falciparum* appears to be random, suggesting that a given subtelomeric *var* gene might potentially recombine with *var* genes from any chromosome end. These results also imply that subtelomeric *var* loci will be close together in the nucleus whereas the constraint of diffusion might prevent interaction with other homologous *var* sequences being located in the central chromosome region (see Fig. 3B). This indicates that the telomere associated *var* genes diverge more rapidly than members located in the central chromosome domain.

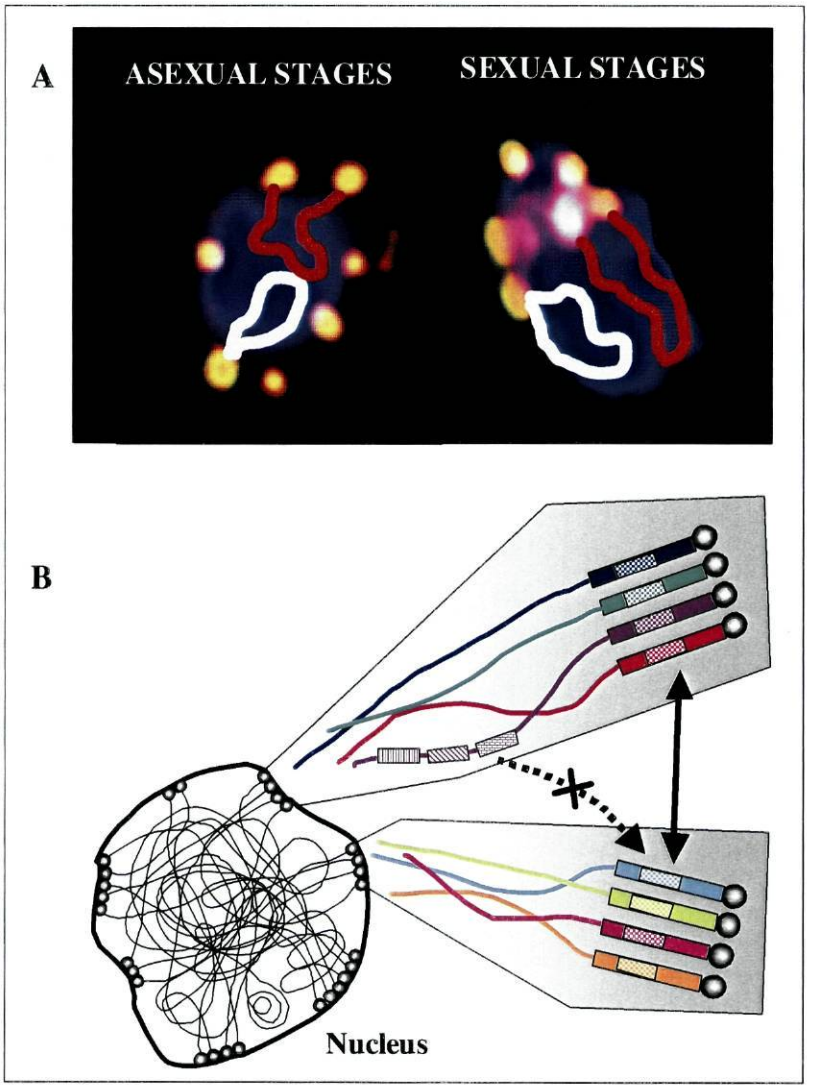


Figure 3. A. Nuclear architecture of *P. falciparum* chromosome ends. FISH analysis of a telomere specific DNA probe showing that the interphase chromosome termini form clusters at the nuclear periphery during trophozoite asexual blood stages (left) and a bouquet like structure in pre-meiotic gametocytes sexual blood stage (right). Parasite nuclei stained with DAPI (in light grey) and telomere clusters (in white). Two hypothetical chromosomes are shown schematically. Both chromosome ends are in the same cluster (white) or are located in distinct clusters (grey). B. *P. falciparum* cluster formation has a random component. Multicolour FISH analysis of individual chromosome ends has demonstrated that a given end can associate with different chromosome ends (Freitas-Junior *et al.*, 2000). A schematic of 2 clusters summarizing these results is shown.

Can the subnuclear architecture of *P. falciparum* be considered to be a blueprint for other malaria species? Although the chromosome tips of different malaria species are bounded by the same type of telomere repeats, the telomere-associated sequences are surprisingly divergent from each other (see below). FISH analysis of the chromosome end organisation of several different malaria species of distinct evolutionary origin form clusters of several chromosome ends during the interphase of blood stage parasites, which are similar to those described first for *P. falciparum* (Fig. 4).

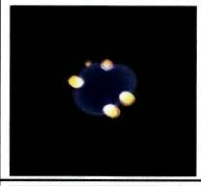
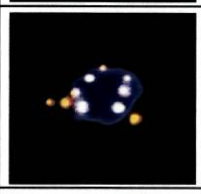
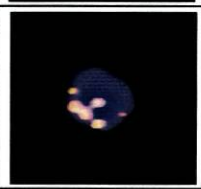
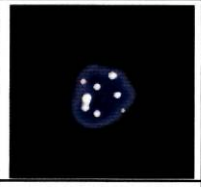
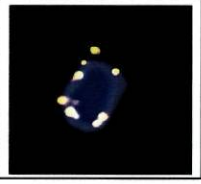
Plasmodium species	Mammalian host	FISH	Number of clusters per nuclei (average)
P. falciparum	Human		4-7 (**5**)
P. vivax	Human		7-13 (**9**)
P. gallinaceum	Bird		4-7 (**5**)
P. berghei	Rodent		3-8 (**5**)
P. yoelii	Rodent		4-12 (**8,1**)
P. chabaudi	Rodent		4-13 (**8,4**)

Figure 4. Chromosome end clustering is a conserved feature of malarial species. FISH analysis of blood stage interphase parasites (young trophozoites) was performed using a fluorescent telomere probe ($(GGGTT^{T}/_{C}A)$). The mean number of clusters per nucleus is indicated in brackets.

However, *P. vivax* and two of the rodent malaria parasites (*P. yoelii and P. chabaudi*) displayed significantly more fluorescent foci than *P. falciparum*, suggesting that the number of chromosomes per cluster is smaller or that several chromosomes are not located in clusters. It will be interesting to see if the heterogeneity of subtelomere organisation observed for some *Plasmodium* species does influence the cluster formation of chromosome ends. In summary, this analysis underlines that the nuclear architecture of chromosome ends is preserved between malaria species and might be related to an evolutionary advantage that this peculiar structure promotes.

Interaction between subtelomeric loci (Recombination frequencies)

What are the biological implications of the *P. falciparum* nuclear organisation on genes located close to telomeres? Does it advance our understanding of the genomic plasticity and recombination processes? It is now well established that plasmodial chromosome ends form a highly dynamic chromosome compartment and most of the chromosome polymorphism is due to DNA rearrangements occurring in the subtelomeric region (reviewed in (Scherf *et al.*, 1999)). The high recombination frequencies observed in subtelomeric regions seem to create an environment that allows the expansion and diversification of gene families located at chromosome ends. Thus, telomeres provide an ideal setting for genes involved in host parasite interactions such as parasite cytoadhesion and antigenic variation.

Our new knowledge about the nuclear organisation points to a strong influence on chromosome interactions (Marshall, 2002). In *P. falciparum* the spatial proximity of heterologous chromosome ends combined with a common higher order structure of subtelomeres seems to be a critical factor in determining the high rate of DNA recombination observed in *var* loci in a genetic cross of *P. falciparum* (Freitas-Junior *et al.*, 2000) (see Fig. 2A). It was shown that chimeric *var* forms were created by gene conversion involving two *var* members located at different chromosome ends. The marked diversity of *var* genes in genetically different laboratory strains and clinical isolates can lead to parasites with minimal overlapping *var* gene repertoires (Freitas-Junior *et al.*, 2000). This demonstrates the important role of the subtelomere in shaping genetically distinct parasite populations. Clusters are also observed during blood stage parasite proliferation and thus might enhance mitotic ectopic recombination events leading to a continual reorganisation of subtelomeric gene families during chronic patient infection.

Recently, a highly conserved subtelomere located *var* gene (*varCSA*), which is expressed during placental malaria, was discovered in parasite isolates from all over the world ((Vázquez-Macías *et al.*, 2002); Scherf et al., data not shown). How does this 'non-variant' *varCSA* gene resist the diversification process? This might be explained by the exceptional organisation of this particular *var* gene, which is orientated in the opposite direction compared to other telomere-associated *var* genes (Fig. 2C). Ectopic recombination events would lead to the formation of dicentric chromosomes that are generally lethal or would yield defective cells. Furthermore, being trapped in a cluster, would constrain the *varCSA* interaction with central *var* gene copies, making it relative inert to ectopic DNA recombination. Further analysis of the *P. falciparum* database suggests that an additional small number of subtelomeric *var* genes exist in the 3D7 genome being transcribed towards the telomere. For example, we identified a cluster of two subtelomeric *var* genes that are transcribed in opposite directions (data not shown). It will be interesting to investigate how this type of *var* genes relate to each other and if they are also conserved between distinct clinical isolates as the *varCSA* gene. In conclusion, this example illustrates the influence of nuclear organisation on important biological events related to parasite virulence.

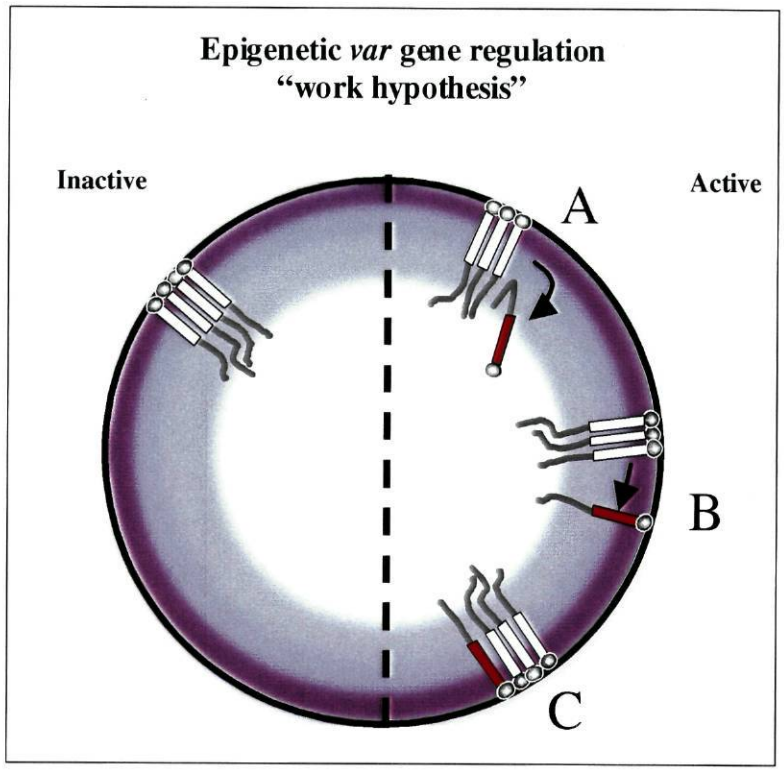


Figure 5. Model of the epigenetic regulation of telomere-associated *var* genes. A cluster of chromosome ends with silent var genes is shown (white). Three alternative activation processes are proposed (subtelomere carrying an activated *var* gene is shown in black). The first two involve chromosome end movements outside the cluster to either a specific chromosome compartment in a more internal region of the nucleus (a) or in the nuclear periphery (b). Alternatively, the activation could occur within a cluster due to local changes in the chromatin organisation of the *var* gene (c).

Epigenetic factors involved in antigenic variation

The telomere position effect brings about a heritable yet variegated repression of genes placed near yeast telomere repeats (Gottschling *et al.*, 1990). This effect has been associated with chromatin organization, as proteins essential for telomeric silencing are found to be concentrated at the telomeres and/or subtelomeric regions (Kyrion *et al.*, 1992). In yeast, a number of telomere-associated proteins involved in telomere-length regulation, cluster formation, telomere anchoring to the periphery and gene silencing have been described and characterized (reviewed in (Gasser, 2001)). The mechanisms that control the regulation of *var* gene expression in *P. falciparum* are still unresolved but many indications point to epigenetic factors being involved in the regulation of *var* gene transcription (Scherf *et al.*, 1998), (Freitas-Junior *et al.*, 2000), (Deitsch *et al.*, 2001). *P. falciparum* orthologues to most yeast telomere-associated proteins have been identified including the genes coding for the "silent information regulators 3 and 4" (LH Freitas-Junior, A Scherf, unpublished data), This finding implies that *P. falciparum* might use a similar mechanism to

silence telomere-associated *var* genes. Gene inactivation studies are in progress to analyse the role of these telomere-associated molecules in *P. falciparum*.

Changes in the intra-nuclear chromosome positioning have been associated with transcriptional activation in mammalian cells and African trypanosomes (Tumbar and Belmont, 2001), (Navarro and Gull, 2001). Thus, a second attractive working hypothesis is that telomere clusters form a repression zone for gene expression. In order to overcome epigenetic *var* gene silencing, the corresponding subtelomere needs to move to a distinct nuclear compartment that would be compatible with gene expression. Figure 5 shows different hypothetical models for *var* gene activation.

Conclusions

The recent discovery of telomere clustering at the nuclear periphery in *P. falciparum* and its role in ectopic recombination between members of the *var* gene families has generated more appeal to a field which was before considered to be a domain of a handful of specialists. Similar nuclear organisation of subtelomeres in clusters has now been observed in other human and rodent malaria species. Other malaria pathogens seem to follow the same strategy in order to create high levels of continual diversity in subtelomeric gene families. Yet, it is clear form the preliminary genome analysis, that each species has developed its own strategy to organize the chromosome ends.

Another important feature of the telomere is its potential role in epigenetic gene regulation. A better understanding of the biology of this specific nuclear region is necessary and might lead to a better comprehension of the chromatin factors necessary to establish repression and the mutually exclusive expression of genes involved in the antigenic variation in *Plasmodium*.

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Research website (www.tigr.org). We wish to thank the scientists and funding agencies comprising the international Malaria Genome Project for making sequence data from the genome of *P. falciparum* (3D7) public prior to publication of the completed sequence. The Sanger Centre (UK) provided sequence for chromosomes 1, 3-9, & 13, with financial support from the Wellcome Trust. A consortium composed of The Institute for Genome Research, along with the Naval Medical Research Center (USA), sequenced chromosomes 2, 10, 11 & 14, with support from NIAID/NIH, the Burroughs Wellcome Fund, and the Department of Defense. The Stanford Genome Technology Center (USA) sequenced chromosome 12, with support from the Burroughs Wellcome Fund. The Plasmodium Genome Database is a collaborative effort of investigators at the University of Pennsylvania (USA) and Monash University Melbourne, Australia), supported by the Burroughs Wellcome Fund.

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CONCLUDING REMARKS

Telomeres have seduced a handful of malarialogists for almost 20 years. The first studies, carried out by Clara Frontali's laboratory, characterized the DNA organisation of *P. berghei* chromosome ends. From this work, we learned that telomeres of different *Plasmodium* species are composed of tandem arrays of short G-rich repeats as is the case for the majority of eukaryotes. Later, subtelomeric regions (or TAS) of malaria parasites were shown to be highly polymorphic, harbouring repetitive elements, undergoing truncations, inversions, interchromosomal exchanges and other genomic rearrangements. In *P. falciparum*, several gene-families encoding virulence factors such as the *var, rifins* and *stevor* genes were shown to be located at subtelomeric regions. This observation raised the first question of my thesis project: why do such gene-families locate proximal to telomeres? Or, in other words, what are the features of telomeres that make them a preferential locus for multigene-families? In parallel, our lab showed in 1998 that *P. falciparum* displayed telomerase activity *in vitro* and that it could be efficiently inhibited by dideoxynucleotides, well-established drugs against viral reverse-transcriptases. My second goal was thus to identify the catalytic component of telomerase and to test whether this enzyme is a valid drug-target.

In view of these goals, the research carried out during the course of my thesis has provided a clearer picture of the organisation of subtelomeric regions in different Plasmodial species. We propose that *P. falciparum* subtelomeric regions are not non-coding "junk" DNA, but are important genetic elements involved in telomere length regulation and nuclear architecture. We have identified putative genes for telomerase reverse-transcriptase (TERT) in *P. falciparum*, *P. berghei*, *P. yoelii* and *P. chabaudi*. Characterisation of the *PfTERT* candidate gene strongly suggests that it encodes the catalytic component of telomerase.

In this final chapter, we attempt to place our observations within the context of the field of telomere biology, and discuss the questions that arise from this thesis.

1. SUBTELOMERIC REGIONS: CONSERVED HIGHER-ORDER STRUCTURE

In the majority of eukaryotes, the chromosome region upstream telomeres is composed of a mosaic of repetitive blocks, which may or not be conserved among all chromosomes. Each block is usually highly variable in its relative position and copy number. Despite such variability, the overall subtelomeric structure appears specific of each species. Cytologically, chromosome ends in a variety of plants and animals are heterochromatic, implying a high degree of chromatin compactation exists at telomeres (Dernburg *et al.*, 1995), (Suja and Rufas, 1994). In *S. cerevisiae*, chromosome ends have been described to exist in a heterochromatin-like state based on the finding that telomeric DNA is late replicating (McCarroll and Fangman, 1988), refractory to DNA methylation, (Gottschling, 1992) and in a fold-back structure that brings the terminal region of the telomere close to a more internal subtelomeric region (de Bruin *et al.*, 2000). On the centromere-proximal side of subtelomeric regions, there are generally gene-families. For example, in *S. cerevisiae*, there are several multigene-families involved in the use of different carbon sources (eg. *MAL, MEL, SUC*, etc) (Pryde *et al.*, 1997). Similarly, members of the human olfactory receptor gene-family are located near the ends of human chromosomes (Trask *et al.*, 1998). It remains to be determined whether the telomeric silencing effect, described in yeast and humans, plays a role in the regulation of these gene-families.

P. falciparum subtelomeric regions are polymorphic and devoid of open-reading frames, consisting of repetitive sequence elements. *Plasmodium* chromosomes are too small to allow heterochromatin to be seen by the same cytological procedures as used for fruit flies and mammals. Nevertheless, given the repetitive structure of the DNA present at chromosome ends, the association of telomeres in foci at the nuclear periphery and preliminary FISH data showing that *P. falciparum* chromosome ends are highly compacted (Freitas-Junior, L.H., unpublished data), it is likely that *P. falciparum* chromosome ends are organised in a heterochromatin-type structure. Studies designed to test the ability of chromatin-altering enzymes (such as histone-acetylases, histone-methylases, etc) to access telomeric / subtelomeric DNA and to measure when in S-phase telomeres are replicated will help address this prediction.

Subtelomeres of *P. falciparum* do show, however, several unique features. The DNA organisation of the six distinct repetitive elements (TARE1-6) seems to be a trait of all chromosome ends; these motifs are always positioned in the same order. Moreover, the inter-

motif sequences are nearly identical among heterologous chromosomes (Figueiredo *et al.*, 2000). As far as we know, such a strict conserved higher-order structure of the subtelomeric regions has not been observed in other organisms (Fig. 5, Introduction of this thesis). In other Plasmodia species, preliminary data also indicate that TAS organisation is not as conserved among chromosomes as in *P. falciparum*. One interpretation of this finding is that functional constraints prevent *P. falciparum* TAS from diverging. Such possible roles are discussed below. Towards the centromere of most chromosomes, as it had been observed for chromosome 2 and 3 (Gardner *et al.*, 1998), (Bowman *et al.*, 1999), we found a member of the *var* gene-family, followed by members of other gene-families, such as *rifins*, *stevor*, etc.

2. ROLES OF *P. FALCIPARUM* SUBTELOMERIC REGIONS

The role of subtelomeric regions in different organisms is not yet clear. When spontaneous chromosome breakages result in the loss of the entire subtelomeric region in humans (Wilkie *et al.*, 1990), *S. cerevisiae* (Murray and Szostak, 1986) and *P. falciparum*, (Pologe and Ravetch, 1988), the viability of these organisms is maintained by adding *de novo* telomere repeats to the truncation site. Thus subtelomeric genetic elements do not seem to be essential. When Telomere Positioning Effect was described by Gottschling et al., subtelomeres were proposed to be act as a "buffer" that separates silenced telomeric regions from actively transcribed internal loci (Levis *et al.*, 1985), (Gottschling *et al.*, 1990). In support of this idea, ACS, an X-core internal genetic element found within all subtelomeric regions, was shown to be involved in silencing at native telomeres by limiting the spreading of repressive chromatin. This process is mediated by telomeric proteins (Rap1-Sir complex) and proteins bound to the subtelomeric core X (Pryde and Louis, 1999). TAS have also been proposed to be important in telomerase-independent processes for maintaining telomeres (reviewed in (Lundblad, 2002)).

Our work has revealed two roles for *P. falciparum* subtelomeric regions. Compared to intact chromosome, chromosome ends that have lost their entire subtelomeric region by a spontaneous truncation event harbour longer telomeric tracts and occupy a different position within the nucleus. These results strongly suggest that subtelomeric regions are implicated in telomere length regulation and nuclear architecture (Figueiredo *et al.*, 2002).

2.1 Telomere length regulation

Telomere length regulation has been extensively studied in yeast and humans. A number of trans-acting factors have been described that bind directly or indirectly to telomeric DNA to act as positive or negative regulators of telomerase. The folding of telomeres in a T-loop or other types of structures could also potentially regulate their access to telomerase. In *P. falciparum*, orthologues to some yeast telomeric proteins (eg. Rap1p, Rif1, Sir2-4, Ku80) have been found in the genome-sequencing database and characterisation is under way to verify their role in telomere maintenance in *P. falciparum*. Preliminary data shows co-localisation of these proteins with telomeres (Scherf *et al.*, 2001), which is a good indication that these are telomeric proteins. Both *P. falciparum* and *S. cerevisiae* display relatively short telomeres (1200 bp and 300 bp, respectively) that are stable with time. In both organisms, telomeres are organised into clusters that localize to the nuclear periphery (Klein *et al.*, 1992), (Freitas-Junior *et al.*, 2000). We predict, therefore, that the above mentioned orthologues to yeast telomere binding proteins will have similar roles in *P. falciparum*.

Our observation that truncated chromosome ends harbour longer telomeric tracts than intact chromosome ends demonstrates that regulation of telomere length in *P. falciparum* will depend not only on the aforementioned telomere-binding protein candidates, but also on the DNA sequences found upstream of the telomeric tract. Since, telomeric proteins are trans-acting factors, their role should theoretically be identical at every chromosome end (if each end looks identical and contains the appropriate binding sites). What distinguishes one chromosome end from another is its subtelomeric sequence (TAS). At intact chromosome ends, TAS are conserved: six repetitive elements are organised in the same order (TARE1-6). Because the "genomic environment" is the same, this would explain the identical telomere length displayed by such chromosomes. At truncated chromosome arms, telomeric tracts are adjacent to a more internal type of DNA (eg. close to a gene). This new "genomic environment" might explain why the telomere length has changed. Although how it would be sensed by the cell remains unknown. We propose three possible mechanisms:

I. TAS may contain genetic elements that act as cis-elements and that may be recognised by specific proteins. Such subtelomeric-binding proteins may interact with some of the trans-acting telomeric factors and participate in the regulation of telomere length. In yeast, it has been shown that the junction between telomere and subtelomeres is important for the "counting" mechanism that "senses" the average length of the telomere (Ray and Runge, 1999). Moreover, a fold-back structure formed by interactions between proteins bound at the telomeric tract and subtelomeric region has been proposed to be necessary for telomere capping and TPE (Grunstein, 1998), (de Bruin *et al.*, 2000).

II. Presence of TAS may be necessary to form a heterochomatin-specific structural state. This hypothesis is supported by FISH preliminary data suggesting that subtelomeric regions form a highly compacted compartment (Freitas-Junior, L.H, personal communication). When TAS is deleted, the chromatin organisation would be affected and consequently the accessibility of regulatory molecules to the telomere would be altered. Due to such changes, a new average telomere length would result.

III. "Genomic environment" might reflect the position in the nucleus occupied by the chromosome end. In fact, we observed that a truncated chromosome end localises preferentially outside telomere clusters, but still at the nuclear periphery. If anchoring to nuclear periphery is mediated by telomeric proteins that interact with the nuclear membrane structural components as observed in yeast (Shore, 1998), (Laroche *et al.*, 1998), (Galy *et al.*, 2000), we speculate that an isolated chromosome does not benefit from the cooperative anchoring that might happen in a cluster of chromosome ends. Thus a longer telomeric tract might compensate for the fact that the telomere is isolated, by providing more sites for anchoring at the nuclear membrane.

2.2 Nuclear architecture

Our observations that TAS are involved in the association of telomeres in foci ("clustering") (Figueiredo *et al.*, 2002) is supported by a recent study from the B.S. Crabb laboratory. They demonstrated that a plasmid carrying the subtelomeric sequence called TARE6 (or rep20), co-localises preferentially to telomere clusters in *P. falciparum* blood stage parasites, whereas a control plasmid lacking this element showed a random distribution in the nucleus (O'Donnell *et al.*, 2002). These results suggest that a function of the subtelomeric element TARE6 is in the formation and/or maintenance of chromosome end

clusters. It is possible that other TAREs are also involved in this process. How "clustering" is promoted remains unknown. Nonetheless, preliminary data from our laboratory indicate that specific proteins bind to TARE6 (rep20) repeats, raising the possibility that these molecules are involved in cross-linking *P. falciparum* chromosome ends (Fig. 15).

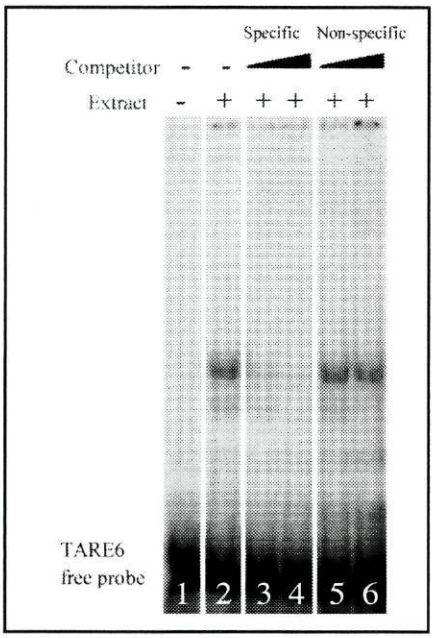


Figure 15. Specificity of TARE6 (rep20) binding protein(s). A 42 bp TARE6 probe was prepared by annealing two complementary single-strand oligonucleotides (5' AGACCTATATTAGTAAAGTTAGGACCTAAGTTAG TTATTATA 3' and 5' TATAATAACTAACTTAGGTCCTAACTTTACTAA TATAGGTCT 3'). Duplex was purified on a G25-Sephadex column and end-labelled using T4-polynucleotide kinase and [γ-^{32}P]adenosine triphosphate. 5 μg of *P. falciparum* nuclear protein extract was incubated with 12000 cpm of probe, for 30 min at room temperature in 1x Binding Buffer (*20mM HEPES pH7.9, 0.1M NaCl, 1mM EDTA pH8.0, 1mM DTT, 5% glycerol* and *0.25 mg/mL BSA, 2μg of PolydI:dC*). Complexes were analysed by electrophoresis through a 4% tris-borate EDTA PAA gel run at 30 mA for 1:30hr. In lanes 3 and 4, binding reaction was performed in the presence of increasing amounts of cold TARE6 duplex. In lanes 5 and 6, increasing amounts of a 17 bp telomeric duplex were used as a non-specific competitor.

We observed that a truncated chromosome end that has lost its subtelomeric region remains at the nuclear periphery, suggesting that TAS are not involved in the localisation of chromosome ends at the nuclear periphery. In *S. cerevisiae* "anchoring" of chromosome ends to the nuclear membrane is mediated by proteins bound to the telomeric DNA (Ku80) that interact with nuclear membrane structural components (MLPs) (reviewed in (Hediger and Gasser, 2002)). Orthologues to Ku80 have been found in the *P. falciparum* genome and immunolocalisation studies show it co-localises with telomeres (Scherf *et al.*, 2001). In agreement with our results, we speculate that "anchoring" is mediated by telomeric proteins. A truncated chromosome end, therefore, localises close to the nuclear periphery via its telomere, but does not "cluster" with other chromosome ends because it lacks a conventional subtelomeric region. "Clustering" and "anchoring" are thus two distinct processes in *P. falciparum*, which are likely mediated by specific proteins as shown in Fig. 16. It will be interesting to investigate if a similar dictotomy exists in other organsims.

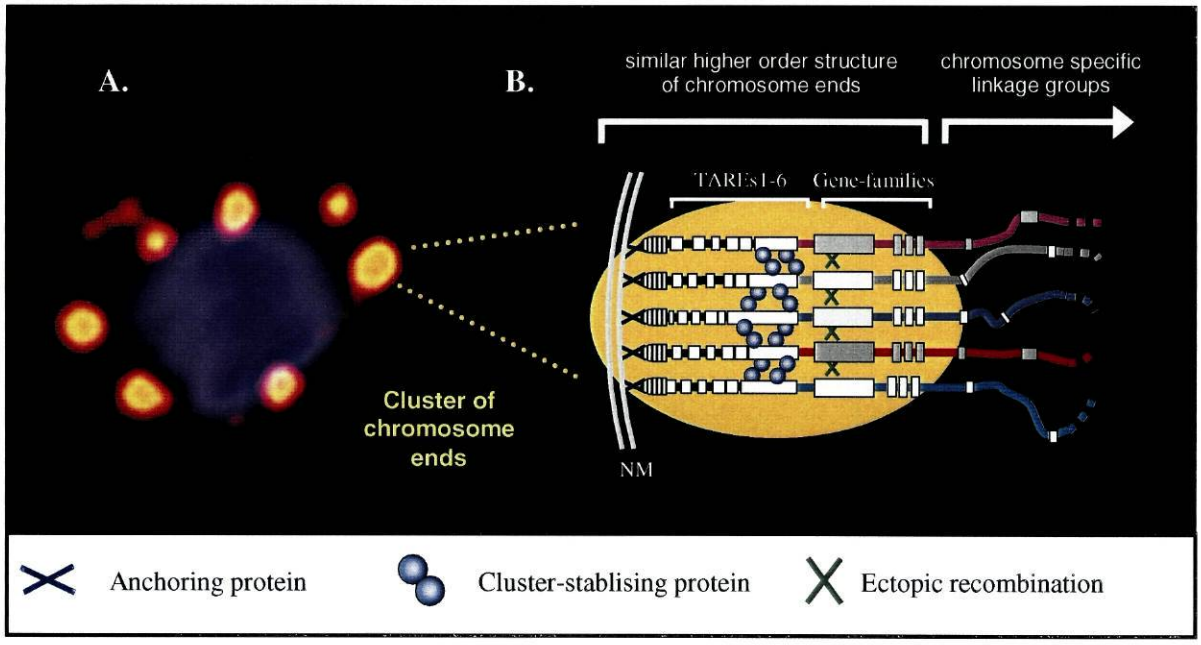


Figure 16. Model of the organisation of chromosome ends in the nucleus of *P. falciparum*, revised from Fig. 14 (of the Introduction) according to the data obtained in this thesis. (A) FISH analysis with a telomere-specific DNA probe (rep20, or TARE6) showing that the chromosome termini form clusters at the nuclear periphery during trophozoite asexual blood stages (kindly provided by Freitas-Junior). (B) The conserved higher order-organisation of chromosome ends consists of six repetitive elements (TARE1-6) and a coding-region, loci of multigene-families (*var, rifins, Pf60.1, stevor*). We propose that the physical alignement of chromosome ends – clustering – is mediated by specific proteins that recognise TARE6 and may be other subtelomeric motifs. Perinuclear localisation of chromosome ends seems to be independent of subtelomeric elements. We suggest that telomeric proteins mediate the "anchoring" to structural components of the nuclear membrane. NM: nuclear membrane.

We need to take into account that telomeres are an integrant part of a whole genome. Thus, telomere clusters need to be put in a context of 14 chromosomes organised within the nucleus, a defined space. In higher eukaryotes, a chromosome localises in a rather discrete region of the nucleus, its "chromosomal territory". Recent studies have shown that during interphase, specific chromosome sites can be highly mobile within a general subnuclear zone (Vazquez *et al.*, 2001); (Heun *et al.*, 2001). In contrast, centromeres and telomeres show much more constraint on their mobility (Marshall *et al.*, 1997), (Heun *et al.*, 2001). Thus in yeast, telomere clusters represent relatively immobile subcompartments.

What's the biological significance of telomere clusters in P. falciparum?

Plasmodial chromosome ends are highly recombinogenic and most of the chromosome polymorphisms among strains are a result of DNA rearrangements occurring in the subtelomeric region (reviewed in (Scherf *et al.*, 1999)). The common higher order structure of subtelomeres combined with the spatial proximity of heterologous chromosome ends in the clusters seem to be a critical factor in determining the high rate of DNA recombination observed among members of the multigene-families located nearby, such as the *var* genes (Freitas-Junior *et al.*, 2000). Therefore, it seems that clustering of chromosome ends creates an environment that promotes the diversification of these subtelomeric gene-families. Since some of these multigene-families have been shown to encode surface antigens, diversification would most likely increase the chances of survival of parasites.

Another possible consequence of chromosome end clustering is on the regulation of telomere-associated *var* genes. Changes in the intra-nuclear chromosome positioning have been associated with transcriptional activation in mammalian cells and African trypanosomes (reviewed in (Borst, 2002)). Likewise, telomere localisation at the nuclear periphery appears to facilitate the formation of a repressed chromatin, a condition necessary but not sufficient for telomeric silencing (TPE). Rap1 (a sequence-specific telomere-binding factor) is seeded first and then recruits heterochromatin-associated non-histone proteins (silencing proteins: Sir2-Sir3-Sir4 complex) to the telomere. The Sir complex also interacts with nucleosomes and thus spreads into subtelomeric regions resulting in a region of "silenced"chromatin that extends several kilobases from the telomere (reviewed in (Grigoryev, 2001)). Since, orthologues of Sir proteins have been found in *P. falciparum* it will be interesting to investigate whether Plasmodial telomere clusters also form compartments of transcriptional repression. If so, in order to activate a *var* gene, its corresponding subtelomere might need to move to a nuclear compartment that would be compatible with gene expression. FISH analysis are under way to compare the nuclear positioning of an active and inactive *var* gene (Freitas-Junior, L.H.). In two studies in *P. berghei*, a selectable marker was introduced in subtelomeric locations and no transcriptional repression was observed (van Dijk *et al.*, 1996), (Pace *et al.*, 2000), refuting the possibility of a general silencing mechanism in malaria parasites. Such results refer to chromosome ends that have either lost the entire subtelomeric region (~60 kb) or in which the selectable marker was inserted among the repetive motifs of *P. berghei* TAS. Therefore, we cannot exclude that such modifications of the subtelomeric region may have led to a displacement of the chromosome end from telomere clusters, which

resulted in a release of transcriptional repression. Alternatively, since telomeric silencing is a variegated phenomen, we would expect that a population of parasites present the selectable marker activated and others present it silenced. Since no single-cell analysis was performed to measure the mRNA levels, the transcription levels detected are an average value of the entire parasite population. Therefore, we cannot exclude that TPE exists in *Plasmodium* species.

3. *P. FALCIPARUM* TELOMERASE: A DRUG TARGET

Telomeres of *P. falciparum* display a constant average length of about 1200 bp, varying ± 400 bp depending on the specific strain. Whereas the majority of chromosome ends displays this average length, a minority harbours longer telomeres (probably corresponding to truncated chromosome arms) (Figueiredo *et al.*, 2002). Telomerase activity has been previously detected in *Plasmodium* (Bottius *et al.*, 1998), and, in this work, a potential *PfTERT* gene has been identified. In order to examine *P.falciparum*'s *in vivo* requirements for telomerase activity, we attempted to disrupt the *PfTERT* gene. However, we were unsuccessful, which suggests that telomerase is essential for the parasite viability. To validate telomerase as a drug target, it will be indispensable to demonstrate that *P. falciparum* does not possess telomerase-independent mechanisms to maintain telomere length. Such mechanisms, in which homologous recombination between chromosome ends avoids fatal telomere shortening have been detected in *S. cerevisiae* and in certain immortalised cell lines and tumours), The transfection system used in this work to attempt to disrupt the *PfTERT* candidate gene requires 30-50 days of drug selection before transfected parasites can be detected (Crabb *et al.*, 1997). If plasmid integration in *PfTERT* locus occurs at Day 1 of transfection, we speculate that if ~1% of telomeric repeats are lost per replication cycle (12nt) (as observed in other unicellular eukaryotes, Table 4 in Introduction), in 50 days all telomeric repeats would have been lost and the telomeres would therefore be rendered non-functional. Assuming functional telomeres are required for viability and that an "efficient" recombination-based 'back-up' method of telomere maintenance does not exist, PfTERTΔ parasites would die prior to detection of drug-resistant parasites. This could potentially explain why we were never able to detect disrupted *PfTERT*, but only parasites harbouring the disruption-plasmid as episomes. We cannot exclude the possibility that the gene-disruption

failed due to problem in the targeting of plasmid to this particular chromosome locus. Ideally, an inducible 'knock-out' system would be necessary to follow the effect of telomerase inactivation immediately after induction.

Human cells have telomeres that are approximately ten times longer than *P. falciparum* (10-15 kb vs. ~1.2 kb)(Harley *et al.*, 1990), (de Lange *et al.*, 1990). In the majority of human somatic cells, no telomerase activity is detected and consequently telomeres shorten progressively with each cell division. In the proliferating cells of renewal tissues (such as the bone marrow progenitor cells) and germ cells, telomerase is active and no progressive shortening is observed. Telomeres are longer and no progressive shortening is observed. Given the difference in size and dynamics of telomeres between *Plasmodium* parasite and human cells, one can envision using Plasmodial telomerase as a target for antimalarial drugs. To be successful, such drugs would cause the shortening of telomeres in malaria parasites until a critical point was reached, triggering cellular senescence and eventually the death of the parasite (green line, Fig. 17). Such a drug should not effect the viability of the majority of human somatic cells, given the absence of detectable levels of telomerase in such cells (blue line, Fig. 17). Toxicity, however, might be a problem for germ cells and cells from highly proliferative tissues. This toxicity may be well tolerated since germline cells generally have very long telomeres and are consequently farther from reaching M1 stage or senescence (yellow line, Fig. 17).

For this strategy to work, two conditions need to be fulfilled: (i) telomere shortening in *Plasmodium* must be sufficiently rapid that the treatment period is relatively brief and therefore effects on germline would be minimized; (ii) Plasmodial telomerase must be the unique mechanism for telomere maintenance, at least during the treatment period. If alternative telomere lengthening mechanisms exist, as described in yeast and human cells, and if such mechanisms are activated immediately after the beginning of the treatment, then the parasite could avoid fatal telomere shortening leading to spontaneous emergence of "survivors".

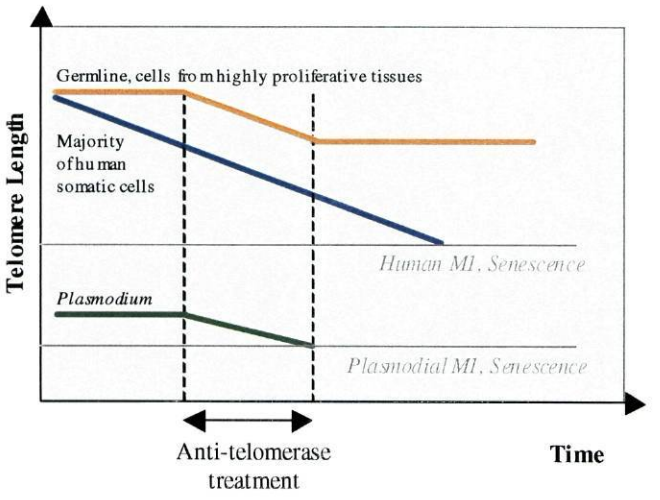


Figure 17. Hypothetical progress of telomere length in *Plasmodium falciparum* and human cells, upon treatment of malaria with anti-telomerase drugs. *Plasmodium* telomeres (green) are shorter than telomeres from human cells (yellow and blue). When Plasmodial telomerase is used as a target for antimalarial drugs, we expect a fatal quick shortening of telomeres in *Plasmodium* (green), no effect on the majority of human somatic cells (blue) and a neglectable telomere shortening effect on germ cells, as well as on cells from highly proliferative tissues. The mortality stage 1 (M1) were defined arbitrarily since we do not know what the critical length is that triggers senescence in *Plasmodium*.

The candidate *PfTERT* gene that we have identified is predicted to encode an unusually large telomerase reverse-transcriptase (280 kDa) which contrasts markedly with the molecular weight of hTERT (120 kDa). In the future, as the PfTERT protein structure/funciton relationship is further dissected, this difference in primary structure may aid in the design of drugs that will specifically inhibit Plasmodial telomerase and will not affect human telomerase.

Bottius et al have shown that Plasmodial telomerase can be efficiently inhibited by reverse-transcriptase drugs (such as AZT) (Bottius *et al.*, 1998).These drugs have been tested in parasites in culture and parasites died after 3-4 days of drug treatment (Figueiredo, L.M., unpublished data), suggesting that only a few replication cycles are necessary to induce fatal telomere shortening. These results, together with the fact that we were unable to obtain *PfTERT*Δ parasites, indicate that Plasmodial telomerase may be considered as a promising drug target.

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