The Cell Biology of Parasitism in *Trypanosoma brucei*: Insights and Drug Targets from Genomic Approaches?

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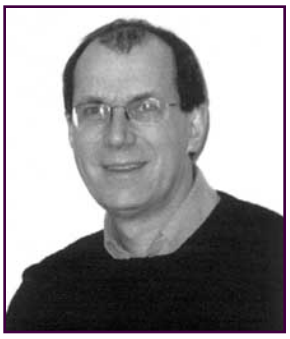

Abstract: The African trypanosome, *Trypanosoma brucei* exhibits a complex, digenetic life cycle that alternates between the tsetse fly vector and the mammalian host. The life cycle is characterised by a complex series of cell type differentiations and variations in metabolism. In addition the trypanosome exhibits a particular cell biology that has become adapted for its role as a parasite. This article places some of these areas in a framework that considers the role of cellular processes in parasitism. I rehearse some conclusions from recent studies and provide hyphotheses and suggestions for future work. Areas debated include: cell surface protein expression, cell differentiation, endomembrane trafficking and protein targeting, the cytoskeleton,flagellum functions in motility, attachment and plasma membrane differentiation, organelle specialisations, control of cell cycle, parasite/host, parasite/parasite and parasite/vector interactions.

The review also focusses on the likely impact of the genome project and reverse genetics in providing greater insight to these cellular processes and how, if coordinated with some élan by scientists and funding agencies, this may provide novel targets for future drug development.

HUMAN AFRICAN TRYPANOSOMIASIS

African trypanosomiasis or sleeping sickness is an ancient disease with reports of classic symptoms attributable to this disease being recorded in the fourteenth century. It is a disease characterised by long epidemic periods and there have been a series of particularly severe examples during the last 100 years, the last began in the 1970s and continues until the present time [1]. This upsurge in cases reversed the view that the disease was all but eliminated by 1960. Sleeping sickness re-emerged as a major health problem in recent years associated with population displacement, collapses of health systems caused by civil wars and other political problems in Africa. WHO statistics for 1998 estimate that there were 500,000 cases with 60 million people currently at risk of contracting sleeping sickness and, of these, only four million have access to diagnosis and treatment. The current epidemic affects people living in 36 Sub-Saharan countries, 22 of which are among the poorest in the world, including southern Sudan, northern Uganda, the Democratic Republic of Congo and Angola. Current information on the prevalence of the disease can be obtained from WHO and Médecins sans Frontières web sites e.g. http://www.accessmed-msf.org/msf/accessmed/accessmed.nsf/html/ 4DTSR2?OpenDocument.

Human African trypanosomiasis is caused by the trypanosome *T. brucei.* Two distinct forms exist, Trypanosoma *brucei gambiense* in West and Central Africa, and *Trypanosoma brucei rhodesiense* in East Africa. *T. b. gambiense* causes a chronic and protracted illness, which may last several years whereas that caused by *T. b. rhodesiense* is acute, and death may arrive in a matter of weeks or months. In essence, both forms of trypanosomiasis are fatal if not treated. An insect vector, the tsetse fly, spreads the trypanosome. There are seven different *Glossina* tsetse fly species, which can transmit the disease, and all are restricted to sub-Saharan Africa. Tsetse flies have a life span of between one and six months and, once infected, appear to maintain the infection [2]. The overlap of the favoured ecology of the tsetse flies (warm, shady, humid areas) with human activity defines, in part, the spatial pattern of disease. The disease progresses from a bite from an infected tsetse fly and moves through an initial stage, where trypanosomes multiply in the bloodstream and lymphatic system. The disease can then progress; the trypanosomes can cross the blood-brain barrier and invade the central nervous system. During this second stage the patient can present a variety of neurological symptoms and often exhibits characteristic signs such as an alteration of the circadian sleep/wake pattern. In the absence of treatment the disease progresses with wasting of body tissue, coma, and ultimately death.

DRUGS

The phenomenon of antigenic variation (see below) produces only a slim hope of a vaccine for human African trypanosomiasis. In the absence of this form of clinical intervention effective drugs offer the obvious major routes forward [3]. Here, the story is also not good (Figure 1). There is a clear need for new drugs.The currently used drugs are often toxic and difficult to administer, yet even with

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1381-6128/02 $35.00+.00

these caveats there is still a sad lack of access to these available drugs [4,5]. In addition, resistance to currently used drugs is a serious problem [6-8].

Drugs for Human AfricanTrypanosomiasis	
Pentamindine	Early stage *T.b.gambiense* Intramuscular injection
Suramine	Early stage *T.b.rhodesiense* Intramuscular injection
Melarsoprol	Advanced stages of disease/CNS involvement Both *T.b.gambiense* and *T.b.rhodesiense* Intravenous injection
Eflornithine	Late stage *T.b.gambiense* Intravenous infusion

Fig. (1). The current list of main drugs available for treatment of trypanosomiasis.

There are four main drugs available for the management of trypanosomiasis, which is a difficult disease to treat, particularly after the parasites have crossed the blood-brain barrier.

Pentamidine

Pentamidine isethionate is used for the treatment of early stage human African trypanosomiasis due to *T. b. gambiense* infections. It has been in use since the 1950s in various formulations and is administered daily or every other day by intramuscular injection, with a regimen of 7 to 10 injections. This puts the drug beyond reach for many.

Suramine

Suramine sodium is used for the treatment of early stage human African trypanosomiasis due to *T. b. rhodesiense* infections. It is administered by a single weekly intramuscular injection for 6 weeks.

Melarsoprol

Melarsoprol is an arsenic-containing compound used for the treatment of the advanced stage of the disease, when the central nervous system is affected, in both *T. b. gambiense* and *T. b. rhodesiense* infections. Again it has been used from the late 1940s and is administered by intravenous injections. Usually, 3 series of 3 injections are given with a 7 to 10 day rest period between each series. There are adverse reactions including the most serious side effect of reactive encephalopathy which occurs in 5-10% of the patients treated; 10-50% of these patients will die.

Eflornithine (DFMO)

Eflornithine is used in the treatment of late stage human African trypanosomiasis due to *T. b. gambiense* infections. It won regulatory approval in 1990. It is the only existing drug available for the treatment of patients not responding to melarsoprol. It is administered by intravenous infusions evenly spread every 6 hours during 7 or 14 days.

In addition to the often very serious side effects of Melarsoprol both Pentamidine and Suramine produce worrying adverse reactions. However, in the case of each drug there is a further major difficulty. This is the difficulty of ensuring continued production and supply of these drugs by manufacturers. These are complex issues and events, involving public/private initiatives and discussions. These have been rehearsed recently in both print and electronic form [9,10].

THE *T. BRUCEI* LIFE CYCLE

Examination of life cycle stages emphasises the specific shape changes that African trypanosomes undergo at particular transitions. The various differentiated forms, however, have such characteristic shapes and organelle positions that original cytological descriptions remain useful even after 100 years of study [11]. During their life cycle African trypanosomes make a series of transitions between three major environments: mammalian host bloodstream, tsetse midgut and tsetse salivary gland. The differentiations in the life cycle produce cell types characterised by particular properties such as shape, cell cycle control, metabolism, surface coat, etc. At two of these transition points, bloodstream to tsetse midgut and tsetse salivary gland to mammalian bloodstream, there is a specific pattern of events. In each case the transmitted parasites (stumpy form and metacyclic, respectively) exhibit particular, differentiated attributes useful to its survival in the next environment. On reaching that environment, the trypanosome then enters a proliferative cell cycle and colonises, respectively, the midgut or bloodstream. In one of the other transitions, tsetse midgut to the salivary gland, the parasite differentiates to an epimastigote form that uses its flagellum for attachment to surfaces. Thus the trypanosome life cycle is characterised by alternating patterns of proliferation and differentiation and a balance between cell cycle entry and exit [12-14].

On entering the mammalian host from the tsetse salivary gland the metacyclic trypanosome possesses a variant surface glycoprotein (VSG) cell coat[15]. These trypanosomes enter the cell cycle, start to express the bloodstream VSG coat and adopt a slender morphology. The population increases and a parasitaemia is produced. These trypanosomes have a suppressed mitochondrion and rely upon glycolytic pathways contained within membrane bound glycosomes. As the parasitaemia progresses long slender forms differentiate to short stumpy forms in an apparently density dependent manner. Short stumpy forms exhibit preadaptions for survival in the tsetse midgut by partial activation of the mitochondrion, exit from the cell cycle and other features [13]. On uptake by the tsetse in a blood-meal the stumpy forms are transformed to proliferative procyclic forms. These tsetse mid-gut forms replace the bloodstream VSG coat with another protein, procyclin [13,16]. From the procyclic population proventricular forms arise and these migrate, invade the salivary glands, attach and become epimastigotes. Recent data on this rather cryptic stage of the life cycle

suggests the presence of another cell cycle block whereby trypanosomes moving from the anterior midgut arrest and differentiate into a long epimastigote which then gives rise, by an asymmetric cell division, to two unequal, diploid daughter cells: a long, probably dead-end epimastigote and a short epimastigote [17]. The latter is envisaged as being responsible for the epimastigote colonization of the salivary glands. The proliferative epimastigotes are packed closely together during colonization of the salivary gland and it has been proposed that it is at this stage that genetic exchange occurs in a non-obligatory fashion. Finally, epimastigotes differentiate to form the detached, non-dividing metacyclic forms, which are infective to the mammalian host [18].

CELL STRUCTURE AND PROCESSES - INSIGHTS AND OPPORTUNITIES

Current research on the cell biology of African trypanosomes is revealing a vast array of intriguing and interesting insights to this organism's lifestyle as a parasite. In the following section I will briefly visit some of these. The list is selective and cannot be comprehensive (apologies in advance to those colleagues who find their favourite cellular process missing!). I have placed certain aspects together since a smaller set of important general themes then emerges. These themes are likely to represent areas of intense research activity over the next few years and ones, which are likely to benefit dramatically from the genome-sequencing project [19]. In some cases therefore these sections represent a rather personal view of opportunities likely to provide both new and deeper insights to the cell biology of the African trypanosome. There is much overlap between the sections. This serves to reinforce the view that our understanding of trypanosome biology is reaching an interesting maturation where it becomes truly integrative. Some areas should undoubtedly provide avenues for translational research in drug target identification and validation.

Antigenic Variation

Early last century classic experiments determined that the population density of parasites in the blood of mammalian hosts exhibited profound undulations of regular periodicity. A key to the biology underlying these events came with the discovery that up to 22 variable antigen types could be recognised from an infection with a single trypanosome. It was thought that a small subpopulation of trypanosomes might be able to alter their antigenicity, thus evading the host's immune response, which would eliminate the majority. This phenomenon is now termed antigenic variation. Elucidation of the molecular mechanisms by which the trypanosome achieves this strategy have provided intriguing insights to parasitism and to fundamental aspects of genomic plasticity and expression [20-25].

The surface of the African trypanosome is completely covered in a cell coat comprising one form of a variant surface glycoprotein (VSG). This is a membrane associated, glycosylated protein with a GPI anchor. *T. brucei* contains around 1000 chromosome internal copies of VSG genes with others positioned close to telomeres. Only one VSG gene is expressed in any one trypanosome at any one time and that is always located in a telomeric expression site (ES). Differential activation of individual VSG genes in the bloodstream trypanosomes occurs as a function of time; hence the population is able to evade the waves of antibody response. A variety of switching mechanisms have been shown to effect variation of antigenic surface coat in the bloodstream parasite population. Many of these mechanisms have been observed in so called "monomorphic" cell lines that fail to undergo the slender to stumpy transition characteristic of the parasite in the bloodstream. Such lines have usually been adapted to laboratory animals by rapid syringe passage without passing through the tsetse fly [26]. Recent evidence implicates a gene duplication process as the predominant switching mechanism used by "pleomorphic" trypanosomes that more closely resemble the natural bloodstream parasite [27]. There are around 20 different telomeric VSG expression sites in the trypanosome nucleus. Although a number (possibly all) of these can be used, only one is active at any one time. Hence a major question exists as to which expression site is chosen and how the trypanosome organises the activation of only one site at a time, leading to expression of a single VSG on the cell surface of that individual parasite [28]. This process provides enormous potential to evade the immune system of the mammalian host and, in essence, disables strategies for clinical control of trypanosomiasis based on vaccine development and mass vaccination strategies.

When trypanosomes are inoculated into the host by the tsetse bite they are already expressing a VSG and already have a surface coat. This VSG sub-type is expressed at the metacyclic stage in the tsetse fly salivary glands in preparation for inoculation into the human bloodstream. As an individual metacyclic parasite develops it initiates VSG expression at random from a small subset of metacyclic VSG (M-VSG) genes. This process arms the parasite for survival in the bloodstream and creates population diversity. M-VSG genes are activated in their telomeric sites as monocistronic transcription units, whereas the bloodstream VSGs are expressed from polycistronic transcription units in their telomeric sites [15,29].

Cell Type Differentiations

The *T.brucei* life cycle rehearsed above involves a series of successive changes of cell type and an alternation between proliferative and non-proliferative forms of the parasite. The cell type differentiations involve alterations in morphology, motility, metabolism (particularly with respect to oxidative phosphorylation and glycolysis) and, in some cases, exchange of the major surface coat protein [11,14]. The cell type differentiations that occur between the tsetse gut and mouthparts, and within the mouthparts, have been the most difficult to reproduce *in vitro* or to study at the cell biology or biochemical level *in vivo* [17].

Two major switches of cell type have been studied in some detail. These are the slender to stumpy bloodstream form differentiation and the bloodstream form to procyclic (tsetse midgut form) differentiation.

The differentiation from the stumpy form to the procyclic form is the most amenable trypanosome differentiation to study *in vitro*. The *in vitro* process is stimulated by the addition of citrate and *cis*-aconitate and a reduction in temperature from 37°C to 27°C. Studies of this differentiation over the last few years have produced a reasonably detailed temporal map of molecular, biochemical and cytological events accompanying the switch of cell type [13]. These include loss of the VSG coat, acquisition of the procyclin coat, kinetoplast positioning, changes in gene expression and metabolism and entry into a proliferative cell cycle from the G0 stumpy form [16]. This temporal map allows specific event markers to be defined for the differentiation process from stumpy forms. Recently these event markers have been useful in defining the influence of differentiation "triggers" on specific aspects of the process [30,31]. Further, recent work on the expression of the procyclin surface coat protein has revealed a hitherto unrecognised complexity. Procyclin exists in two forms encoded by separate gene families; each form being characterised by the nature of the peptide repeat within the molecule, EP or GPEET. Interestingly, there appears to be some expression and functional distinctions between the two forms of the coat protein. When the EP genes were deleted the resulting parasites behaved essentially as wild type in culture but showed a phenotype of reduced infectivity for tsetse flies [32-34].

The use of genetically modified cell lines to study this differentiation has been extended in other work with trypanosome clones selected for their inability to initiate differentiation to the procyclic form. Further modification of one such clone, DiD1, revealed that it was possible to re-acquire the pleomorphic form and that these trypanosomes were able to differentiate efficiently to procyclic cells [35].

The slender to stumpy form differentiation represents the second differentiation for which we have a reasonable understanding of some of the main events. It has its own particular set of characteristics that require explanation [13,36-41]. The differentiation does not involve a change of cell coat protein type but it does involve the change from a proliferative cell type to a non-proliferative cell type. A set of cytological and metabolic changes accompany these events. Unfortunately, the process has, until recently, been difficult if not impossible to study *in vitro*. The process of differentiation of the parasite population proceeds from mainly slender forms at the early stages of the parasitaemia to stumpy forms at late stages. Models of the acquisition of the stumpy phenotype in populations of different trypanosome cell lines have focussed on suggestions that the differentiation could be inherent or induced or at least influenced by population density.

We have concentrated on questioning the events of this differentiation at an individual cell level in order to reveal the type of division that might give rise to a non-proliferative stumpy trypanosome. We have used a variety of antibodies recognising a panel of proteins whose expression is altered in the process of the slender - stumpy differentiation. Examination of individual cells suggested that a `differentiation-division' exists in bloodstream trypanosomes. In this division a slender cell that has committed to differentiation undergoes a cell division to produce two daughter cells, at least one of which becomes blocked in the next cell cycle, does not divide further but continues on to undergo the full differentiation to a stumpy form [38].

Progress has been made in developing systems to interrogate this differentiation *in vitro*. These involve the ability to maintain bloodstream pleomorphic cell lines *in vitro* and the ability to differentiate such lines to stumpy forms *in vitro*. A long series of experiments suggest that a density sensing mechanism is involved in this differentiation and initial steps have been made in the

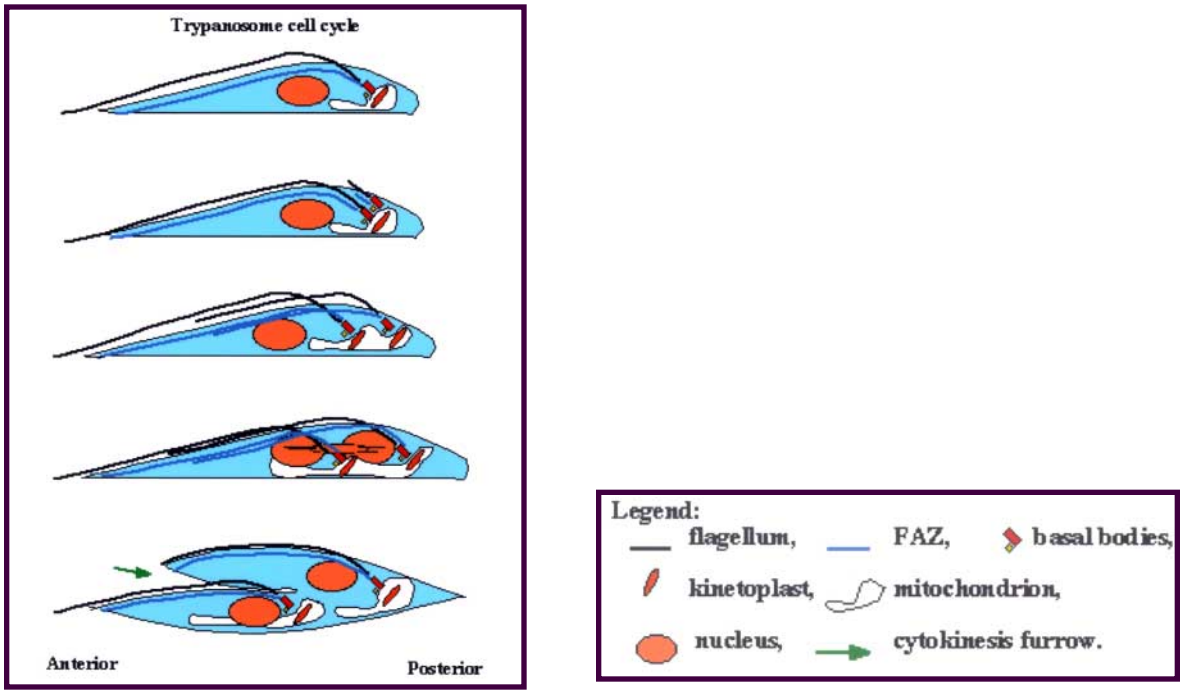

Fig. (2). Cartoon of the main structural events of the procyclic cell cycle.

definition of a putative "stumpy induction factor" that may orchestrate such events [36,37].

Microtubule Cytoskeleton

The overall shape of trypanosomes is defined by an internal microtubule cytoskeleton, whose most obvious feature is a sub-pellicular corset of microtubules [42]. These microtubules are cross-linked both to each other and to the inner face of the plasma membrane. Microtubules have a defined polarity and we have shown that those of the microtubule corset are orientated with their plus ends towards the posterior of the cell [43]. The trypanosome has an overall morphology that exhibits a wide central portion of the cell body, narrowing towards the posterior and anterior end, with the anterior end being the thinnest. Given this morphology and the fact that the inter-microtubule distance is constant at any point along the cell, microtubules clearly stop and start at different positions along the cortical array. New microtubules are inserted between old ones during the cell cycle resulting ultimately in a semi-conservative pattern of inheritance of the cortical array to each daughter at division [44-47].

In contrast to this the flagellum is inherited by the daughter cells in a conservative manner (Figure 2). This inheritance pattern is invariant. Trypanosomes at the start of their cell cycle possess a single flagellum and a new flagellum is formed alongside the old during the cell cycle. The new flagellum is positioned at the posterior of the trypanosome such that the daughter formed at this end of the original cell inherits the new flagellum and the daughter formed mainly at the anterior inherits the old flagellum. These events and their precise cell cycle timings have been studied in detail [45,48]. The flagellum of the African trypanosome protrudes from the cell through the flagellum pocket area and is attached to the cell body along its length.

The final cytoplasmic microtubule structure in the trypanosome is a set of four specialised microtubules that, in contrast to the others in the sub-pellicular array, originate close to the basal bodies in the flagellar pocket area, run around the flagellar pocket and then participate in the sub-pellicular array under the point of attachment of the flagellum [42,43,45]. These are specialised microtubules in both structural terms, in that they are closely associated with a smooth membrane vesicle, and in terms of their biochemistry. They are highly resistant to high salt treatments that depolymerise the other microtubules of the subpellicular array. These four specialised microtubules together with a complex filament constitute the flagellar attachment zone (FAZ). The FAZ defines a particular position in the sub-pellicular corset and the FAZ filament underlies the precise position where the plasma membrane of the cell body and the flagellar membrane are closely opposed [49,50].

These structural features of the cytoskeleton are now being defined in terms of their molecular components. Thus, in this as in other areas of trypanosome cell biology a molecular cytology is emerging [42,49]. This molecular cytology has often been aided by the use of monoclonal antibodies to define particular structures and then their use to identify proteins and their encoding genes. Definitions of structures will ultimately lead to functional interrogations via use of reverse genetics. In this respect we have been able to identify a number of components of the FAZ filament and the area that connects the flagellum and the cell body membrane (Figure 2). The connection between the cell body and flagellum occurs via a series of 25 nm diameter junctional complexes on the cytoplasmic side of each membrane. They are spaced with a centre-to-centre periodicity of 95 nm along a line defined by the FAZ filament. The FAZ filament contains proteins possessing repetitive motifs that appear to be very immunogenic. Immunological approaches reveal a number of proteins that locate to this region of the *T. brucei* cytoskeleton. We have used antibodies to a 200kDa protein to define the morphogenesis of the FAZ filament in relation to other cell cycle events in *T. brucei* [50]. Since the FAZ filament extends from the basal body region to the anterior tip of the cell we have predicted that it may be responsible for defining the required positional information at the "distant" anterior end of the cell where cytokinesis must be initiated between the tips of the old and new daughter flagella [42,43,51].

Microtubules are composed of repeating subunits of alpha and beta tubulins that are encoded in a cluster of tandemly repeated alpha/beta pairs [52,53]. Our recent high resolution imaging of DNA spread directly from the nucleus has shown a maximum of 19 repeats [54]. Both alpha and beta tubulins undergo post-translational modifications. The description below details the position in *T. brucei,* however, these modifications are found in many organisms and there has been widespread interest in their occurrence and possible function [42]. In many cases our understanding of the modifications has been greatly assisted by the use of monoclonal antibodies that recognise the modified form of tubulin. An acetylation of alpha tubulin at lysine 40 was identified initially because of the concomitant shift of the alpha tubulin spot on 2D gels [55,56]. Acetylated alpha tubulin is associated with both the sub-pellicular and the axonemal microtubules and acetylation occurs coincidently with assembly of axonemal microtubules and the modification can be reversed upon microtubule disassembly[57].

The genome encoded C-terminal tyrosine of alpha-tubulin can be removed by a carboxypeptidase and replaced by a tubulin tyrosine ligase enzyme [46]. Detyrosination of alpha-tubulin occurs after microtubule assembly and therefore tyrosinated alpha-tubulin acts as a molecular marker for new microtubules. Monoclonal antibodies specific for tyrosinated alpha tubulin reveal a cell cycle related modulation of microtubule assembly, shown most clearly in some cells in mid-cycle where the old flagellum is not detected but the extending axoneme of the new flagellum is [46]. This monoclonal antibody approach also allowed us to define the pattern of assembly of microtubules in the sub-pellicular cortex, showing that new microtubules invade the cytoskeletal array between old microtubules [44].

Protein sequencing and mass spectrometry has shown that beta tubulin can be detyrosinated and that both tyrosinated and detyrosinated alpha and beta tubulins are

extensively glutamylated. Polyglutamylation adds multiple negative charges in the already very acidic carboxy-terminus of tubulins. Glutamate residues are added to the γ-carboxyl group of a defined glutamate near the carboxy-terminus of both tubulins (residue 445 of alpha tubulin and 435 of beta tubulin) via its α-carboxyl group (γ/α linkage). The maximum numbers of glutamyl residues in the lateral chain are 15 and 6 for alpha- and beta-tubulin respectively [58]. We have little idea of the function of these modifications in trypanosome biology. Identification of the enzymes responsible will offer opportunities to reveal the rather cryptic functions of these enigmatic modifications.

Until recently we knew of three tubulins in *T.brucei*: alpha, beta and gamma. Alpha and beta constitute the subunits of the microtubule wall and gamma is a minor tubulin responsible for microtubule nucleation [59-61]. A fourth member of the tubulin family (delta tubulin) was originally described by analysis of a mutation in *Chlamydomonas* [62]. The *uni3-1* mutation in the delta tubulin gene produces cells which exhibit zero, one or two flagella instead of the consistent two of wild type *Chlamydomonas.* Electron microscopy revealed that the basal bodies of the mutant possessed doublet rather than triplet microtubules at their proximal end. We were interested to determine whether trypanosomes possess a delta tubulin and mounted a PCR based search for this gene using knowledge of conserved sequences in the tubulin super-family. This led to the identification of not only the trypanosome delta tubulin gene but also of two completely novel tubulin genes: epsilon and zeta [63]. *T.brucei* epsilon tubulin was recognised to have homologues in the human EST databases, whilst zeta tubulin seems so far to be found only in the genome databases of only kinetoplastid protozoa [63,64]. Thus, so far *T. brucei* is the only organism in which our bioinformatics analysis indicates the presence of six tubulins. No doubt this picture will develop as we get more full genome sequence data on *Leishmania* and other organisms. We are currently engaged in analysis of the position of these tubulins in the trypanosome cytoskeleton and their functions. Given that *S. cereviaiae* and *C. elegans* contain genes for only alpha, beta and gamma tubulin in their genomes then these appear to be the minimal set of tubulins required by eukaryotic cells. The trypanosomes express a flagellum as well as the set of four specialised microtubules and one might predict that the delta, epsilon and zeta tubulins will turn out to have functions related to these differentiated microtubule structures [63].

Microtubule mediated functions rely upon an additional set of microtubule associated proteins (MAPs) that are diverse in nature and position. They include the kinesin and dynein motor proteins. There is some experimental evidence for the identity of MAPs in the microtubule cytoskeleton. Often this has come from the fact that they appear to include repetitive elements that are highly immunogenic. The current catalogue of MAPs has been reviewed recently [42,49].

Microtubule inhibitors have been useful in defining the mechanisms whereby microtubules orchestrate events in the trypanosome. In addition biochemical analyses have shown that there are intriguing differences between the drug sensitivity of mammalian tubulin and that of trypanosomes. *T. brucei* tubulin has been purified and assembled into microtubules *in vitro* [65]. This allowed a direct test of the effectiveness of microtubule inhibitors. Both colchicine and the benzimidazoles, classical and potent inhibitors of mammalian tubulin polymerisation, had only very slight effects on the polymerisation of trypanosome tubulin. This result fits with the well-established view that protists are often remarkably resistant to the action of colchicine. The benzimidazoles represent a range of compounds with often-discrete effects on different organisms [66,67]. The trypanosomatids are rather resistant to the action of a range of benzimidazoles, although some protozoa such as *Giardia* and *Trichomonas* are susceptible to some of these inhibitors.

Another group of inhibitors, the Vinca alkaloids (vinblastine and vincristine) and the macrolides (maytansine, ansamitocin and rhizoxin) are effective inhibitors of trypanosome tubulin [43,65,68]. Vinblastine and maytansine have been shown to be good inhibitors of *T. brucei* tubulin polymerisation *in vitro.* Molecular analysis of rhizoxin sensitive and resistant organisms and mutants has shown that sensitivity to this drug depends on the location of an asparagine residue at position 100 in beta-tubulin The trypanosome beta-tubulin sequence has an asparagine at this site and studies with *T. brucei* have shown that this compound is a powerful anti-microtubule agent. Other inhibitors, with a range of effectiveness against trypanosome tubulin, are the range of herbicides including trifluralin [42].

In contrast to these anti-microtubule agents that effect cytoskeletal systems by inhibiting tubulin polymerisation, depolymerising or destabilising microtubule arrays the compound taxol acts by stabilising microtubule arrays. It is effective against trypanosomes and produces interesting and informative effects on cytokinesis.

The Flagellum

T. brucei is characterised, throughout its life cycle, by the possession of a flagellum attached along its length to the cell body. The flagellar axoneme has the classical 9+2 microtubule structure. It is subtended by a basal body at the posterior end of the cell and exits the cell body through the flagellar pocket. Cells at early stages of the cell cycle possess a single flagellum and a single basal body with an associated probasal body. During the G1 to S transition in the cell cycle the probasal body matures to nucleate the assembly of the new flagellum, and two new probasal bodies are elaborated, one associated with each basal body (Figure 2). During the next period of the cell cycle the new flagellum extends and the basal bodies move apart to define the particular positions influencing cell cleavage and kinetoplast segregation [45,69].

Trypanosome flagella are characterised by the possession of a paraflagellar rod (PFR). This large, complex, highly organised structure runs alongside the axoneme from the point the flagellum emerges from the cell body [70]. The overall diameter of the PFR (150 nm) is constant throughout its length. In cross-sections, 3 different zones termed proximal, intermediate and distal, can be defined by their position relative to the axoneme (Figure 3). The proximal

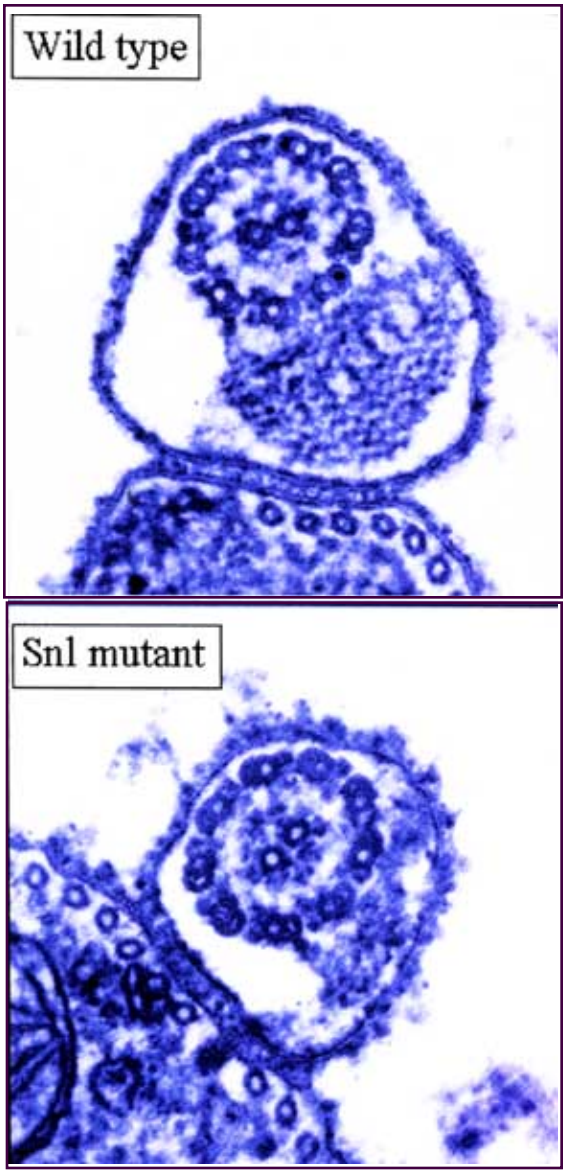

Fig. (3). Electron micrographs of transverse sections of the flagellum of wild type and a paralysed Snl mutant in *T. brucei*. The wild type cell shows the paraflagellar rod and the axoneme, whilst the paraflagellar rod struct. is ablated in the mutant cell.

and distal domains are characterised by filaments having a diameter of 7-10 nm and which intersect at an angle of 100 degrees, explaining the lattice-like aspect of this zone of the PFR. In the intermediate domains, filaments are thinner (5 nm) and intersect with an angle of 45 degrees. The proximal domain is connected to the axoneme, at a constant position between microtubule doublets numbers 4 to 7, and specific filament connections can be seen to these doublets. Other filaments connect the proximal domain to the inner face of the flagellar membrane at the point of the flagellum attachment zone (FAZ) connection to the cell body [49,50].

Initial isolation of the PFR from kinetoplastid cells showed the major protein components to be a doublet of electrophoretic motility (Mr) of 68,000 and 76,000 [70,71]. In *T. brucei* this pair of protein constituents have been termed PFR-A and PFR-C. Molecular characterisation of their genes showed that PFR-A and PFR-C share extensive similarity throughout their length [72]. There are undoubtedly other minor components of the PFR and some of these are known. Minor PFR proteins have been identified by the use of antibodies: the ROD-1 antigen and the I2 antigen. Both are large proteins (Mr 180,000-200,000 and Mr 300,000 respectively) with a varying number of repeated sequences showing no similarity to other known proteins. The antigen recognised by the ROD-1 monoclonal antibody is localised only in the distal domain whereas I2 is found throughout the PFR structure. The catalogue of other PFR proteins in *T. brucei* and other kinetoplastid parasites has been reviewed recently [42,70].

We have recently studied the morphogenesis and function of the PFR in some detail. To do this we have taken advantage of the extensive techniques now available for reverse genetic analysis in trypanosomes. These studies also led us to understand and exploit the new phenomenon of RNA interference whereby effective gene silencing permits the production of mutant phenotypes without resort to gene deletion [73-76]. Time-course inducible expression of epitope-tagged PFR-A protein [77] provided an excellent system to follow recently synthesised PFR-A subunits. When expression of the tagged PFR-A protein was induced for a short period in a trypanosome growing a new flagellum, subunit addition sites could be visualised by subsequent staining with an anti epitope-tag monoclonal antibody. Two addition sites were identified in the elongating flagellum, one at the distal tip and the other one along the length of the PFR. Mutant analysis of the PFR protein allowed a molecular dissection of these events [70,73,74,77-79].

The PFR-A protein is encoded by a set of 4 tandem genes, with two such clusters being present in the diploid genome. The use of antisense technology to down-regulate the expression of PFR-A protein and so affect the structure and function of the PFR itself was not successful. PFR-A antisense constructs expressed from “ectopic” sites in the genome produced no phenotype in the transfected trypanosomes. However, in one such experiment we identified a rare clone (the Snl1 mutant) that exhibited a very strong phenotype [73]. It was viable but almost completely paralyzed. Biochemical analysis revealed that expression of the PFR-A RNA and protein was essentially ablated. Structural analysis by electron microscopy and immunofluorescence microscopy revealed that the Snl1 mutant lacked most of the PFR structure (Figure 3).

Our molecular analysis revealed that in the Snl1 mutant the transfected antisense construct had inserted in the genome in one of the two PFR-A gene clusters [73]. We conjectured that the result of this insertion, that produced a strong promoter operating in the opposite direction and driving transcription overlapping normal PFR-A expression, could indicate "antisense interference with early processing". Recently, the description of a phenomenon called RNA interference (RNAi) has extended conventional views about antisense mechanisms. The introduction of double-stranded RNA into organisms leads to a specific, potent and rapid degradation of the corresponding mRNA [74,76,80-90].

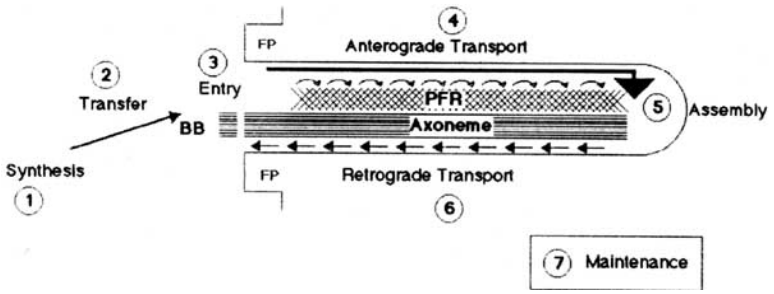

Fig. (4). Cartoon of the main events likely to be occurring during morphogenesis and maintenance of the trypanosome flagellum (from Bastin and Gull, 1999. Ref 72.).

Our analysis of the genotype of the paralysed snl-1 mutant [73] suggested a number of possibilities whereby expression of the antisense constructs from this genomic environment would provide possibilities for local dsRNA formation. We tested and verified this hypothesis by generation of a new cell line (*snl*-2) expressing an RNA containing linked copies of sense and antisense PFR-A from a tetracycline-inducible promoter [74]. Induction of expression of this PFR-A dsRNA reproduced PFR-A ablation, disappearance of the PFR itself and cell paralysis as in the *Snl1* mutant. However, because of the inducible vector the wild-type population phenotype was recovered upon removal of the inducer (tetracycline). These results indicate the usefulness of a heritable and inducible RNAi system for interrogation of gene function in parasitic protozoa. We envisage that this method [74,76] should be extremely useful in the post-genomics analyses referred to later.

Our analysis of the PFR mutants in *T. brucei* has been instructive in terms of understanding both PFR morphogenesis and function. However, it has also provided insight to more general aspects of flagellum assembly and motility.

For perhaps too long the flagellum of parasites has often been viewed mainly as an organelle for cellular locomotion. Undoubtedly motility is important at times in the life cycle of parasites such as (in the African trypanosome) during invasion of the bloodstream from the chancre at the site of the tsetse bite, also in the movement from the tsetse midgut to the salivary glands. But I suggest that the flagellum is much more than a motility organelle.

The flagellum is a ribosome-free zone! Thus flagellar precursors are synthesised in the cytosol and then targeted to the flagellum compartment. We have recently outlined (Figure 4) a series of steps that are likely to be involved in flagellum morphogenesis, maintenance and turnover in the trypanosome (and other organisms). After synthesis in the cytoplasm proteins are presumably transported to the basal body area and imported into the flagellum compartment. This is followed by anterograde transport towards the addition site (the distal tip or along the already elaborated flagellum length) where assembly occurs. In addition, there is evidence for retrograde transport of components and presumably some longer-term turnover mechanism exists for refurbishing the completed flagellum [72]. The molecular motors operating to move materials in the anterograde and retrograde directions during a process of intraflagellar transport have started to be recognised. We can recognise the action of these motors by analysing the phenotypes of the *Snl1* and *Snl2* mutants in *T. brucei.* These paralysed mutants, referred to earlier, lack most of the paraflagellar rod and do not express the PFR-A protein. A particular feature of their phenotype is the occurrence of a blob of unassembled PFR components that accumulates at the tip of the new but not the old flagellum during the cell cycle. This material appears to be transported to the growing tip of the flagellum but does not assemble in the absence of the PFR-A protein [70,73,74,79]. The genome project has already identified many flagellar proteins, recognised by homology to genes previously studied in other systems. The use of the inducible RNAi system that proved so useful for dissection of PFR function is likely to be useful in providing insight to the functions of particular genes. It will also be useful in uncovering the molecular mechanisms that orchestrate the series of flagellar targeting, transport and assembly phenomena outlined above.

An often-ignored function of the flagellum is that it provides the trypanosome with a mechanism to differentiate the environment of its surface membrane. Because of the ciliary necklace connections at the basal body region of cilia and flagella, the membranes of the flagellum and the cell body are contiguous, yet able to be differentiated. In trypanosomes the major VSG or procyclin coat proteins cover both the cell body membrane and the flagellum membrane. There is evidence that, as in other systems, the flagellar membrane enables the specific localisation of particular proteins. One example of this localisation is that of adenylate cyclase on the flagellar membrane. [25,91]

The ability to locate a specific translocator, receptor and signalling system in a membrane domain may have particular benefits in host/parasite, parasite/parasite and parasite/ environment interactions. One could imagine reasons for requiring differential transport of small molecules, mediated by translocators that vary between the flagellum membrane and the cell body membrane, yet in

both locations are hidden from the host immune response by the surface coat. Receptors for macromolecules such as transferrin and lipoproteins from the surrounding plasma are hidden to some extent from the immune response by being located in the flagellar pocket. The flagellum allows *T. brucei* to develop these three distinct plasma membrane domains: the cell body, the flagellar pocket and the flagellum. Molecular mechanisms and signals likely to be important in targeting of particular proteins to particular regions of the three major plasma membrane domains of the trypanosome and the flagellum cytoskeleton are becoming clearer, but general paradigms have yet to emerge [92-102]. I suggest that there are really two questions here. First, why are certain proteins/structures targeted to particular regions of the plasma membrane? Second, why are these proteins/structures not allowed to locate to other parts of the plasma membrane? A specific illustration of this point is the question of why microtubules are located on the inner face of the cell body membrane but not that of the flagellar pocket?

The intimate relationship of the surface membrane and the microtubule cytoskeleton, both in the cell body and the flagellum, suggests that these microtubules may assist in directing membrane protein movement. The existence of the intraflagellar transport phenomenon has been referred to earlier in the context of matrix molecules such as PFR-A. However, it is likely that such motility functions may mediate membrane protein targeting and movement. Likewise the fact that we have been able to define specific polarities for the microtubule sub-sets of the subpellicular array provides a structural basis for the action of plus end and minus end directed motors in the flagellum pocket area. When we determined the polarity of the trypanosome microtubules we recognised the importance of the fact that the four specialised microtubules have the opposite polarity to the main microtubule cohort and that they are more intimately associated with the basal body/flagellar pocket area [43]. We recognised that these points suggest mechanisms for setting up directed membrane trafficking and other polarity dependent phenomena during interphase and division [43]. Apart from membrane organelle positioning, this architecture could underlie phenomena such as the observed capping and internalisation of antibody molecules, receptor cycling, directed access and egress from the flagellum pocket, etc [14,42,103].

The *T. brucei* flagellum is used for attachment of the parasite to the tsetse mouthparts at the epimastigote stage of the life cycle. Obviously the microtubules underlying the cell body membrane prohibit the formation of complex "hemidesmosomal-type" plaque structures between the parasite and the host surface. Attachment of *T. brucei* epimastigotes to the salivary gland epithelial brush border is mediated by rather elaborate, branched outgrowths of the flagellar membrane. These outgrowths diminish but the attachment plaques are maintained as the parasite differentiates to the nascent metacyclic form before the eventual release of the metacyclic parasite. Epimastigote forms are proliferative and therefore able to divide whilst attached and it has been suggested that this mode of attachment facilitates cell division [42]. Detergent extraction reveals the detailed sub-structure of filaments and electron dense attachment plaques [104] but the biochemical nature of the components of both are unknown.

Membrane Bound Organelles and Metabolic Specialisations

Two organelles have received considerable attention: the mitochondrion and the glycosome [105-114]. Kinetoplastids are exceptional with respect to the structural arrangements for glycolysis. In other eukaryotes the enzymes of the glycolytic pathway are cytoplasmic. However, in trypanosomes the first nine enzymes of glycolysis are localized within a single membrane bound organelle, the glycosome. Since compartmentalized glycolysis appears highly efficient, it is surprising that it is limited solely to kinetoplastids. Import of nuclear encoded proteins to glycosomes has shown that the import signals bear similarities to those of peroxisomes.

Both organelles are heavily reliant on proteins encoded in the nuclear genome and, in the case of the mitochondrion, RNA molecules also have to be imported from the cytoplasm. Various studies have detailed this import process for both organelles and specific targeting sequences have been identified and characterised. The metabolic compartmentalisation and specialisations of these organelles have long been suggested as providing opportunities for novel drug targets [115,116].

Classic descriptions of the biology of *T. brucei* revealed a life cycle stage modulation of mitochondrial activity [11]. The fully active respiratory chain of the procyclic form mitochondrion contrasts with the inactivity of the bloodstream slender form parasite. Mitochondrial enzyme activity modulation is an intrinsic component of the cell-type differentiations referred to earlier. The single mitochondrion of *T.brucei* displays a number of peculiarities common to other kinetoplastids. The organisms are defined by the presence of a highly organised mitochondrial DNA structure, the kinetoplast. The kinetoplast is composed of a network of catenated circular DNA molecules - minicircles and maxicircles. There are approximately 50 maxicircles which are essentially identical and resemble conventional mitochondrial DNAs, however unlike many other mitochondrial genomes no tRNAs are kinetoplast encoded. The *T.brucei* kinetoplast also possesses many thousand copies of minicircles which fall into some 400 different classes. Minicircles are only 1Kb to 1.5Kb in length and encode multiple small guide RNAs involved in the post-transcriptional process of RNA editing [114,117,118]. This mass of DNA molecules is segregated as a unit and is replicated in a unit period of the cell cycle [43,48,69]. This must involve a high level of nuclear, mitochondrion and cytoskeletal coordination in the cell cycle: I have termed this the "two unit-genome replication / segregation problem" [14].

The genome of the trypanosome mitochondria is expressed via the process of RNA editing. The uridine insertion/deletion RNA editing is a post-transcriptional RNA maturation process that involves the addition or removal of uridine residues at precise sites, usually within regions transcribed from maxicircles. The process is achieved

by use of many short, overlapping complementary guide RNAs encoded in both the maxicircle and the minicircle molecules and it proceeds by a series of enzymatic cleavage-ligation steps. Whilst the details of the process have become clear over the past decade there is still little known about the high order organization of the editing complexes and protein biochemistry. Studies in this area illustrate the increasing integration that I alluded to previously in our understanding of *T. brucei* cell biology. It has been known for some time that kinetoplast mRNAs are edited differentially during the life cycle of the parasite. Modulation of the editing pattern suggests that editing assists in regulating the two modes of mitochondrial metabolism referred to above. Thus, studies of regulation of this process speak also to issues of both metabolic control, cell differentiation and parasitism.

The Trypanosome Nucleus and Genome

Chromatin of the trypanosome nucleus is clearly arranged in nucleosomes and both conserved and divergent histones have been identified [119]. The use of reporter gene constructs has enabled the identification of monopartite and bipartite nuclear import signals in the sequence of *T. brucei* nuclear proteins [120]. *T.brucei* has a diploid genome with an estimated haploid DNA content of around $3.5 - 4 \times 10^7$ bp. Whilst the chromosomes do not condense at mitosis the molecular karyotype can be visualised by pulsed field gel electrophoresis. The nuclear genome consists of three chromosome classes, megabase, intermediate and minichromosomes (Figure 5). The megabase chromosomes of this diploid genome contain the expressed genes and form at least 11 homologous chromosome pairs [19,121]. Strain dependent chromosomal length polymorphisms exist for this chromosomal set. The ploidy and function of the intermediate chromosomes is unclear, though they contain variant surface glycoprotein (VSG) gene expression sites. The 100 or so minichromosomes, which are between 30 and 150 kbp in size, constitute the group of smallest chromosomes. The sequence of a minichromosome mainly consists of a 177-bp tandem repeat, with the remaining sequence consisting of other repeats and a silent subtelomeric *VSG* gene. The presence of VSG genes on minichromosomes has led to the suggestion that they can act as a reservoir of these genes that may be transferred to expression sites.

The *T. brucei* genome

Megabase chromosomes:
- 11 pairs at least
- diploid
- 1-6 Mb
- length polymorphisms
- haploid VSG gene expression sites at telomeres
- expressed genes and repetitive sequence blocks
- interior VSG gene clusters

Intermediate chromosom es:
- 200-900kb
- number and size variation
- ploidy?

Minichromosomes:
- Linear
- 30-150kb
- 177bp repeat (~90% of sequence)
- GC and AT rich repeats
- silent telomeric VSG gene
- 10-20% genome
- ploidy?

Fig. (5). The various classes of chromosomes of the *T. brucei* genome and their properties.

Light and electron microscopy of the mitotic nucleus clearly shows the presence of a microtubule spindle enclosed within a closed nuclear envelope [122]. The organisation of megabase chromosomes and minichromosomes in interphase nuclei, and their subsequent behaviour during mitosis, has been characterised by fluorescence *in situ* hybridisation [122-124]. Electron microscopy has revealed kinetochores with attached microtubules. However, it is not clear how the large number of megabase chromosomes are segregated with what appears to be a small number of visible kinetochore structures. A model has been proposed to explain how a faithful segregation of large numbers of minichromosomes could occur along the central mitotic spindle [125]. In this model, minichromosomes are assumed to form lateral associations with the spindle microtubules instead of the terminal associations seen in classical kinetochore-mediated chromosome segregation.

The megabase chromosomes have telomeric sites for the expression of both metacyclic and bloodstream VSG genes (see earlier). The main internal stretches of these chromosomes however, are organised with closely packed genes expressed as large polycistronic units. Processing to mRNA involves trans splicing and poly (A)+ addition[24]. Rare cis-splicing events are now known to occur during the processing of some genes[126].

Whilst we have learnt much about the trypanosome genome and its expression we have little information pertaining to the high order structure of the nucleus. This is becoming a serious deficiency. There are suggestions that new insight to the control of VSG expression and the recombination events surrounding VSG switching may come from a clearer understanding of the location of individual telomeric sites. Whilst expression sites exist on the ends of many megabase and intermediate chromosomes there is only one actively expressed telomeric site in a trypanosome at any one time. This raises the possibility of a unique nuclear structure, capable of housing only one chromosome telomere at a time, and representing the site from which expression can be orchestrated. Whilst we have some insight to chromosomal arrangements in the interphase and mitotic nucleus a new level of detail is much needed. In a similar manner a much more detailed understanding of chromosomal segregation at mitosis would assist in understanding chromosomal dynamics and polymorphisms in wild type populations of parasites.

I include sex in this discussion of the requirement for a better understanding of high order organization of the trypanosome nucleus! A view that a form of meiotic recombination exists in the African trypanosome is

reasonably well established [127-130]. The best evidence suggests that this non-obligatory aspect of the life cycle occurs in the tsetse fly after the midgut stage. Given this, the phenomenon is unlikely to be of great use in providing a genetic route to unraveling the role of particular genes in cell biological processes. However, a better understanding of the cell biology of the process would provide a clearer view of its significance in the acquisition, spread and evolution of phenomena directly relevant to parasitism and parasite fitness.

Protein Targeting and Trafficking

This is an area of long standing interest in *T. brucei* as researchers have sought to understand how the surface coat proteins in particular are transferred through the secretory system, the flagellum pocket and onto the parasite surface. It has been reviewed above in other sections and here I wish only to draw attention to a few particular results to emphasize the general concepts that are emerging. It is an area of increasing activity since these mechanisms of targeting and trafficking underpin some critical parasite functions.

T. brucei has a well-developed endomembrane system responsible for secretion, uptake and recycling of molecules. The flagellum pocket is the site of this activity. Both bloodstream and procyclic trypanosomes are capable of internalizing macromolecules from the surrounding medium. Whilst it has been generally recognized that structures such as clathrin-coated vesicles are more prevalent in bloodstream forms it is now clear that receptor-mediated endocytosis does operate in procyclic forms. Evidence has been presented that the flagellar pocket associated cysteine-rich acidic transmembrane protein (CRAM) may function as a high-density lipoprotein receptor in the procyclic form trypanosome and that uptake of anti-CRAM IgG in procyclic trypanosomes occurs via receptor- mediated endocytosis of the CRAM protein [102,131]. There is increasing evidence, both experimental and from database homology searches that *T. brucei* possesses most of the general enzyme/receptor/signaling and processing functions required for an extensive array of targeting, secretion, endocytosis and recycling phenomena [70,103,110-112,120,132-136]. The complexity of these systems in trypanosomes is probably more extensive than that in microbes such as yeast.

A number of studies of kinetoplastids in general have discovered a series of molecular events and signals that locate proteins to particular locations in the flagellar pocket/flagellum or cell body membrane. In *Leishmania* the major glucose transporter exists in two isoforms differing only in their cytosolic NH2-terminal domains [101]. One (iso-1) localizes to the flagellar membrane, while iso-2 localizes to the plasma membrane of the cell body and is associated with the underlying microtubules. Deletion of an N - terminal region from iso-1 targets the protein from the flagellum to the cell body plasma membrane and an association with the cytoskeleton. These results suggest cytoskeletal binding serves as an anchor to localize the iso-2 transporter within the cell body plasma membrane, and that the flagellar targeting signal of iso-1 diverts it into the flagellar membrane.

Cell Cycle Control and Organelle Interactions

The trypanosome possesses two unit-genomes - a nuclear genome and, in contrast to many other eukaryotes, a mitochondrial unit-genome in the kinetoplast. Both genomes are replicated periodically in the cell cycle [48]. Immunofluorescence detection of bromodeoxyuridine incorporation into replicated DNA allowed us to determine the timings of such events in the *T. brucei* cell cycle. The configuration of the nucleus and kinetoplast allows one to separate the cell cycle into four morphologically discrete periods, classifying cells as 1KIN; 2KIN; 2KmitoticN and 2K2N. The cell cycle timing data suggests that DNA synthesis is triggered in the mitochondrion at essentially the same time as in the nucleus, and suggests a unique regulation of events within the trypanosome cell cycle. The segregation of the two genomes takes place in a specific temporal order by interaction with microtubule-based structures, the spindle for nuclear DNA and the flagellum basal bodies for the kinetoplast DNA [43,69,122,124].

There are three major questions relating to the replication timing of the mitochondrial genome. First, how is the periodic replication orchestrated and second, is it co-ordinately regulated with nuclear S phase or merely coincidental? Finally, what (if any) regulatory and checkpoint steps operate to provide links to other cytoplasmic events such as basal body replication and cytokinesis?

This is the first (replication) aspect of the "two unit-genome replication/segregation problem". The second (segregation) involves the challenge to understand how the kinetoplast is linked across two mitochondrial membranes to the flagellum basal body [42] and how the new basal body acquires a kinetoplast connection. Finally, there is a need to understand what regulatory pathways/checkpoints exist to connect basal body segregation with cell cycle events such as nuclear division and cytokinesis?

Since we provided the detailed mapping of timings and event markers in the *T. brucei* cell cycle a number of studies have addressed the regulatory links and the molecules that might orchestrate them. In addition to experimentally derived evidence on the identity of cell cycle genes such as cyclin-dependent kinase complexes, both kinases and cyclins can be identified in the genome project as homologues of those with known functions from other organisms [137].

We have recently used drug inhibition studies to reveal how cell cycle events are co-coordinated. We have shown that *T. brucei* cytokinesis is not dependent upon either mitosis or nuclear DNA synthesis, suggesting that there are novel cell cycle checkpoints in this organism. These and other experiments raise a possible dominant role in trypanosomes for kinetoplast/basal body segregation in control of later cell cycle events such as cytokinesis [68].

Genomic and comparative genomic analyses are likely to prove of great value in identifying nuclear encoded gene products likely to be involved in mediating and regulating kinetoplast DNA synthesis as well as those coordinating these events with nuclear S phase.

Parasite/Host and Parasite/Vector Interactions

Whilst there are reasonable descriptions of clinical events related to disease progression in African trypanosomiasis we still require a better understanding of the molecular cell biological events that underpin the interaction of the parasite with the mammalian host and tsetse vector.

These processes are too extensive to review fully here. However, they include the determination of virulence factors, receptors, and recognition factors important for attachment, invasion and survival. We already have a good catalogue of surface receptors, channels and transporters. There is no doubt that the genome projects are likely to assist in identifying missing proteins. I suggest that these may include receptors for such events as the attachment of the trypanosome flagellum to the salivary gland surface, receptors for possible directed, tactic movements between different sites in the tsetse and those that direct movements (traversing the blood / brain barrier) in the mammalian host.

VSG antigenic switching represents a highly developed attribute that has enormous impact in the virulence and pathogenicity of *T. brucei*. We know little about the virulence factors other than recent work on the resistance to lysis by human serum [138-142]. This area of defining virulence factors should be assisted by the descriptions of the *T. brucei* genome by the genome-sequencing project. In addition comparison with the *Leishmania* genome project should be illuminating. Here experimental assessments of aspects of virulence in different *Leishmania* species have led to intriguing results [143-145]. Amongst other areas of the cell biology of trypanosomes that await clarification are the molecular identification and significance of the putative "stumpy induction factor" whereby there is a possibility of a trypanosome: trypanosome interaction biology being defined. Of course, one of the most significant areas is the interaction of the trypanosome with the host's immune system. Although much progress has been made in this area recently one imagines that there is still much to clarify that is of direct clinical relevance [146-151].

The Genome Project, Genomic, Proteomics and Bioinformatics

I have referred earlier to aspects of the biology of trypanosomes that I expect to be assisted by being able to scan the complete genome. Thus the present *T. brucei* sequencing project is a critical step in understanding the biology of these parasites. It needs to be completed with all haste! The heavy investment, made over the past 15 years by a number of laboratories, in developing molecular techniques applicable to *T. brucei* is now key to the identification of gene function. Many technical approaches to these studies (gene-knockouts, RNAi and conditional expression) are now in place. These technologies are important for academic studies of parasite functions but will be critical to the development of target validation studies in drug therapy initiatives. Starting out to design an effective drug for a non-essential gene function is clearly a waste of effort! It is now possible to address these questions of gene product inhibition directly and early in a drug discovery programme. This has consequences for the management of such programmes (see below).

The application of global gene expression surveys by genomic array technologies could be extraordinarily powerful. It has potential for providing insight to unique areas of the parasites biology, particularly those occurring in the tsetse stages. The caveat however, is that the organism's differing reliance on both transcriptional and post-transcriptional levels of gene regulation may complicate these approaches. I have rehearsed previously some of the opportunities and caveats of these developments [14,152]. They will need careful and rigorous registration of growth conditions, physical and media factors, strains and clones used, parasite cell types, life cycle stage, etc. Without such registration of the experimental conditions we are likely to amass a lot of isolated data sets that provide little added value and much uninterruptible data! I suspect that major developments that will come from greater emphasis on the concept of "wild type" and better, more defined *in vitro* and animal models of African trypanosomiasis.

Dealing with the mass of orphan genes that will emerge from the *T. brucei* sequencing project is a major challenge since within this may lie the new and specialised biology that defines some of the best drug (and just possibly) vaccine targets [14,152].

Drug Targets, Identification, Validation and Project Management

Many drug targets have been rehearsed for the African trypanosome [3]. This list includes targets based on analysis of existing anti-parasite drugs such as tubulin and a range of metabolic targets defined by specificity or differential sensitivity in the parasite. The scientific challenge is to define first line leads to realistic targets. With some notable exceptions this has not been the case in the past. Too often parasite drug targets have been rehearsed within narrow and selective information sets. More realistic and early insight to target protein function, expression and effect of deletion will be needed, coupled with realistic toxicological and pharmacological appraisals. In essence there is a need for academic laboratories to adopt industry-standard analyses for assessment of target validation. There will also be a need for industry-standard approaches to project management if such long-term programmes are to end in successful regulatory approvals. I have argued previously that ensuring that the basic science read-outs of the parasite sequencing projects translate through to drug targets and drug searches will need changes in academic science and funding agencies [14,152]. In this area, much will rest on the success of public: private strategic alliances. Recognition of this is clear in a recent

WHO report on incentives and disincentives for new anti-tuberculosis drug development. The report concludes:

"These include the need for the public sector to lead efforts to build relationships with industry and other stakeholders, provoke discussion and drive forward the process of anti-TB drug development by helping build, define and protect markets for new drugs. Governments should also be encouraged to strengthen their health infrastructures, lower the developmental barriers and build financing mechanisms so that the private sector sees fewer market barriers ahead".

One could apply these conclusions to virtually every tropical parasitic disease. The key is achieving excellent science and translating the discoveries through to development. Making use of the sequence information from the *T. brucei* genome project will provide major opportunities and challenges to scientists and science administrators!

ACKNOWLEDGEMENTS

Work in my laboratory has been supported by funding from the Wellcome Trust and the BBSRC.

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