Differentiation and division of *Trypanosoma brucei* in the mammalian bloodstream.

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Kevin Tyler

School of Biological Sciences

Division of Biochemistry

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ABSTRACT

In the bloodstream of mammalian hosts, the African trypanosome *Trypanosoma brucei*, undergoes a life cycle stage differentiation from a long slender form to a short stumpy form. The slender form is proliferative and meets its energy requirements solely by glycolysis. The stumpy form is non-proliferative and partially preadapted for survival in the midgut of its vector, the tsetse-fly. This preadaption includes mitochondrial enlargement and activation of enzymes associated with Krebs cycle. Thus, three major events characterize the slender-to-stumpy differentiation:

- Exit from the proliferative cell cycle.
- Morphogenesis.
- Partial mitochondrial biogenesis.

This project sought, to provide markers associated with each of these events and, to use these markers to discriminate between theoretical models of the slender-to-stumpy differentiation. In this way, I aimed to determine the manner in which differentiation occurs, with respect to the co-ordination of its three major events. In the course of the project, organellar segregation and outgrowth of the new flagellum were characterized as markers of the slender form cell cycle; this facilitated the discrimination of cells committed to cell division. Flagellar length was found to be a good morphological marker of the slender-to-stumpy differentiation and expression of dihydrolipoamide dehydrogenase, hsp60 and mhsp70 were established as markers of mitochondrial biogenesis.

When cells committed to division were investigated, evidence was found of heterogeneity between slender populations and differentiating populations. In differentiating populations many cells which were committed to division showed

increased expression of the mitochondrial marker mhsp70 and possessed a shortened new flagellum. This was taken as evidence that the slender-to-stumpy differentiation incorporates a round of cell division; a differentiation-division which is distinct from previous proliferative divisions.

ABBREVIATIONS

5’ UTR	5’ untranslated sequence
5’SL	5’ splice leader sequence
3’UTR	3’ untranslated sequence
BrdU	5-bromo-2-deoxyuridine
DAPI	4,6-diamidino-2-phenylindole
df	degrees of freedom
DHLADH	dihydrolipoamide dehydrogenase
dH_20	(double) distilled water
DIG	digoxygenin
EATRO	East African Trypanosomiasis Research Organization
E.M.	electron microscope
ES	expression site
ESAG	expression site associated gene
EST	expressed sequence tag
FCS	foetal calf serum
FITC	fluoroscein isothiocyanate
gRNA	guide RNAs
GUP	Glasgow University Protozoology
GUTat	Glasgow University *Trypanozoon* antigenic type
hsp	heat shock protein
J	β-D-glucosyl-hydroxymethyluracil
kDNA	kinetoplast DNA
MAP	microtubule associated protein
mhsp	mitochondrial heat shock protein
MOPS	3-(N-Mrpholino) propanesulphonic acid
MS	mean square
MTOC	microtubule organising centre
NIH	National Institute of Health
PARP	procyclic acidic repetitive protein
PBS	phosphate buffered saline

PCR	polymerase chain reaction
PFR	paraflagellar rod
PSG	Puck's saline glucose
RAPD	randomly amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
slender form	long slender form
SS	sum of the squares
s	standard deviation
s^2	variance
S.E.	standard error
SDS-PAGE	polyacrylamide gel electrophoresis using buffers containing sodium dodecyl sulphate
stumpy form	short stumpy form
TAO	Trypanosome alternative oxidase
Tris	Tris(hydoxymethyl)aminomethane
TRITC	tetramethylrhodamine isothiocyanate
UTR	untranslated region
VAT	variable antigenic type
VSG	variant-specific surface glycoprotein
WHO	World Health Organization
(w/w)	weight per weight
(w/v)	weight per volume

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Chapter 1

General introduction.

1.1 *Trypanosoma brucei*.

Diseases causing wasting in cattle (Ngana) and sleeping sickness in humans have been long linked to the bite of the tsetse-fly. Research, on the pathogen which causes these diseases, began with the identification of trypanosomes both in the bloodstream of infected cattle and in the tsetse-fly (Bruce, 1895). Early papers described these trypanosomes as polymorphic (later pleomorphic) and characterized the morphologies present in the mammalian bloodstream and in different compartments of the tsetse-fly. These early studies provided insight into the way in which trypanosomes replicate and the way in which they adapt to new environments by differentiation. Since the turn of the century the species complex of trypanosomes that causes Ngana and can cause sleeping sickness has been named *Trypanosoma brucei* (Plimmer and Bradford, 1899). For reviews of many of these early studies see Duggan (1970), Cox (1996) and Vickerman (1997).

T.brucei consists of three subspecies: *T.b.brucei* is defined by its non-infectivity to humans. *T.b.gambiense* causes the chronic or West African form of sleeping sickness, a disease sometimes resolved by the host and that is fatal only after involvement of the central nervous system. *T.b.rhodesiense* causes the acute or East African form of sleeping sickness; this disease is generally fatal between 20 and 50 days post-infection and follows a relapsing fever, jaundice and anaemia. In both forms of sleeping sickness behavioral change can precede coma. For an overview of pathology see Manson (1996).

The bite of an infected tsetse-fly introduces trypanosomes into the mammalian host and at the bite site a chancre is formed. Once in the bloodstream trypanosomes multiply freely. Trypanosomes are flagellated protozoons, the bloodstream forms are elongate and taper towards the anterior end. They are highly motile and are longer and narrower than the (approximately) 10μm diameter erythrocytes which surround them. Trypanosomes possess two masses of DNA, a

nucleus and a kinetoplast. The kinetoplast, the genome of the trypanosome's single mitochondrion, is visible at the level of the light microscope and is situated at the posterior end of the cell close to the region which subtends the flagellum.

1.2 Phylogeny and evolution

Possession of a kinetoplast places *T.brucei* within the Order Kinetoplastida. As well as African trypanosomes, the Order Kinetoplastida encompasses *Trypanosoma cruzi* and *Leishmania* species. *T.cruzi* causes the chronic but fatal Chagas' disease which, by recent estimates, currently infects some 12 million people in South and Central America. Leishmanoses vary in pathology from self-healing tropical ulcers, to non-healing mucocutaneous lesions, to severe visceral involvement. Leishmanoses are widespread and some 18 million people may be infected. As an Order encompassing pathogenic protozoons, Kinetoplastida is second in importance only to the Order Eucoccidiida (which encompasses the malarias) in terms of world health. For recent estimates of the incidence of protozoan parasitic disease affecting humans see the WHO report (1995), for the effects of trypanosomoses and leishmanoses on public heath see Molyneaux (1997).

T.brucei (*sensu lato*) causes a widespread zoonosis and includes the only African trypanosomes pathogenic to humans. Trypanosomes of the *T.brucei* complex are not, however, the primary cause of Ngana. Ngana is a disease of huge economic importance which has rendered the tsetse belt of sub-Saharan Africa, a region of some 10 million square kilometers, unsuitable for rearing domestic cattle. Two other tsetse transmitted trypanosomes, *T.congolense* and *T.vivax,* are responsible for most of the cases of Ngana in African cattle. Other pathogenic trypanosomes include *T.equiperdum,* which causes disease in horses and which although closely resembling *T.brucei,* is transmitted venereally. *T.evansi*, which also resembles *T.brucei,* causes Surra. Surra is an important disease of camels but also infects horses and cattle. *T. evansi* is found in South America and Africa, it is transmitted mechanically by biting flies and has no vector-specific life cycle stages. *T.equinum* is believed to be a variant of *T.evansi* which lacks a detectable kinetoplast. Finally, *T.suis* is a tsetse-fly transmitted trypanosome that can cause disease in swine. For an overview of trypanosomes that cause pathology see Cox (1992).

From an evolutionary perspective, the Kinetoplastida are one of the most divergent Orders of eukaryotes from the multicellular eukaryotes (or metazoans). That this is so has become obvious with the advent of molecular phylogenetic techniques which can be used to compare mutational rates in conserved sequences such as ribosomal RNAs (for a review of molecular clocks see Woese, 1981). When Sogin (Sogin et al., 1989) applied these techniques to the eukaryotic lineage, the Order Kinetoplastida was found to be situated close to the root of the eukaryotic lineage and was the most divergent group of organisms to possess organelles such as peroxisomes and mitochondria (Fig. 1.1a). Molecular clocks have also been used to review the relatedness of the species within the Kinetoplastida (Fig. 1.1b) which comprises free living Bodoniids and monogenetic insect parasites such as *Crithidia spp.* in addition to the digenetic *Leishmania* and *Trypanosoma* (Lake et al., 1988; Fernandes et al., 1993; Maslov et al., 1996). Kinetoplastids diverged from the eukaryotic lineage at a point very close to the initial construction of a eukaryotic cell. Consequently, the study of kinetoplastids from an evolutionary perspective has led to refinements of, and insights into, endosymbiotic theory (Cavalier-Smith, 1993, 1995, 1997).

As a kinetoplastid, *T.brucei* is evolutionarily highly divergent from the metazoan lineage. As a parasite with ancient origins, which presumably co-evolved first with both insects and with mammals - perhaps several times (Maslov et al., 1996), the trypanosome represents a highly evolved cell with an enormous degree of specialization. It is presumably for these reasons that some specialized features of the trypanosome are unique. Other features, shared with other members of the Order but which are unique to kinetoplastids, have been predominantly

Figure 1.1A

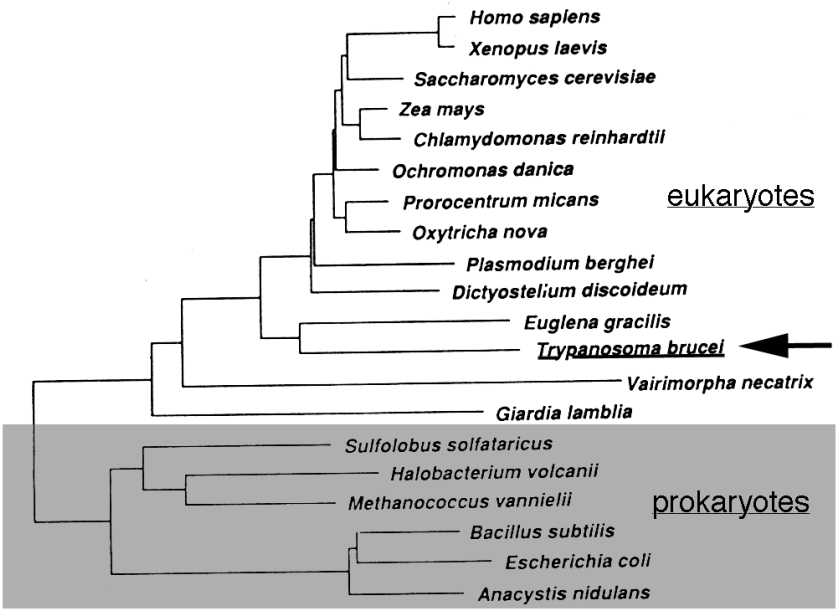


Figure 1.1B

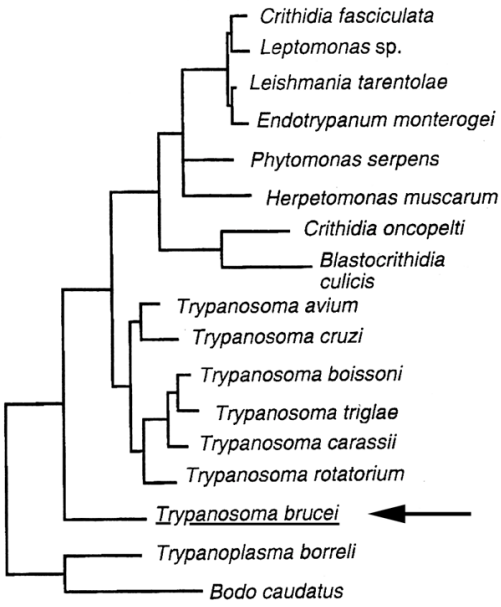


Fig. 1.1. Phylogenetic trees based on a comparison of small subunit rRNAs.
Fig. 1.1a Trypanosomes diverged early in the eukaryotic lineage; the kinetoplastids which incorporate *T.brucei* are the most divergent branch to possess mitochondria and peroxisomes (reproduced with permission Sogin et al., 1989. Copyright AAAS).
Fig. 1.1b Phylogenetic trees of the Kinetoplastida reveal the divergent lineage of salivarian African trypanosomes such as *T.brucei* compared with other members of the genus (reproduced with permission Maslov et al., 1996).

characterized in trypanosomes. Most cellular components are, however, common to trypanosomes and metazoans. An argument can be made that conserved features of such an evolutionarily divergent organism must be effectively universal. It seems likely, however, that due to convergent evolution some of these features, that appear to be conserved between trypanosomes and metazoans, have been evolved independently (perhaps on multiple occasions) and may not have been present in a common progenitor or be universal features of eukaryotes.

T.b.brucei is lysed by human serum but *T.b.gambiense* and *T.b.rhodesiense* are not. This difference in phenotype is rooted in the differential uptake of human haptoglobin related particles containing trypanosome lytic factors (Rifkin, 1978; Hager et al., 1994; Raper et al., 1996; Hager and Hajduk, 1997). *T.b.gambiense* isolates do show some consistent differences in isoenzyme (Reichner et al., 1989; Gibson, 1994), restriction fragment length polymorphism (RFLP) and randomly amplified polymorphic DNA (RAPD) (Gibson, 1994; Mathieu-Daude et al., 1995) profiles when compared to *T.b.rhodesiense* and *T.b.brucei.* At these and every other level tested (including by comparison of various genetic sequences) *T.b.rhodesiense* and *T.b.brucei* isolates are, however, indistinguishable (reviewed by Baker, 1995). In addition there have been various reports of human infections by stocks of *T.b.brucei* and resistance to human serum is clearly variable (reviewed in Hajduk et al., 1994). It remains likely that *T.b.rhodesiense* is a convenient way of referring to some strains of *T.b.brucei* which have acquired infectivity to humans, but which are easily accommodated inside the genetic heterogeneity of *T.b.brucei*.

1.3 Life cycle of *T.brucei*

Survival of *T.brucei* is dependent on successful transmission between, and colonization of, three radically different environments: the tsetse salivary gland, the mammalian bloodstream and the tsetse midgut. It is now generally held that to accomplish this the trypanosome has evolved a six stage life cycle with a non-dividing transitional form and a proliferative colonizing form for each environment (reviewed Vickerman, 1985).

Briefly, (and as shown in Fig. 1.2.) proliferative long slender forms multiply in the mammalian bloodstream. These forms have a suppressed mitochondrion and utilize the host's blood glucose, by glycolysis, as their primary energy source. At high parasitaemia, long slender forms differentiate to non-dividing short stumpy forms in an apparently density dependent fashion (Seed and Sechelski, 1989; Vassella et al., 1996). Short stumpy forms are preadapted for survival in the tsetse midgut by partial activation of the mitochondrion, which allows some metabolism of the major tsetse metabolite, proline. When taken up in a blood-meal the stumpy forms are transformed (preferentially to slender forms) to proliferative procyclic forms. From the procyclic population proventricular forms arise that are thought to be non-proliferative and which migrate, invade the salivary glands, attach and become epimastigotes. This transition, which takes place in the tsetse-fly, is cryptic. Consequently, outside of the initial dissections and cellular descriptions, studies on transition between these tsetse stages of the life cycle are scarce. The proliferative epimastigotes pack close 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 (Jenni et al., 1986). Finally, epimastigotes differentiate to form the detached non-dividing metacyclic forms which are infective to the mammalian host. Once in the mammalian host metacyclics re-enter the cell cycle and readopt a slender morphology.

Figure 1.2

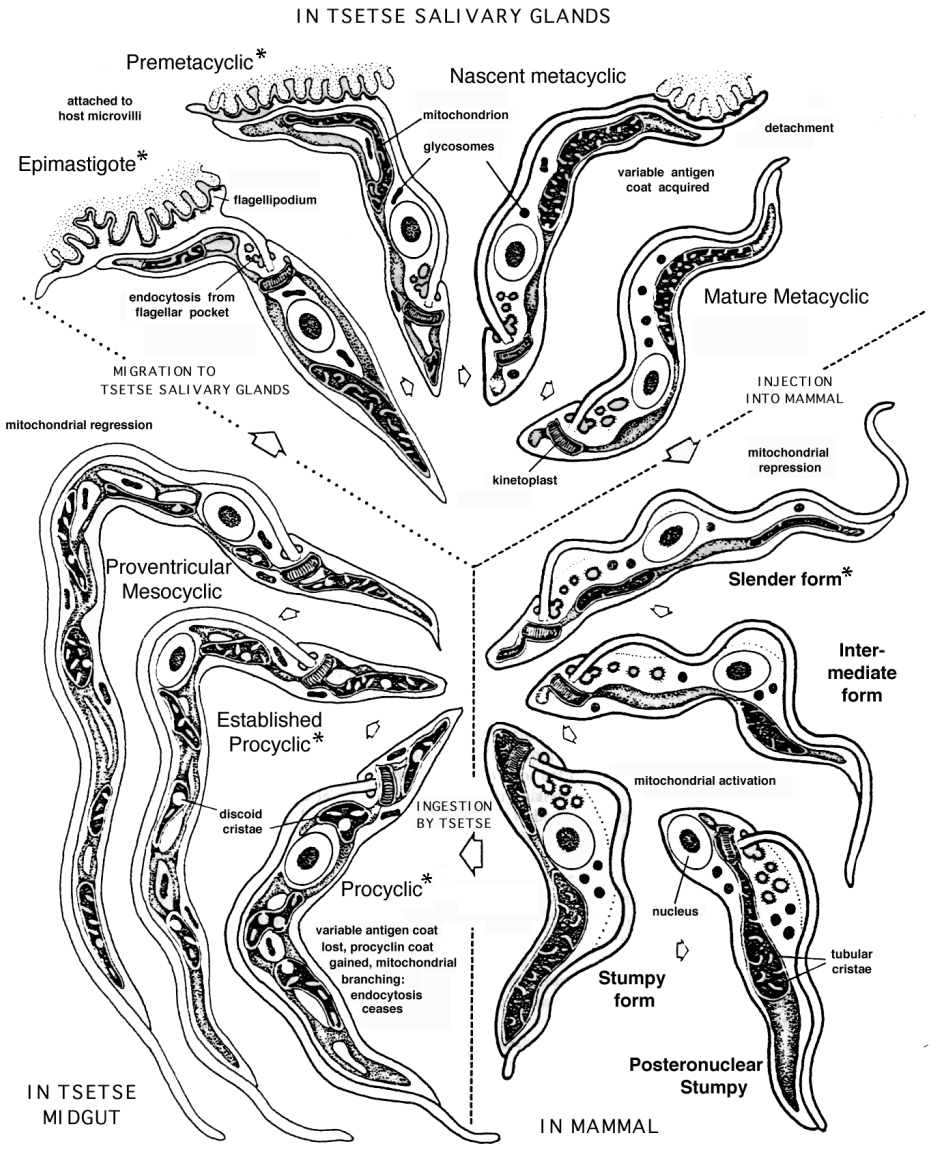


Fig. 1.2 The life cycle of Trypanosoma brucei is digenetic, alternating between the mammalian host and tsetse-fly vector, but involves adaptation to three environments: the mammalian bloodstream and lymphatic system, the tsetse-fly midgut and the tsetse-fly salivary gland. These adaptations are believed to occur by unidirectional differentiation between six stable life cycle stages, which alternate between proliferative colonizing forms (denoted by *) and division arrested transitional forms. The figure also shows changes in morphology, relative abundance of endosomes and glycosomes and mitochondrial activation and repression during the life cycle. (Reproduced, from Vickerman, 1985 with permission).

1.4 Trypanosome cell structure

Trypanosomes have a characteristic morphology that varies throughout the life cycle. It is by the variation in this morphology that life cycle stages were first defined. Of primary importance in the maintenance and modulation of trypanosome morphology is the cytoskeleton. The most obvious feature of the cytoskeleton is the subpellicular array of microtubules. This array is constructed from a corset of parallel microtubules along the length of the trypanosome and underlying the plasma membrane, which defines the cell body. In contrast to other microtubule structures the subpellicular array is particularly stable and remains polymerized throughout the cell cycle. The subpellicular array is itself cross-linked by microtubule associated proteins -MAPs (for reviews focusing on the trypanosome cytoskeleton see Seebeck et al., 1988, 1990; Robinson et al., 1991; Bouck and Ngo, 1996; Kohl and Gull, In press)

A number of microtubule associated proteins have now been found in trypanosomes including the microtubule associated repetitive proteins or MARPs. These are two large (320 KDa) proteins localized to the plasma membrane facing side of microtubules in the subpellicular array (Hemphill et al., 1992; Affolter et al., 1994). Another protein that has been associated with the membrane-skeletal complex, p41 (Schneider et al., 1988), may be a Ca^{2+} binding protein. Other proteins have been associated with the anterior tip of the trypanosome cell body, where the microtubules and plasma membrane form a capping structure and, where Gb4 has been associated with the ring-shaped opening in the microtubule array (Rindesbacher et al., 1993). Other MAPs cross-link the subpellicular array, such as the antigen recognized by WCB (whole cell body) antibody, p240 (Woods et al., 1992), the immunogenic Ca^{2+} binding protein I/6 (Detmer et al., 1997), p15 and p52 (Balaban and Goldman., 1992) and the life cycle stage specific CAP 5.5 (Matthews and Gull, 1994a; R. Gerke-Bonet, A. Bagerzadeh and K. Matthews, unpublished observations). Finally, a leishmanial kinesin homologue has been characterized(Burns et al., 1993) and a number of putative kinesin homologues are now being characterized in trypanosomes (K. Ersfeld, unpublished).

The other major microtubule structure is the flagellar axoneme which has the classical flagellar 9+2 microtubule structure. The flagellum exits the cell body through the flagellar pocket and is subtended by a basal body which is normally associated with another probasal body. The basal body is the only defined microtubule organizing centre in the trypanosome cell. Associated with the external flagellum is the paraflagellar rod, a complex semi-crystalline structure which is composed of several proteins including the PFR A and PFR C gene products (reviewed in Bastin et al., 1996). Molecular ablation of PFR A expression, by an antisense mechanism, severely disrupts the paraflagellar rod but leaves the flagellar axoneme intact. Disrupting the paraflagellar rod gives rise to a phenotype with severely restricted motility (Bastin et al., 1997).

Associated with the flagellum and four specialized microtubules of the subpellicular array is the flagellar attachment zone. The flagellar attachment zone is apparently composed of several structural proteins resistant to extraction by detergent (Woods et al., 1988a,b; Nozaki et al., 1996).

In trypanosomes, tubulin is the primary cytoskeletal protein. Actin, although apparently expressed (Ben Amar et al., 1988), has not been localized in a filamentous form. Intermediate filaments have never been demonstrated. The tubulin locus is well defined and consists of alternating α-tubulin and β-tubulin genes in tandem array (Seebeck et al., 1983; Thomashow et al., 1983; Kimmel et al., 1985; Imboden et al., 1986, 1987). A third tubulin gene, the γ-tubulin gene, has also been cloned in trypanosomes (Scott et al., 1997). In other organisms, the homologues of γ-tubulin have been localized to microtubule organizing centers (Joshi, 1994; Lee and Joshi, 1996). In *T.brucei*, γ-tubulin appears to localize to the only well defined microtubule organizing centre, the basal body, but may also localize to other structures at the anterior tip of the cell body, within the nucleus and along the quartet of microtubules associated with the flagellar attachment zone (Scott et al., 1997). Trypanosome tubulins can be post-translationally modified either by acetylation, by glutamylation or by detyrosination of the carboxyl terminus after assembly (Stieger et al., 1984; Schneider et al., 1987; Sherwin et al., 1987; Sasse and Gull, 1988; Schneider et al.,

1997). Tyrosination is a marker of newly assembled tubulin and therefore marks regions of cell growth and microtubule mediated events in trypanosomes (Sherwin et al., 1987; Sherwin and Gull, 1989b). It has been proposed that variation in post-translational modifications may be responsible for morphological changes associated with differentiation in kinetoplastids (Gallo et al., 1987). Certainly, newly tyrosinated α-tubulin detected with the monoclonal antibody YL1/2 (Kilmartin et al., 1982) has been used to demonstrate microtubule mediated repositioning of the kinetoplast/basal body complex during the stumpy-to-procyclic form differentiation (Matthews et al., 1995).

1.5 The mitochondrion

The size, structure and many of the metabolic functions of the *T.brucei* mitochondrion are under stage specific control. In addition, the mitochondrion of *T.brucei* displays a number of peculiarities common to other kinetoplastids. Kinetoplastids are defined by the presence of a sub-organellar DNA structure, the kinetoplast. The kinetoplast is composed of a network of concatenated circular DNA molecules. These are the multiple copies of the mitochondrial genome joined together as a single unit. Since there is only one kinetoplast per cell, it probably follows that there can be only one mitochondrion per cell. Nevertheless, the kinetoplastid mitochondrion remains a dynamic structure, able to fuse and branch syncitia. It can form a network of processes (reticulum) throughout the cell, showing at least superficial structural similarity to the fused and cage-like mitochondria of organisms like *Chlamydomonas reinhardii* (for the structure of the fused mitochondrion of a *Chlamydomonas reinhardii* gamete see Blank and Arnold, 1984. Compare with Vickerman's drawings of the procyclic form, trypanosome mitochondrion Fig. 1.2).

Some other eukaryotic cells may, from time to time, possess a single mitochondrion as a response to environmental change or as a result of differentiation. Single mitochondria in the cells of non-kinetoplastids, however, result from fusion of multiple mitochondria in the cell. Multiple mitochondrial genomes are therefore present and distributed throughout the fused mitochondrion. Consequently, unlike the kinetoplastid mitochondria, under

appropriate conditions the fused mitochondria of non-kinetoplastids are fissile and can produce cells with multiple mitochondria each containing mitochondrial genomes (for general reviews of mitochondrial behavior see Bereiter-Hahn, 1990; Bereiter-Hahn and Voth, 1994).

1.5.1 The kinetoplast

Interphase trypanosomes possess a single kinetoplast. Therefore, and as with the nucleus, the kinetoplast must be replicated and segregated with fidelity once per cell cycle. The kinetoplast is a complex structure composed from a concatameric network of minicircles and maxicircles (reviewed Simpson, 1987; Englund et al., 1995). Typically there are approximately 50 maxicircles (Hajduk et al., 1984) which are essentially identical and resemble conventional mitochondrial DNAs. The maxicircle of *T.brucei* is completely sequenced and encodes 18 mRNAs, the 9S and 12S mitochondrial rRNAs and a further stable transcript of unknown function (reviewed in Stuart, 1995). Unlike other mitochondrial genomes, however, no tRNAs are kinetoplast encoded and so mitochondrial tRNAs must be imported to the kinetoplastid mitochondrion (Hancock and Hajduk, 1990; Mottram et al., 1991).

In addition to the maxicircles, the *T.brucei* kinetoplast is composed of about 5,000 minicircles which fall into some 400 different classes (Steinert and Van Assel, 1980). Minicircles are only 1Kb to 1.5Kb in length and encode multiple small guide RNAs involved in the post-transcriptional process of editing (see Section 1.5.2). All of the minicircles and maxicircles are topologically linked as a network into one structure which, when spread out, forms “an elliptical sheet of interlocked rings 10 *x* 15μm in size”. In the trypanosome this structure is packed into a disc 1μm in diameter and 0.3μm thick (Englund et al., 1995).

Clearly, replication of such a complex structure represents a unique set of problems. Replication of the minicircles has been studied in some detail. As shown in Fig. 1.3 (upper diagram), minicircles detach from the kinetoplast network a few at a time, rather than all at

once, then replicate and reattach at the disc periphery (Simpson et al., 1974). Newly replicated minicircles contain nicks, which are believed to mark them as different from unreplicated minicircles. These nicks are repaired after kinetoplast segregation is completed. Minicircle replication is mediated by enzyme complexes, including primase. These complexes are localized above and below the disc (Li and Englund, 1997) and at opposite poles, where at least one DNA polymerase is localized (Fig. 1.3; lower diagram).

Kinetoplast replication is most studied in *Crithidia fasciculata*, a species within the Order Kinetoplastida (Fig 1.1b) which is a parasite of insects and which is readily grown in culture. In *C. fasciculata*, the distribution of the minicircles around the kinetoplast is thought to be achieved by the spinning of the kinetoplast between the two stationary poles of the polymerase/attachment complex. It is not yet clear whether some form of shuffling mechanism exists to ensure equal distribution of the different minicircles (reviewed Shapiro and Englund, 1994; Englund et al., 1995). In *T. brucei*, however, the kinetoplast does not appear to spin and the reattached minicircles remain polar, rather than distributed around the kinetoplast periphery (Robinson and Gull, 1994).

Figure 1.3

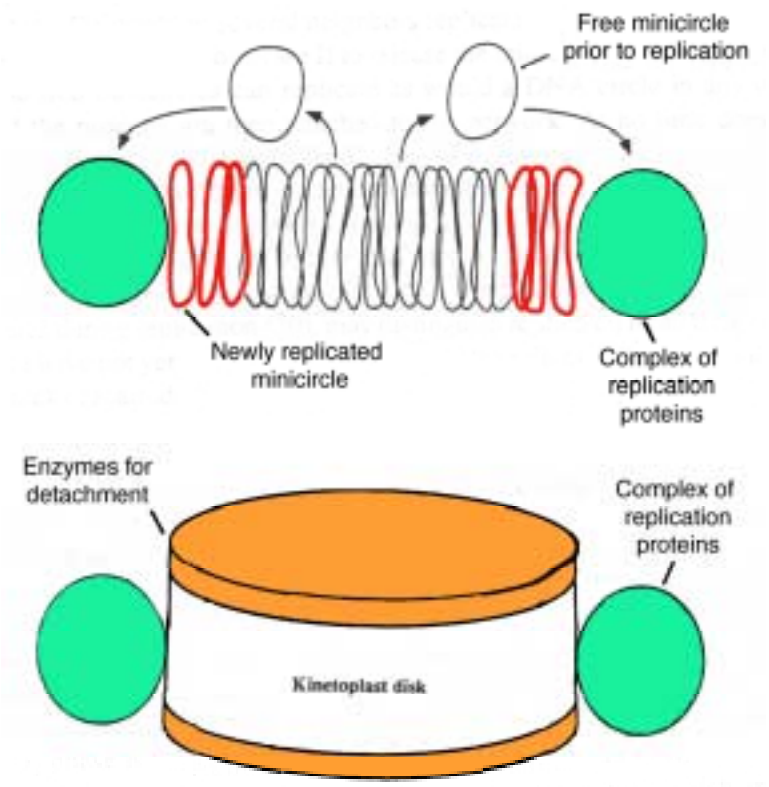


Fig. 1.3 The kinetoplast is surrounded by machinery for its replication. Minicircles are detached from the kinetoplast by enzymes present above and below the kinetoplast and are replicated and then reattached to the kinetoplast periphery at polar complexes (reproduced with some modification from Englund, 1995 with permission).

Maxicircle replication is less well studied. It is believed that replication occurs at a non-random location within the disc, not at the poles. During replication maxi-circles remain attached to the network, replicating in a θ type formation. Once replication is complete the maxicircles are transiently released and then reattached to the network, within the kinetoplast disc (Hajduk et al., 1984).

The kinetoplast is linked directly to the basal body by a filamentous network that penetrates the mitochondrial membranes (Robinson and Gull, 1991; Robinson et al., 1995). This hardwiring to the cytoskeleton may provide both positional information and a degree of torsion during segregation. It remains unclear how the application of cytoskeleton mediated segregational force coordinates with the completion of kinetoplast replication.

1.5.2 Editing of mitochondrial pre-mRNAs

Like more conventional mitochondrial genomes, the maxicircles of *T.brucei* encode some genes associated with oxidative phosphorylation. The transcription of maxicircle encoded genes is promoter driven and polycistronic. Maxicircle transcripts may be encoded on either DNA strand and may contain overlapping genes in different reading frames. Unlike other mitochondrial encoded pre-mRNAs, the pre-mRNAs transcribed from maxicircles do not always contain sequence which can be translated into full length proteins. In the case of 12 of the 18 mRNAs encoded by the mitochondrial genome of *T.brucei*, the pre-mRNA must be amended post-transcriptionally (Stuart, 1995). This occurs by the addition and occasional deletion of uracil residues, producing mature mRNAs encoding complete functional proteins. This mechanism is unique to the mitochondria of kinetoplastids and is referred to as RNA editing. Since the discovery of RNA editing in trypanosomes, other processes which involve the post-transcriptional modification of coding sequence in pre-mRNAs have been brought to light (reviewed Benne, 1993). While these analogous processes involve different mechanisms than that employed by kinetoplastids, they are also now referred to as editing.

Editing proved an exception to the accepted dogma of "DNA makes RNA makes protein" and its discovery prompted initial skepticism, followed by widespread interest. Consequently, the mechanism has been explored in considerable molecular detail (for reviews see Simpson, 1990; Benne, 1993, 1994; Adler and Hajduk, 1994; Stuart, 1995; Simpson and Emeson, 1996). Briefly, maxicircle sequencing established that genes homologous to cytochrome oxidase subunit II (CO II) and cytochrome b (Cyb) were present, but could not encode a functional protein. The corresponding mRNAs were then cloned (as cDNAs) and sequenced (Benne et al., 1986). The mRNA sequences obtained, did encode functional proteins. These mRNA sequences were complementary to the maxicircle sequences, except that some regions contained stretches of additional uracils while occasional uracils were deleted. These additions and deletions typically overcame frame shifts, stop codons, created initiation codons and added additional amino acids to the

encoded proteins (Shaw et al., 1988). In some cases, such as cytochrome oxidase subunit III (CO III), the maxicircle sequence was only obvious once the cDNA had been cloned (by homology to non-kinetoplastid genes) and then sequenced, since over half of the nucleotides present were as a result of editing (Feagin et al., 1988a). It was also found that editing of these mRNAs was regulated in a life cycle stage dependent manner, but that the manner of regulation was transcript dependent (Feagin et al., 1985ab, 1987, 1988b; Feagin and Stuart, 1988; Simpson and Shaw 1989; Koslowsky et al., 1990).

The function of minicircles had remained enigmatic for many years. Minicircles produce two classes of transcript: the first class of transcripts are approximately 200bp long and are thought to act as primers for replication. The second class contains multiple different transcripts of 50bp to 70bp in length (Simpson et al., 1989; Sturm and Simpson, 1990). Regions of complementarity between the second class of transcripts and sequences in edited mitochondrial mRNAs were established (Blum et al., 1990; Blum and Simpson, 1990). This complementarity suggested a role in editing for these short heterogeneous minicircle transcripts, which were named guide RNAs (gRNAs). Guide RNAs are encoded in cassettes which lie between flanking 18bp inverted repeats (Jasmer and Stuart, 1986; Sturm and Simpson, 1990; Pollard et al., 1990). Only 240 gRNAs are required to produce all the editing necessary in *T.brucei*, but approximately 1200 gRNAs are encoded by 400 classes of minicircle, implying considerable redundancy (Stuart, 1995).

Guide RNAs have a region of 3'oligo-U tail which is thought to be added by a mitochondrial (terminal uridyl transferase) TUTase: an enzyme capable of the untemplated addition of uracil residues to the 3' end of RNA transcripts. The 3'oligo-U tail may act as a reservoir for the addition or subtraction of uracils during editing (Bakalara et al., 1989). There is also a 5' anchor region which is complementary either to edited or unedited mRNA sequence and allows the formation of a mRNA/gRNA hybrid. Editing is at least partly processive and proceeds in a 3' to 5' direction. Guide mRNAs from the 3' limit of the editing domain have an anchor region which is complementary to the unedited mRNA. Some gRNAs can also form a partial duplex with unedited mRNA. Other gRNAs form a

duplex only with edited mRNA. Duplex formation with edited mRNA is dependent on editing having occurred 3' to the new editing site and ensures that editing proceeds in the 3' to 5' direction (Blum et al., 1990, 1991).

Figure 1.4

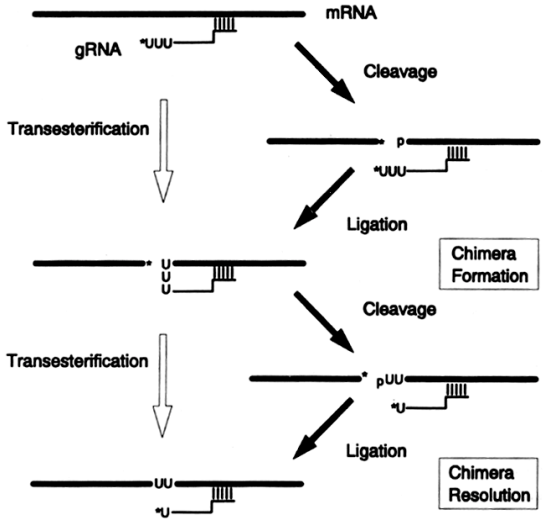


Fig. 1.4 The incorporation of additional uracils during editing may proceed by one of two molecular mechanisms: either by two sequential transesterification reactions, or by the successive cleavage and ligation of both mRNA and gRNA (reproduced from Stuart, 1995 with permission).

Much of the enzyme activity associated with editing is believed to be mediated by a multi-subunit ribonucleoprotein assembly, named the editosome by analogy to the spliceosome. Evidence for such an assembly comes from co-sedimentation studies with gRNA and mRNA. In these studies RNA ligase and TUTase activities were isolated in two bands of 20S and 35-45S. Analysis of the other components in these bands has led to the characterization of several associated proteins. The gRNA/mRNA hybrid is thought to direct the enzymatic activities of the putative editosome (Pollard et al., 1993; Goringer et al., 1994).

Fig. 1.4 summarizes two models of RNA editing which have been proposed (Blum et al., 1990; Decker and Solner-Webb, 1990). The first model proceeds by transesterification only. The second, by successive cleavage and ligation reactions. Reconstitution experiments have been performed on gRNA/mRNA chimeras, in vitro, using fractions of mitochondrial extract which lacked any endoribonuclease activity. These experiments were successful in adding uracil residues to the mRNA substrate (Koslowsky et al., 1992). The results of these experiments, although not definitive, imply a transesterification model.

The reasons for the evolution of editing in this lineage and the consequent splitting of information into two or more different loci (maxicircle mRNA and minicircle gRNAs) are unclear. Editing may be an artifact from the adaptation to a nutritionally deficient environment of an ancestral kinetoplastid. Editing might form part of a defence mechanism against introns, transposons and viruses. Alternatively, editing may just confer a useful extra tier of control of mitochondrial gene expression (Cavalier-Smith, 1997). Whatever the reason for editing, once it had evolved, there was a need to ensure that the separate loci carrying protein coding information remained together during replication. This need may have led to the evolution of a single kinetoplast, by catenation of all the circular DNAs in the mitochondrion, and hence a to single mitochondrion.

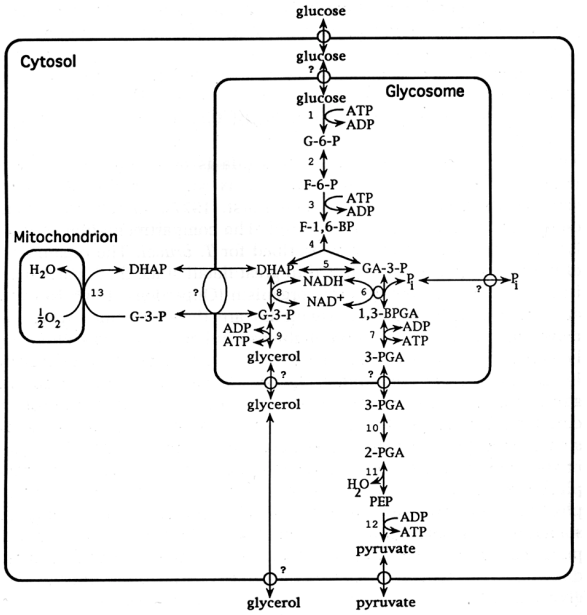
1.6 Glycosomes

Like the erythrocytes which surround them, the slender form trypanosome meets its energy requirements primarily by the glycolysis of the abundant (approximately 5mM) blood glucose. Glycolysis yields energy by the cleavage of a hexose carbon chain into two three-carbon chains which are then excreted. Although the complete oxidation of one glucose molecule potentially yields 36 ATP molecules and glycolysis yields only 2 molecules of ATP, the ready availability of blood glucose to trypanosomes makes glycolysis a viable alternative to oxidative phosphorylation.

Kinetoplastids are exceptional with respect to glycolysis. In other eukaryotes the enzymes of the glycolytic pathway are found in the cytoplasm. However, in trypanosomes the first nine enzymes of glycolysis are localized, at high concentration in a membrane bound organelle, the glycosome (Opperdoes and Borst, 1977). This contributes to the high efficiency of glycolysis in *T.brucei* which has been estimated to be some 50 times more efficient than in cultured mammalian cells (Fairlamb and Opperdoes, 1986).

Trypanosome glycolysis is also unusual insofar as twice as much ATP is produced by glycolysis under aerobic conditions as under anaerobic conditions. This is because of the glycerol-3-phosphate shuttle from the glycosome to the mitochondrion. Under aerobic conditions the trypanosome alternative oxidase, (TAO) which is localized in the mitochondrion, oxidizes glycerol-3-phosphate to dihydroxy acetone phosphate rather than allowing its excretion as glycerol. The accumulation of dihydroxy acetone phosphate drives glycolysis down the glutaraldehyde-3-phosphate route to the production of pyruvate yielding a second ATP. Under these aerobic conditions pyruvate is the main excretion product. Under anaerobic conditions equal amounts of glycerol and pyruvate are produced (Fig. 1.5). For reviews of trypanosome glycolysis see Opperdoes (1987) and Michels et al.(1997).

Figure 1.5



G-6-P: Glucose-6-phosphate
F-6-P: Fructose-6-phosphate
F-1,6-BP: Fructose 1,6 biphosphate
DHAP: Dihydroxyacetone phosphate
GA-3-P: Glyceraldehyde-3-phosphate
PGA: Phosphoglycerate
BPGA: Biphosphoglycerate
PEP: Phosph*enol*pyruvate
G-3-P: Glycerol-3-phosphate

1 Hexokinase
2 Glucosephophate isomerase
3 Phosophofructokinase
4 F-1,6-BP aldolase
5 Triosephosphate isomerase
6 GA-3-P dehydrogenase
7 PGA kinase
8 G-3-P dehydrogenase
9 Glycerol kinase
10 PGA mutase
11 Enolase
12 Pyruvate kinase
13 Trypanosome alternative oxidase

Fig. 1.5 Glycolysis in trypanosomes takes place in predominantly membrane bound organelles - glycosomes. The first nine enzymes of the pathway are localized in glycosomes where there is no net production of ATP. In the cytoplasm 3-phosphoglyceraldehyde dephosphorylation yields one molecule of ATP per molecule of pyruvate. Mitochondrial oxidation of glycerol-3-phosphate to dihydroxy acetone phosphate drives the equilibrium towards the production of two pyruvates and a second molecule of ATP. ? denotes proposed but unidentified glycerol transporters. (Reproduced with permission from Michels et al., 1997).

Since compartmentalized glycolysis is highly efficient, it is surprising that it is limited solely to kinetoplastids. Work on import of proteins to the glycosomes has shown that the import signals bear similarities to those of peroxisomes (reviewed Clayton et al., 1995; Sommer and Wang, 1995). Glycosomes seem to be one of a family of related organelles that include the glyoxysomes of plants and the microbodies of fungi. Glycosomes have a similar morphology to these organelles and possess enzymes involved in β-oxidation and ether-lipid synthesis that are common to all of the peroxisome family.

It has been proposed that glycosomes, glyoxysomes and microbodies have a common endosymbiotic origin (Cavalier-Smith, 1997). This origin must have occurred at a time close to the divergence of the kinetoplastid line. The endosymbiotic precursor would have initially possessed all of the enzyme activities currently present in peroxisome-like

organelles as well as many others. During the course of evolution those activities which did not require a compartmentalization to provide selective advantage would be lost. In aerobic organisms compartmentalization of glycolysis would be lost. However, in facultative anaerobes, akin to the Bodoniids, which may have been the earliest kinetoplastids, the compartmentalization would have provided a selective advantage: a benefit maintained to the present day in their specialized parasitic descendants.

1.7 The trypanosome nucleus

The nuclear organization of the trypanosome is very different to that of metazoans (reviewed El-Sayed and Donelson, 1997; Ersfeld et al., submitted). In common with other protozoans, termed mesokaryotes, the DNA does not condense at mitosis to a level where discrete chromosomes are visible, nor does the nuclear membrane break down. It is thought that the condensation ratio of metazoan genomes during mitosis cannot be reached in trypanosomes because of an inability to form 30nm fibrils. Furthermore, although nucleosomes are clearly present (Hecker and Gander, 1985; Schlimme et al., 1993), higher order structures such as scaffold attachment regions, where the DNA might interact with the nuclear matrix, have not yet been identified (Hecker et al., 1994). It is thought that the inability to form 30nm fibrils may reflect the quite divergent nature of the trypanosome H1 homologue (Schlimme et al., 1993; Burri et al., 1993,1994,1995).

The *T.brucei* nucleus is diploid in all life cycle stages so far characterized (Tait et al., 1989). It has a haploid DNA content of 3.5 - 4 x 10^7 bp (Borst et al., 1982; Van der Ploeg et al., 1989). Chromosomes can be visualized by pulsed field gel electrophoresis (Van der Ploeg et al., 1984) which resolves chromosomes into two size classes. The exact numbers of chromosomes varies between isolates but there are approximately 11 pairs of large chromosomes (1 Mb to 6 Mb), 1-5 intermediate chromosomes (200-900Kb) and approximately 100 minichromosomes of between 50 and 150Kb (Van der Ploeg et al., 1989; El-Sayed and Donelson, 1997; Ersfeld et al., submitted).

Chromosome segregation presents a unique set of problems in trypanosomes. While the trypanosome nucleus clearly contains kinetochores (Vickerman and Preston, 1970; Ogbadoyi, 1997) there are too few to carry even the 11 pairs of large chromosomes independently, let alone the minichromosomes. Recent work has used DNA in situ hybridization, with independent probes for each chromosome class, to highlight a kinetochore independent method of segregation for minichromosomes (Ersfeld and Gull, 1997).

Unlike metazoans, trypanosome genes are packaged very close together, separated by hundreds rather than thousands of base pairs. Introns are apparently absent. Promoters are sparse and to date, none are of an RNA polymerase type II variety. Strikingly, large numbers of genes are organized into multicistronic units often containing tandemly repeated gene families, for example: tubulin (Imboden et al., 1987); calmodulin (Tschudi and Ullu, 1988); phosphoglycerate kinase (Gibson et al., 1988) and heat shock protein 70 - hsp70 (Lee and Van der Ploeg, 1990a). This has implications for the post-transcriptional modification of trypanosome mRNA.

1.8 Trans-splicing of nuclear encoded pre-mRNA

In the monocistronic transcription of metazoan protein encoding genes, the pre-mRNA is processed by addition of a poly$(A)^+$ tail to the 3'end and by capping of the 5' end. These modifications confer stability to the mature mRNA and allow recognition of mRNA by the ribosomes for translation. Capping is specific to RNA polymerase type II transcripts and involves modification of the 5' end by methylation. The process of capping is mediated, co-transcriptionally, by the RNA polymerase II complex.

Figure 1.6

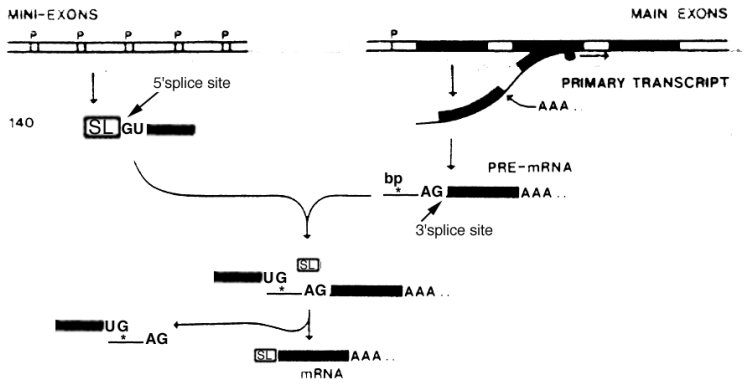


Fig. 1.6 Mature mRNA synthesis in trypanosomes is discontinuous. 140nt transcripts are derived from loci encoding tandemly repeating mini-exons. Once capped the short transcript is trans-spliced onto polyadenylated pre-mRNA transcripts. The pre-mRNAs are processed cotranscriptionally from nascent primary transcripts, frequently of polycistronic units. Trans-splicing occurs first by the cleavage of the 140nt transcript at the 5'splice site. The newly generated 5'end attaches to the pre-mRNA at a branchpoint (bp) upstream of the 3' splice site. Cleavage then takes place at the 3'splice site and the new 5'end of the pre-mRNA is ligated to the spliced leader sequence, generating mature mRNA and a forked product which is degraded. (Adapted from Laird, 1989 and Tschudi, 1995).

In trypanosomes capping cannot occur co-transcriptionally since transcription is polycistronic. Trans-splicing is employed, by trypanosomes, as a post-transcriptional mechanism to provide a homogeneous 5' end and cap (reviewed Tschudi, 1995; Vanhamme and Pays, 1995).

Trans-splicing, in trypanosomes, involves the acquisition of a 39nt sequence by the 5' end of all nuclear encoded mRNAs (Walder et al., 1986; Freistadt et al., 1988). Discovery of trans-splicing stemmed from sequencing of variant-specific surface glycoprotein cDNA (see Section 1.9) which was found to include this 39nt sequence not encoded at the genomic VSG locus (Boothroyd and Cross, 1982; Van der Ploeg et al., 1982; Kooter et al., 1984; Kooter & Borst, 1984). The 39nt sequence is referred to as the spliced leader sequence and is encoded, in a 1.35Kb tandemly repeating sequence, at one or two discrete genomic locations with a copy number of approximately 200. Each 1.35Kb repeat encodes a single transcribed RNA of 140nt which contains the spliced leader sequence (Kooter et al., 1984).

During the maturation of the 5’ end of trypanosome mRNA, the 140nt transcript donates the 39nt spliced leader sequence in a splicing reaction analogous to that used in many eukaryotes for excising introns (Murphy et al., 1986; Sutton and Boothroyd, 1986; Laird et al., 1987). The reaction takes place in the nucleus, on the nascent pre-mRNA, and involves the assembly of a ribonucleoprotein complex sometimes referred to as a spliceosome. The spliceosome consists of short nuclear RNA (snRNA) transcripts complexed with polypeptides to form ribonucleoprotein particles called snRNPs (Bruzik et al., 1988). In cis-splicing of introns these particles are well defined as U1, U2, U4, U5 and U6. Homologues of U2, U4 and U6 have been shown to be involved in trypanosomal trans-splicing reactions (Mottram et al., 1989; Tschudi and Ullu, 1990). It is thought that U1 and U5, which recognize the 5’splice site, may be replaced by activity from the 140nt transcript (Bruzik and Steitz, 1990).

The spliceosome facilitates the formation of a mature 5’end by removing sequence 3’ to the splice site of the 140nt transcript (leaving the spliced leader), and by removing sequence 5’ to the splice site for the coding sequence. The spliceosome then ligates the newly formed ends together. The mechanism can be described in two steps (Fig. 1.6). First the 5’ splice site is cleaved and the new 5’end ligated to a branch point upstream of the 3’ splice site. This is analogous to the characteristic lariat formation in cis-splicing. In trans-splicing, however, a branched “Y”- structure is generated. Second the spliced leader becomes covalently attached at 3’splice site to yield a mature 5’end and eliminates a branched structure that is degraded. Four spliced leader bases that were previously modified, (in the 140nt transcript) by methylation, form a cap 4 structure. The cap 4 structure is thought to be unique to the nuclear encoded mRNAs of kinetoplastids. It is essential for recognition and translation by the cytoplasmic ribosomes of the trypanosome (Perry et al., 1987; Frestadt et al., 1988; Ullu and Tschudi, 1991; Bangs et al., 1992).

Maturation of the 3’end is by cleavage and subsequent addition of a poly(A) tail. Processing of the 3’ and 5’ ends of trypanosome pre-mRNAs has been shown to be interdependent since abolition of trans-splicing also abolishes polyadenylation of mature

mRNA (Ullu et al., 1993). An explanation for this may be that the polypyrimidine tract which marks the 3'splice site may have a dual role also delineating the 3'end of the preceding gene. Alternatively, the polypyrimidine tract may bind a common factor used in maturation of both the 3' and 5' ends (LeBowitz et al., 1993; Hug et al., 1994; Matthews et al., 1994; Schurch et al., 1994).

1.9 Antigenic variation

After Bruce's identification of the African trypanosome as the causative agent of tsetse-fly sickness (Bruce, 1895), the parasitaemia during the time-course of chronic human disease was found to show profound undulations of regular periodicity (Ross and Thomson, 1910; see Fig. 1.7). The explanation for this dramatic rise and fall in the levels of trypanosomes sometimes seen in the host bloodstream was proposed by Ritz (1914, 1916). Ritz found resistance to serum lysis between original and relapse strains and eventually distinguished 22 variable antigen types from an infection with a single trypanosome. Ritz thought that a small subpopulation of trypanosomes might be able to alter their antigenicity, thus evading host immunity. The host immune system then eliminated the vast majority of trypanosomes before they were able to reach a lethal level. This mechanism, now called antigenic variation, is an essential component in the maintenance of chronicity of African trypanosomiasis. The elegance of a strategy in which the trypanosome can itself adapt to evade an adaptive immune response has attracted much scientific interest. Elucidation of the molecular mechanisms by which the trypanosome achieves this strategy have contributed, not only to the understanding of the disease but, to fundamental aspects of molecular biology such as genomic plasticity and gene expression.

Progress towards understanding the mechanism of antigenic variation continued with discoveries that the bloodstream form trypanosomes possessed a glycoprotein coat surrounding the plasma membrane (Vickerman, 1969), and that this coat contained the variable antigen (Vickerman and Luckins, 1969). It was then found that for any given trypanosome this coat was composed exclusively of a single glycoprotein (Cross, 1975),

but that the coat proteins expressed by trypanosomes in successive parasitaemic waves were antigenically distinct. The coat protein was therefore given the name variant-specific surface glycoprotein (VSG). A single trypanosome was found to possess a repertoire of VSG genes (Hoeijmakers et al., 1980) differing radically in nucleotide sequence. By sequentially expressing VSG genes from this repertoire, trypanosomes alter their antigenicity and evade the immune response.

From an estimated repertoire of about 1000 VSG genes (Van der Ploeg et al., 1982), only one VSG is expressed at a time. While the mechanism of this restriction is not fully worked out, it is known that only VSG genes which occupy telomeric expression sites can be expressed (Hoeijimakers et al., 1980). Expression sites are polycistronic transcription units located subtelomerically (De Lange and Borst, 1982; Kooter et al., 1987). Expression sites contain a promoter for a type 1 (α-amantin resistant) RNA polymerase and encode eight to ten expression site associated genes (ESAGs) which are situated 5' to a VSG gene (Fig. 1.8) (Kooter and Borst, 1984; Cully et al., 1985; Zomerdijk et al., 1990, 1991a,b,c).

Figure 1.7

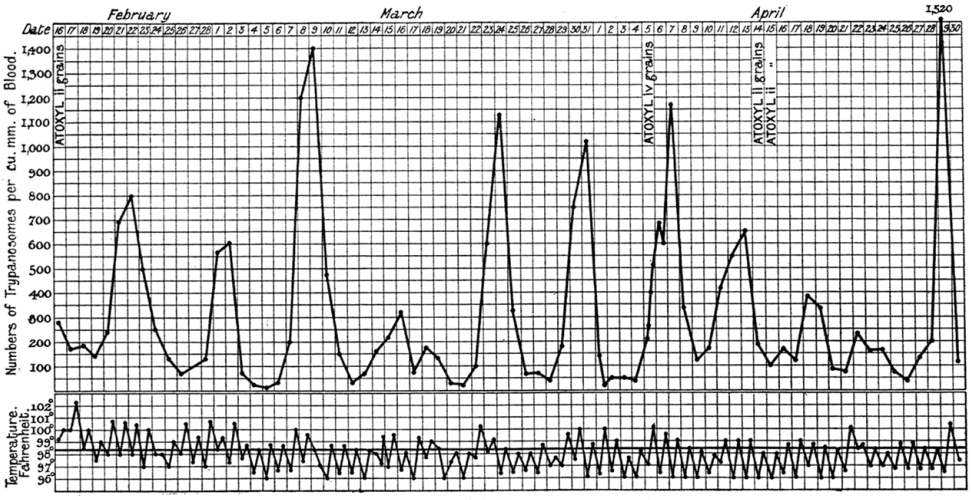


Fig. 1.7. The parasitaemias measured during the course of a human infection with *T.b.gambiense*. Counts were prepared daily. Graph shows a regular series of parasitaemic peaks and troughs, a pattern which led to the hypothesis of antigenic variation. Graph also shows patients temperature and periodic attempts at chemotherapeutic intervention. The drugs used, however, were clearly ineffective in this case. (reproduced from Ross and Thompson, 1910).

It seems that each of the trypanosome's l1 pairs of large chromosomes has an expression site situated at each telomere, so there are presumably 44 expression sites. A subset of these expression sites are, however, associated with metacyclic VSG expression as distinct from the bloodstream form expression sites. The metacyclic expression site contains a stage specific polymerase 1 type promoter and VSG gene only at the telomere and lacks expression site associated genes (Graham and Barry, 1995). It has been estimated that there are, of the order of, 25 bloodstream expression sites (Cully et al., 1985, Alexandre et al., 1988), however, intermediate chromosomes may also serve as an additional reservoir of expression sites (discussed in Ersfeld et al., submitted).

Figure 1.8

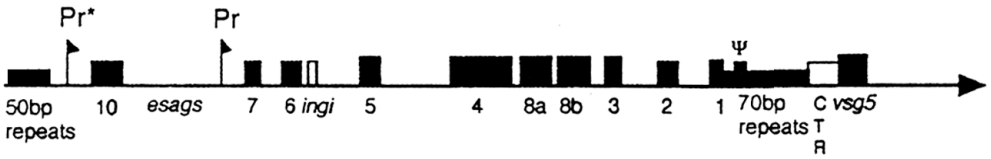


Fig. 1.8. The architecture of an expression site. Individual expression sites show some variation, conserved features being the expression site promoter (Pr), 50bp repeats, 70bp repeats, ESAGs 1 to 8, a cotransposed region (CTR) and a VSG gene. Alternative promoters (Pr*) and additional ESAGs such as ESAG 10 are found only at some expression sites. ψ is a VSG pseudogene (adapted from Cross, 1996).

Only one of these expression sites is active at a time, so that the trypanosome only ever expresses one VSG. How this is achieved remains unclear. However, the discovery of a novel nucleotide (β-D-glucosyl-hydroxymethyluracil) named "J" has indicated a possible mechanism (Bernards et al., 1984; Gommers-
Ampt et al., 1991, 1993ab). The presence of J is specific to bloodstream forms and appears concentrated at the telomeric repeats. Expression site activity has been correlated with the presence or absence of J. That is, J is present in expression sites which are inactive but absent from the active expression sites. It is not clear whether this observation is part of a mechanism, with J actively inhibiting expression site transcription, or whether J is merely an incidental marker of the inactive sites.

Antigenic variation relies on some trypanosomes in a population changing their expressed VSG before the immune system eliminates the population. The mechanism by which the expression of one VSG is arrested and a new VSG commenced is known as switching. Switching has been studied in some detail (reviewed Van der Ploeg et al., 1992; Vanhamme and Pays, 1995; Borst et al., 1996, 1997; Cross, 1996) and can occur by different mechanisms (Fig. 1.9). Basically, switching can occur in situ by inactivation of one expression site and activation of another. Alternatively, switching can occur by introduction of a new VSG gene into the active expression site.

Figure 1.9

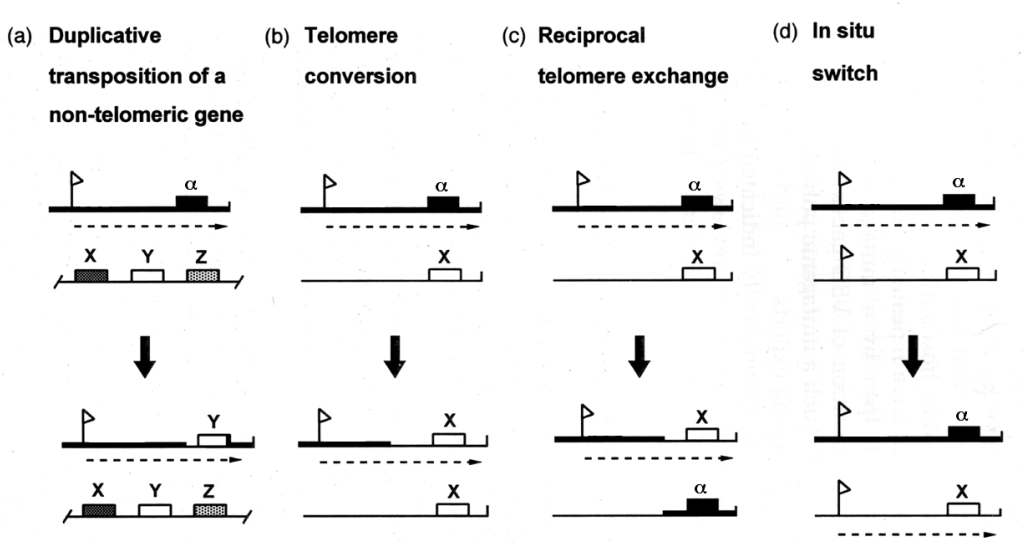


Fig. 1.9 The major mechanisms of VSG switching. The flag indicates the expression site promoter, the black box indicates the initial VSG (type α) and the dashed line with arrow indicates the active expression site with direction of transcription. Following gene conversion the VSG gene α is lost and replaced by a VSG gene copied either from an internal locus (a) or another telomeric expression site (b). Alternatively telomeric VSG genes may be exchanged so that VSG α is not lost, merely transferred to an alternative expression site (c). The active transcription site may also be changed to an alternative expression site without obvious genetic recombination (d). (Modified from Borst et al., 1997).

Introduction of a new VSG into the active expression site is usually achieved by duplicative transposition (gene conversion) from a silent locus (either internal, minichromosome or silent expression site). Gene conversion is generally mediated by two regions of homology; by the 70 bp repeat region which is 5' to the VSG gene, and by the conserved 3' end of the VSG gene (Laurent et al., 1983; Pays, 1985; Pays et al., 1985b; reviewed by Borst and Greaves, 1987). Occasionally, only part of the VSG gene is duplicated and such partial gene conversions may contribute to additional diversity in the VSG repertoire (Roth et al., 1986; Thon et al., 1989; Kamper and Barbet, 1992). In cases where the new VSG is obtained from a silent expression site, a much larger part of the expression site may be duplicated and this is often referred to as telomere conversion (De Lange et al., 1983; Kooter et al., 1988). Sometimes, telomeres are actually exchanged and this conserves both VSG genes in their expression sites (Pays et al., 1985a).

ESAG genes have been characterized in some detail and most of their expression products are associated with the plasma membrane. Some, like ESAG 4 which encodes a putative adenylate cyclase, may have a role in signal transduction (Paindavione et al., 1992; Ross et al., 1994). Of particular interest are ESAG 6 and ESAG 7 which have been shown to encode the heterodimeric components of a transferrin receptor (Steverding et al., 1994; Salmon et al., 1994). Recent work has indicated that the transferrin receptor may select for an in situ switch of expression site upon the introduction of trypanosomes into a new mammalian host (Borst et al., 1997).

ESAG 6 and ESAG 7 are polymorphic between expression sites. Individual expression sites can therefore encode transferrin receptors of different affinities. Some expression sites will encode transferrin receptors with higher affinities than those transferrin receptors encoded by other expression sites. Differences in the affinity of transferrin for its receptor may lead to growth differentials between trypanosomes with different active expression sites. This provides a basis of selection for an optimal expression site. The structure of the transferrin available to the trypanosome varies with the species of the host. Consequently, if the host is changed, then selection for a different optimal expression site may result. This change of expression site occurs by an in situ switch and being accompanied by a concomitant change in VSG type.

Expression site activity is also under developmental control. There is only minimal (about 1%) transcription of expression sites in non-bloodstream forms. The expression site promoter demonstrates life cycle stage specificity which is, in part, a quality of the promoter itself and in part due to its (sub-telomeric) chromosomal context. This mechanism of stage specific transcriptional silencing is different from the silencing of inactive expression sites in the bloodstream form. When a ribosomal promoter replaces the expression site promoter at an active expression site, the site is actively transcribed in both the bloodstream and procyclic form. When a ribosomal promoter replaces the expression site promoter at an inactive expression site, however, the site remains inactive in the

bloodstream form but transcription is activated in the procyclic form. (Rudenko et al., 1995; Horn and Cross, 1995)

Although considerable progress has been made as to the control and mechanisms of gene-switching at the molecular level, it is not clear how these events relate to the maintenance of a chronic relapsing parasitaemia at the population level. It is known that during the course of a relapsing parasitaemia the vast majority of trypanosomes in each wave express the same VSG coat, i.e., that they have the same variable antigenic type (VAT). This VAT is known as the homotype. Within a serodeme (trypanosomes with the same VSG repertoire) trypanosomes express homotypes in a semi-predictable fashion. In addition to the homotype, trypanosome parasitaemias contain minor subpopulations of trypanosomes expressing different VATs. These are known as heterotypes and generally arise by switching. When a homotype is eliminated by the immune system a heterotype grows up to replace it as a new homotype.

For a relapsing parasitaemia it might be expected that one new VAT should arise per wave. However, even in monomorphic lines which after years of laboratory passage have very low switching rates - 10^{-4} to 10^{-7} per trypanosome per generation (Lamont et al., 1986; Turner, 1997) - the switching rate is too high not to have generated several heterotype populations. In pleomorphic strains which are closer to the wild-type, switching rates may be as high as 10^{-2} to 10^{-3} per trypanosome per generation (Turner and Barry, 1989; Turner, 1997). Explanations
have been sought in terms of differential growth rates between VATs and in preferential switching to more stably expressed VATs (reviewed Vickerman, 1989), however, what determines these VAT specific properties remains unclear.

1.10 The trypanosome cell cycle

The trypanosome shows a high degree of spatial order and when it divides it must replicate and segregate several single copy structures: the nucleus, the mitochondrion and kinetoplast, the flagellum, basal body and probasal body. The spatial relationships alter

during division enabling a longitudinal plane of division to be established. Repositioning occurs so that original spatial relationships can be subsequently adopted by the daughter cells. This is achieved with fidelity, so a high degree of control is implied. Since kinetoplastids possess a single nucleus and a single kinetoplast which are both replicated during the course of one cell cycle, a description of the trypanosome cell cycle must incorporate a description of both nuclear and kinetoplast S-phase (Cosgrove and Skeen, 1970).

The cell cycle of *T. brucei* has been described for logarithmically growing cultures of the procyclic form (Woodward and Gull, 1990). It consists of a conventional nuclear cell cycle with G_1, S_N, G_2 and mitosis phases and a second kinetoplast cycle with its own G_1, S_K, G_2 and segregation phases, which are temporally superimposed on the nuclear cell cycle (Fig. 1.10). Work by Woodward and Gull (1990) analyzed incorporation of the thymidine analogue 5-bromo-2-deoxyuridine (BrdU) by immunofluorescence assay to establish the timing, initiation and duration of S_N and S_K and then, to relate this to previous detailed analysis of the division events during the cell cycle (Sherwin and Gull, 1989a).

Figure 1.10

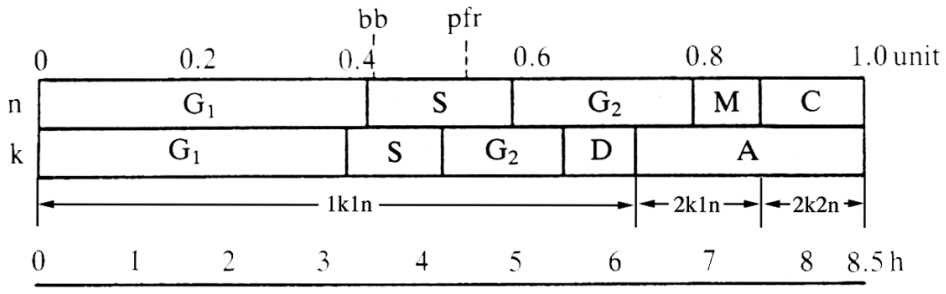


Fig. 1.10. The 8.5 hour cell cycle of the procyclic form of T.brucei. The times of basal-body duplication (bb) and initiation of synthesis of a new paraflagellar rod (pfr) are indicated. The phenotype of the cell by phase and DAPI fluorescence is also shown for each part of the cell cycle: one nucleus and one kinetoplast (1k1n), two discrete kinetoplasts and one nucleus (2k1n) or two kinetoplasts and two nuclei (2k2n). Kinetoplast segregation occurs during the nuclear G_2 phase and is denoted D. The 2k1n forms are defined by the possession of two discrete kinetoplasts using DAPI fluorescence. 1k1n forms will therefore contain some forms which possess two kinetoplasts in close proximity and which cannot be resolved at this level. There are prolonged repositioning phases prior to cytokinesis when single copy structures are correctly positioned (C and A). (Modified from Woodward and Gull, 1990).

The result of this work is that, for the procyclic forms, fluorescent dyes and immunofluorescence probes can be used, in relation to the spatial arrangement of the structures they recognize, to identify the position of a trypanosome cell in the cell cycle. In particular, the observation that kinetoplasts segregate prior to mitosis, in G_2 of the cell cycle, means that it is possible to identify cells in the latter part of the cell cycle. Such cells are known classically as dividing-forms and can be identified by the possession of two kinetoplasts and one (2k1n) or two nuclei (2k2n). Identification of dividing-forms can be achieved conveniently using the DNA staining fluor, 4,6-diamidino-2-phenylindole (DAPI) and fluorescence microscopy. It is also worth noting that in procyclic forms, the onset of S_N is concurrent with the first detectable structural changes in division, the maturation and elongation of the probasal body (Woodward and Gull, 1990). Thereafter, elongation of the new flagellum in procyclic forms continues at an approximately constant rate (Bastin, unpublished observations) until cytokinesis.

Studies of the molecular mechanisms which control the cell cycle are at an advanced stage in genetically tractable models such as *Schizosaccharomyces pombe*. This has allowed the establishment of temporal mapping of checkpoints to the conventional cell cycle and of a point of commitment to cell division in G_1 of the cell cycle designated START (reveiwed in Woolard and Nurse, 1995; D'Urso and Nurse, 1995). In dissecting these temporal events, by the identification of specific mutants, two pivotal families of regulatory molecules have been discovered: the mitotic and G_1 cyclins, and the cyclin associated CDC (cell division cycle) protein kinases. These families show a high degree of evolutionary conservation among eukaryotes but do not appear to be involved in prokaryotic replication (see Donachie, 1993). In the evolutionarily divergent trypanosome, homologues of CDC2 have been found and these have been named CRK 1-4 (Mottram and Smith, 1995). A putative cyclin homologue has also been reported (Affranchino et al., 1993) but appears not to be cell cycle regulated (Hua et al., 1997). Definitive roles for these homologues have not yet been established in the trypanosome cell cycle (reveiwed in Boshart and Mottram, 1997) but, recently, histone proteins have been demonstrated to show cell cycle specific gene expression (Ersfeld et al., 1996).

1.11 Differentiation

Cellular differentiation is characterized by profound changes in cellular morphology, biochemistry, gene expression and behaviour. In multicellular organisms differentiation allows for specialization of cell types, whereas in protozoons, analogous differentiation can allow an organism to adapt to diverse environments. Differentiation may be programmed to occur after a certain number of divisions or may be a response to changes in the cell's environment. Differentiation is a highly controlled process which can affect multiple fundamental biological processes within the cell. Consequently, differentiation normally involves the variation of gene expression, from a number of separate loci and even separate genomes, in such a way that the gene products impact, in a coordinated fashion, on the cellular processes involved. One established approach to

understanding such a complex process, in any given cell type, involves first identifying what is changing during the course of differentiation and then identifying the mechanisms by which such change is brought about.

The mechanism of cellular differentiation is a cascade which links the impetus to differentiate with the multiple effects of differentiation. Many of the intermediates of such cascades have been discovered during recent years. Some show a surprising degree of conservation between both the various cellular differentiations occurring in the same organism and between divergent species throughout evolution. Intermediates of such cascades include:

- Kinases and phosphatases, which directly control the activity of many cellular proteins including transcription factors.
- Second messenger metabolites such cAMP, cGMP, inositol triphosphate and diacylglycerol.
- GTP binding proteins.
- Ionic fluxes, such as Ca^{2+}, which in addition to activating calcium binding proteins, like calmodulin, can directly affect the degree of nuclear condensation affecting transcription factor access.

At the biological level, differentiation may take a number of forms. In animal cells differentiation generally follows an ontogeny from a pluripotent stem cell through to a cell which is said to be terminally differentiated. Terminally differentiated cells are arrested in the cell cycle and are programmed to die rather than to divide again. During the course of ontogeny pluripotent cells become progressively more specialized, losing the potential to form different types of cell. This is the case in haematopoiesis where terminally differentiated blood cells are generated through a series of differentiations (reviewed in Testa & Dexter, 1990; Bockamp et al., 1994). This sort of differentiation is unidirectional and, once differentiated, a B-cell cannot again become a pre-B cell let alone a stem cell. Plant cells, however, maintain potency as they differentiate. Consequently, if environmental conditions are changed appropriately, even differentiated phloem cells,

which would not normally divide again, will dedifferentiate and proliferate to form a callous and then a new plant (Steward et al., 1958).

Figure 1.11

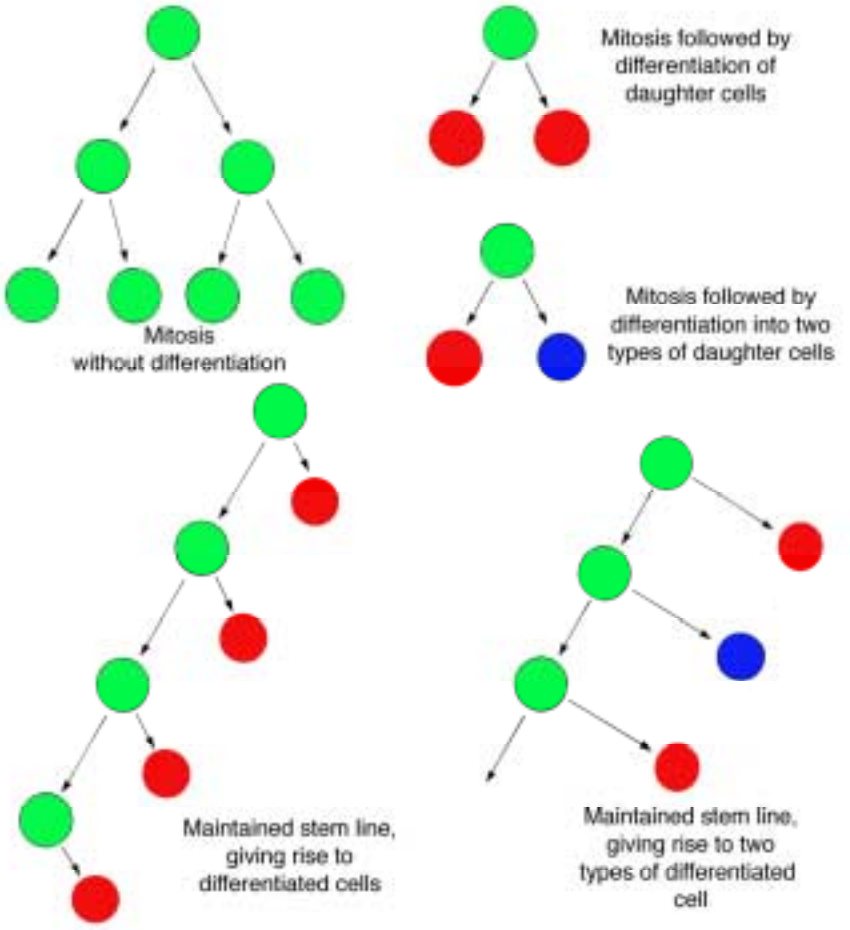


Fig. 1.11 General models for proliferation and differentiation applied to metazoan cells (reproduced from Smith and Wood, 1997, with permission).

Differentiation can therefore be unidirectional or reversible. In both cases important, transitional, temporal landmarks can be established when all of the mRNA and, then, all of the protein necessary for completion of differentiation has been synthesized by the cell. Where differentiation is unidirectional, however, a point of commitment must

occur after which the cell must go on to complete differentiation. Commitment is likely to occur at a point where cellular phenotype has not yet changed substantially, but the molecules responsible for coordinating the early differentiation have been synthesized.

Cellular differentiation is often, but not necessarily, linked to the cell cycle. Although some microorganisms, such as *Naegleria gruberi*, apparently undergo differentiation in a cell cycle independent fashion (Fulton, 1977), differentiation may be associated with exit from the cell cycle into a quiescent state (G_0). This is seen in the diploid spore formation of *Dictyostelium discoidum* (Greg, 1971), or during differentiation of somatic tissue. Alternatively, differentiation may accompany a cell's re-entry into a new cell cycle; this is probably best characterized in trypanosomes (Section 1.12.1), but is paralleled by examples such as spore germination and arguably the expansion of memory B-cells. There is good reason for interaction between the cell cycle and differentiation, since the machineries (such as the cytoskeleton) which mediate these two processes are often shared and so the changes involved in both processes must be coordinated. Differentiation can be related to division by a number of models which predict the possible fates of cells from the preceding division (Fig. 1.11).

1.12 Differentiation in trypanosomes

The life cycle of *T.brucei* (Section 1.3) is composed of successive cellular differentiations between proliferative and non-proliferative forms. Consequently every differentiation event is linked either to cell cycle arrest or re-entry. Additionally, trypanosome differentiation involves alterations in morphology, motility, metabolism (particularly with respect to oxidative phosphorylation and glycolysis) and, in some cases, exchange of the major surface coat protein.

1.12.1 The stumpy-to-procyclic differentiation

Although there are possibly six differentiation events in the trypanosome life cycle, only two are readily tractable. The differentiation from the stumpy form to the procyclic

form is the most amenable to study. This is because 10^{10} stumpy forms, propagated in vivo, can be harvested from a single rat with a high degree of purity. Under appropriate conditions (27^{o}C and in the presence of cis-aconitate), a rapid and very high efficiency differentiation takes place. This differentiation is synchronous with respect both to the acquisition and loss of well defined markers and with respect to re-entry to the cell cycle (Czichos et al., 1986; Zeigelbauer et al., 1990; Pays et al., 1993; Matthews and Gull 1994, 1997; Matthews et al., 1995).

Down regulation of VSG expression, accompanied by shedding of the VSG coat and the gain of procyclin (or PARP) - the procyclic specific coat protein, are the markers most amenable to study. Although VSG and PARP promoters show constitutive activity (Pays et al., 1990), expression is controlled, at least in part, transcriptionally. Transcriptional control is exerted both, by inhibition of initiation at the promoter and, by premature termination of transcripts (Pays et al., 1989, 1990; Rudenko et al., 1994). Control is also exerted at a post-transcriptional level, at the level of mRNA stability, (Ehlers et al., 1987; Pays et al., 1990; Jefferies et al., 1991; Graham and Barry, 1996) and this is the case for the vast majority of differentially expressed genes, including glycosomal enzymes and hexose transporters (Gibson et al., 1988; Vijayasarathy et al., 1990; Brigaud and Baltz, 1993). For many such stage specific transcripts, differential mRNA stability has been shown to be regulated at the 3'UTR (Jefferies et al., 1991; Hug et al., 1993; Berberof et al., 1995; Hotz et al., 1995). For reviews on regulation of gene expression see Clayton (1992), Hehl and Roditi (1994), Graham (1995) and Vanhamme & Pays (1995).

Slender forms can also differentiate to procyclic forms (Balber, 1970; Bass and Wang, 1991) but, as a population, they do so in an asynchronous manner (Matthews and Gull, 1994a). It has been proposed this asynchrony could be accounted for in a model where differentiation to the procyclic form could only be initiated in a receptive window of the cell cycle. The window in G_1 would be the point at which the stumpy form had arrested and so stumpy form differentiation would be rapid and synchronous. Initiation of slender

differentiation would, however, be spread through the course of one cell cycle, explaining the asynchronous kinetics (Matthews and Gull, 1994b).

The coincidence of cell cycle re-entry and initiation of the stumpy to procyclic differentiation has led to speculation as to the degree of interdependence between the two events. Experiments to uncouple the two events have involved drug induced disruption of cytokinesis (Markos et al., 1989) and S phase (Matthews and Gull, 1994a) and have shown an uncoupling of these processes relatively early in the differentiation from stumpy to procyclic forms. These experiments also demonstrated commitment, to the production of a procyclic form, occurring approximately 4 hours through the time-course of differentiation (Matthews and Gull, 1994a).

The trigger for the stumpy to procyclic differentiation is unknown in vivo, but in vitro cis-aconitate has been shown to be an excellent trigger. Addition of cis-aconitate initiates differentiation even in the absence of a decrease in temperature (Overath et al., 1986; Matthews and Gull 1997). It is an enigma as to why this should be; cis-aconitate is a transient intermediate in Krebs cycle between citrate and isocitrate and is not present in the tsetse midgut at high concentration. The enzyme aconitase is responsible for mediating both cis-aconitate formation from citrate and its transition to isocitrate. The gene for an aconitase enzyme was recently cloned and “knocked out” in pleomorphic trypanosomes (Boshart, Harden conference report, unpublished). The disruption of this gene had no effect on the role of cis-aconitate in triggering the stumpy-to-procyclic differentiation in the transgenic line produced. There have been reports of other triggers for the stumpy to procyclic differentiation. Treatment with pronase (Hunt et al., 1994) and mild acid stress (Rolin et al., 1996) both trigger differentiation, perhaps by direct action on an unknown surface membrane receptor. Another preliminary report has shown lack of glucose to have a potential triggering role, a role which may reflect the rapid depletion of glucose in the tsetse-fly midgut after a blood meal (Ferguson, Harden conference report, 1997, unpublished).

1.12.2 The slender-to-stumpy differentiation

Two morphologically distinct stages of the trypanosome coexist in the mammalian bloodstream, the stumpy and the slender form. The slender form is proliferative and predominates at low parasitaemia, the stumpy form is non-proliferative and is predominant just after the parasitaemic peak. Bloodstream stages are themselves heterogeneous, but also contain cells of intermediate morphology; such populations are said to be pleomorphic. This is in contrast to monomorphic lines, which have lost the ability to produce stumpy forms through multiple syringe passage.

The slender-to-stumpy differentiation is characterized by three major events:

1) Arrest in G_1 of the cell cycle (Shapiro et al., 1984; Czichos et al., 1986; Zeigelbauer et al., 1990; Pays et al., 1993; Matthews and Gull, 1994a,b).
2) Change in morphology - the change which allowed the initial discrimination of each life cycle stage (Bruce, 1912; Robertson, 1912a; Hoare, 1960, 1972).
3) Partial elaboration of the mitochondrion - as a preadaptation for survival in the tsetse-fly midgut (Vickerman, 1965; Bowman and Flynn, 1968; Flynn and Bowman, 1973; Brown et al., 1973; Bienen et al., 1991, 1993. Reviewed by Vickerman, 1985; Priest and Hajduk, 1994).

These characteristics make *T.brucei* an interesting system in which to study the coordination of three fundamental and biochemically distinct processes. With regard to the cell cycle, during the slender-to-stumpy differentiation, trypanosomes arrest during G_1 of the bloodstream form cell cycle. Stumpy forms can therefore be considered to occupy a G_0 state since, although stumpy forms retain potency, stumpy forms will not divide in a mammalian host. Alternatively, stumpy forms can be considered to be in G_1 of the procyclic form cell cycle, since stumpy forms will continue to progress through the cell cycle under conditions in which procyclic forms grow well.

Increase in mitochondrial activity is accompanied by changes in expression of kinetoplast as well as nuclear encoded genes, implying a mechanism for the coordination of expression from these two genomes during differentiation. The large size of the trypanosome's single mitochondrion facilitates microscopic analysis of this organelle and makes the trypanosome an accessible system for investigating the coordination of mitochondrial and nuclear gene expression during mitochondrial biogenesis.

Although in vitro studies indicate that the slender form can differentiate to the procyclic form without an intervening stumpy stage (Balber, 1970; Bass and Wang, 1991; Matthews and Gull, 1994a), earlier studies found that stumpy forms preferentially established tsetse midgut infections (Robertson, 1912b; Ashcroft, 1957; Wijers and Willet, 1960). The increased activity of the stumpy mitochondrion and the position occupied in the

cell cycle preadapt the stumpy form trypanosome for survival in the tsetse midgut. The stumpy form is apparently poised for rapid transition to the procyclic form and seems able to metabolize proline, the major metabolite available in the tsetse midgut, after the rapid depletion of glucose from the bloodmeal.

Differentiation to the stumpy form also slows the rate of increase in the parasitaemia, and may restrict peak parasitaemia to a non-lethal level. If slender forms do not arrest and differentiate to stumpy forms, then at high parasitaemia the host expires from hypoglycemia before an effective immune response is mounted (Herbert et al., 1975). In fact, it has been suggested that it is primarily the stumpy form rather than the slender form which triggers the immune response (Sendashonga and Black, 1972). The stumpy form is, however, more resistant to antibody mediated killing than slender forms (Mclintok et al., 1993), perhaps because of a higher rate of antibody clearance at the flagellar pocket (Russo et al., 1993). This resistance to antibody allows a prolonged period of potential transmission without jeopardizing the next parasitaemic wave.

Since the stumpy form is arrested in the cell cycle, differentiation can be modeled with respect to the division which precedes formation of a stumpy form (Fig. 1.12). In their simplest form, models envisage differentiation commencing prior to division. This implies that division is heterogeneous to previous proliferative divisions from which a final differentiation-division can be distinguished. Alternatively, differentiation may not be initiated until after division and this would mean that slender form division was homogeneous, consisting solely of proliferative divisions (models proposed by Matthews and Gull, 1994b).

Figure 1.12

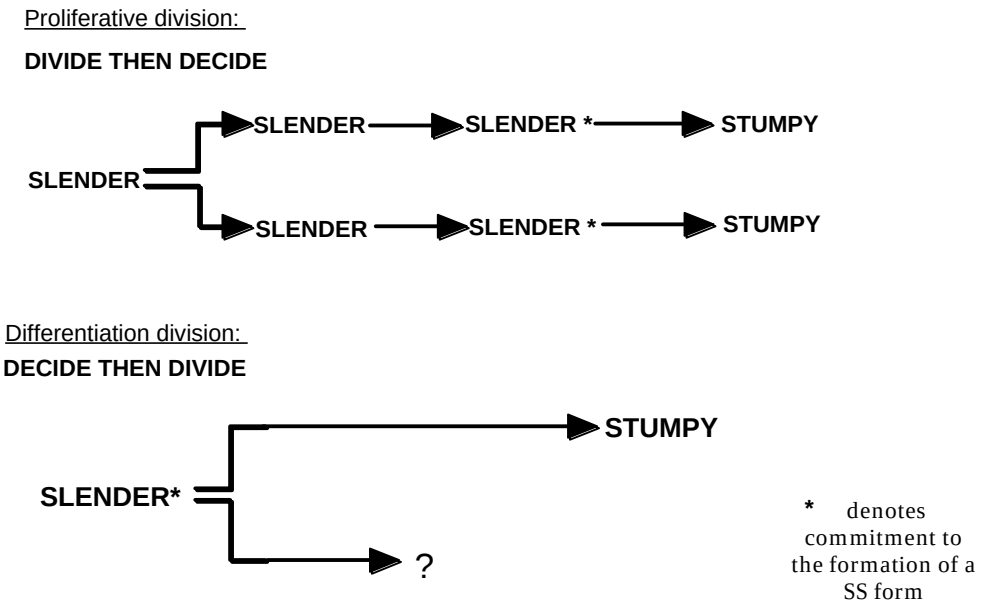


Fig. 1.12 Models of the slender-to-stumpy differentiation. The choice to produce a stumpy form can be made before or after the division from which the stumpy form arises. * denotes the commitment to produce a stumpy form. ? indicates that there is more than one possible fate for this trypanosome.

1.13 The aims of the project

Although the slender-to-stumpy differentiation is associated directly with pathogenesis and trypanosome virulence, systematic examination of this differentiation as a whole has largely been neglected for some years. The reasons for this lie in the intractability of a system which (at the beginning of this project) could not be followed in vitro and lacked defined molecular markers. Nevertheless, it was felt that the time was opportune to begin to apply some of the technology developed in the last twenty years to the investigation of this differentiation. The research summarized in this thesis are the initial studies in an ongoing project on the slender-to-stumpy differentiation at the University of Manchester. This aspect of the project was concerned specifically with the coordination of commitment with the three major events of differentiation: cell cycle arrest,

morphogenesis and mitochondrial biogenesis. The project aimed to discriminate between models previously proposed (Matthews and Gull, 1994) to account for these events.
In order to do this the project sought:

1) To develop reliable and reproducible systems, in vivo and in vitro, with which to investigate bloodstream form division and differentiation.

2) To provide a basic analysis of division in the bloodstream form facilitating examination of cell cycle arrest.

3) To establish well characterized molecular and structural markers of the slender-to-stumpy differentiation.

4) To relate the acquisition of stumpy form specific markers to cell cycle position within the differentiation system developed and, hence, develop a working hypothesis for the temporal order of the major events during the slender-to-stumpy differentiation.

Chapter 2

Materials and methods.

2.1 Materials

2.1.1 The suppliers of consumables

Except where specified:

Disposable plastics from Costar (High Wycombe, Buckinghamshire, UK) or

Sigma Chemical Company Ltd. (Sigma; Poole, UK).

Solvents and chemicals from BDH Ltd. [Merck]. (Liverpool, UK),

Boehringer Mannheim [BCL] (Lewes, UK),

Gibco-BRL (Gibco; Uxbridge, UK) or Sigma.

Bacterial culture medium from Oxoid (Basingstoke, UK) and Gibco.

2.1.2 Trypanosomes

The bloodstream form trypanosomes that were used in these studies were gifts from Dr. C.M.R.Turner at the University of Glasgow, U.K.

- These bloodstream forms were derived from a stabilate of EATRO 2340.
- EATRO 2340 is a line of *Trypanosoma brucei rhodesiense*, which was isolated from a human infection in Nyanza, Kenya.
- Two lines were used, the pleomorphic GUP 2962 and the monomorphic GUP 2965. Both these lines are of GUTat 7.2.
- GUP 2962 was obtained via a clonal metacyclic infection of EATRO 2340 which was passaged through mice 29 times, with 7 recloning events. This generated a pleomorphic line of GUTat 7.13 (Cornelissen et al., 1985). GUP 2962 was derived from a relapse population of this GUTat 7.13 line, following two rounds of cloning (McLintock et al., 1990).
- The monomorphic line GUP 2965 was derived from cloned EATRO 2340, following 40 rounds of rapid passage and recloning, which yielded a monomorphic line of GUTat 7.1. GUP 2965 was then cloned following the induction of a relapse population in a murine infection with the monomorphic GUTat 7.1 line (Barry et al., 1985).

Procyclic forms were obtained from logarithmically growing cultures of *Trypanosoma brucei brucei* strain 427. This strain is a commonly used laboratory strain which has been maintained by in vitro culture for many years.

2.1.3 Animals

Immunocompetent adult female Balb/c and CD1 mice, and Sprague-Dawley rats, were used for raising all parasitaemias. Animals were derived from Charles River (Manston, Kent) and were either supplied directly to, or bred domestically by, the biological services unit (BSU) at the University of Manchester.

2.1.4 Bacteria

Only one strain of *Escherichia coli* was used for these studies:

XL1-Blue was supplied by Stratagene (Stratagene, Cambridge, UK).

Genotype: *Sup*E44, *hsd*R17, *rec*A1, *end*A1, *gyr*A46, *thi*, *rel*A1, *lac*$^{-}$.

F' [*pro*AB^{+}, *lac*I^{q}, *lac*ZDM15, Tn10(*tet*^{r})].

2.1.5 Antibodies

Part of this project involved the screening of a large number of antibodies, from various laboratories. I screened antibodies raised to antigens which I considered potentially stage regulated between the long slender and short stumpy form. These antibodies are detailed and discussed in Chapter 3 (see table 3.2).

Six additional antibodies were also used during the course of this project, these were:

- TAT :- a murine IgG monoclonal antibody which recognizes trypanosome α-tubulin (Woods et al., 1989b).
- Anti-procyclin:- a commercially available murine IgG monoclonal antibody raised to the surface coat protein of the procyclic form (Cedar lane laboratories, Spain).
- Anti-7.2 :- a rabbit polyclonal antibody (gift of Dr. C. M. R. Turner, Glasgow, UK).
- Anti-CAP 5.5:- a murine IgG monoclonal antibody (raised by Dr. R. Docherty at the university of Manchester, Unpublished).
- ROD:- a murine IgM monoclonal antibody which recognizes a minor protein of the paraflagellar rod (Woods et al., 1989).
- Anti-PFR:- a murine monoclonal IgG which recognizes both the PFR-A and PFR-C of the paraflagellar rod (Raised by Dr. L. Kohl at the University of Manchester, Unpublished).

All secondary antibodies used during this project were conjugates supplied by Sigma.

2.1.6 Solutions and buffers

Agarose gel electrophoresis buffer for DNA (1 *x* TAE buffer)

40mM Tris-acetate, 1mM ethylenediamine tetra-acetic acid disodium salt (EDTA); (pH 8.0).

Agarose gel electrophoresis buffer for RNA (1 *x* MOPS buffer)

40mM MOPS, 10mM sodium acetate, 1mM EDTA;(pH 7.0).

Agarose gel loading buffer for DNA

40% (w/v) Ficoll 4000, 5mg/ml bromophenol blue.

Agarose gel loading buffer for RNA

50% glycerol (v/v), 1mM EDTA, 0.4% bromophenol blue (w/v), 0.4% xylene cyanol (w/v).

Ampicillin stock (1000 x)

50mg/ml ampicillin in distilled water, stored at -20^{o}C.

Bovine haemin stock

2.5mg/ml bovine haemin made up in 0.05N sodium hydroxide solution.

Buffer 1 (for northern blotting using the DIG system)

100mM maleic acid, 150mM NaCl; pH 7.5.

Buffer 2 (for northern blotting using the DIG system)

1% blocking reagent made up in Buffer I - Blocking reagent is provided in the DIG labelling kit.

Buffer 3 (for northern blotting using the DIG system)

100mM Tris-Cl (pH 9.5), 100mM NaCl, 50mM $MgCl_2$.

Coomassie staining solution

Coomassie brilliant blue in 25% (v/v) propan-2-ol/ 10% (v/v) acetic acid.

4,6-diamidino-2-phenylindole (DAPI) 1000 *x* stock solution

10mg of DAPI (Sigma) was dissolved in 1ml of distilled water and stored at -20^{o}C in a light-proof container.

Denaturing solution

4M guanidium thiocyanate, 25mM sodium citrate pH 7.0, 0.5% N-lauroylsarcosine (v/v), 0.1M β-mercaptoethanol.

Diethypyrocarbonate (DEPC) treated water

0.1% (v/v) DEPC added to distilled water, shaken and autoclaved.

Ethidium Bromide (EtBr) 1000 *x* stock solution.

10mg of EtBr (Sigma) was dissolved in 1ml of distilled water and stored at room temperature in a light-proof container.

Hybridization solution for RNA FISH

50% formamide, 2 *x* SSC, 10% dextran sulphate (w/v), 50mM phosphate buffer (pH 7.0), 200μg/ml herring sperm DNA and 200μg/ml yeast tRNA.

Phosphate buffered saline (PBS).

200mg/ml KCl, 8g/l NaCl, 114mg/l NaH_2PO_4, 900mg/l Na_2HPO_4.

Adjusted to the required pH with 5N NaOH or 2N HCl.

PEME

100mM piperazine-N,N'-bis (2-ethane sulphonic acid) (PIPES), 2mM ethylene glycol-bis(b-amino ethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 1mM $MgSO_4$, 0.1mM EDTA; pH 6.9.

Plasmid preparation solutions

Solution I

50mM glucose, 25mM Tris-Cl pH 8.0, 10mM EDTA.

Solution II

0.2M NaOH, 1% (w/v) sodium dodecyl sulphate (SDS).

Solution III

5M CH_3COOK, 11% (v/v) glacial acetic acid.

Ponceau's solution

0.25% (w/v) Ponceau stain, 40% (v/v) methanol, 15% (v/v) acetic acid.

Protease inhibitor mixture

50µg/ml chymostatin, 5µg/ml pepstatin A, 50µg/ml leupeptin.

Prehybridization solution for northern blotting

50% (v/v) deionized formamide, 5 *x* SSC, 2% DIG system blocking reagent (Boehringer Mannheim), 0.1% (w/v) N-lauroylsarcosine, 0.02% (w/v) SDS.

Pucks saline G (PSG).

488.8mg/l NaH_2PO_4, 2.55g/l NaCl, 8.08g/l Na_2HPO_4, 15g/l D-glucose.

Adjust pH to 7.8 with 5N NaOH.

Sequencing gel electrophoresis buffer (TBE)

1 *x* TBE is 0.09M Tris-borate, 0.002M EDTA.

Sodium Dodecyl Sulphate/Polyacrylamide gel electrophoresis (SDS-PAGE) buffer

1.4% glycine(w/v), 0.3%(w/v) Tris, 0.1% SDS(w/v).

SDS-PAGE sample buffer

1% SDS (w/v), 0.25M sucrose, 0.125M Tris-Cl; (pH 6.8).

SDS-PAGE Lower gel buffer (4 *x* stock)

1.5M Tris-Cl (pH 8.8), 0.4% SDS.

SDS-PAGE Upper gel buffer (4 *x* stock)

0.5M Tris-Cl (pH 6.6), 0.4% SDS.

SSC (20 *x* stock)

3M NaCl, 0.3M sodium citrate.

STE buffer

0.1M NaCl; 10mM Tris-Cl pH 8.0; 1mM EDTA.

Tetracyclin stock solution (100 x)

5mg/ml tetracyclin in 50% ethanol stored at -20^{o}C in a light-proof container.

Tris buffered saline (TBS)

10mM Tris-Cl (pH 7.4), 140 mM NaCl.

Western blotting buffer

0.58% Tris (w/v), 2.9% glycine (w/v), 0.1% SDS (w/v), 20% methanol (v/v).

2.1.7 Bacterial culture media

Luria-broth (LB)

1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl; (pH 7.0).

LB agar

1% (w/v) bacto-tryptone, 0.5% (w/v) Yeast extract, 1% (w/v) NaCl 1.8% (w/v) Bacto-Agar (pH 7.0).

S.O.C. medium

10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose 2% Tryptone (w/v), 0.5% yeast extract (w/v).

2.1.8 Trypanosome culture media

Sera and media supplements

Supplied by Gibco:

All amino acids, bovine haemin,

Heat inactivated foetal calf serum (HIFCS),

N-2-Hydroxyethylpiperazine-N'-2-Ethane Sulphonic Acid (HEPES),

50 *x* stock solution of Hypoxanthine and Thymidine (H.T.),

Iscove's modified Dulbecco's medium (IMDM),

Minimum Essential Medium (MEM) B14 powder,

MEM amino acids w/o L-Glutamine,

MEM non-essential amino acids,

Medium 199 TC 75 powder,

p-aminobenzoic acid, biotin, folic acid,

adenosine, guanosine and glucosamine.

Supplied by Sigma:

3-(N-morpholino)propane sulphonic acid (MOPS),

Bathucuprione sulphonate, β-mercaptoethanol,

Sodium pyruvate, D-Glucose and NaHCO.

Supplied by Sera-labs(Crawley Down, Sussex, UK):

Serum plus and heat inactivated equine serum.

Bloodstream form culture medium.

HMI-9 (Recipe of Hirumi and Hirumi, 1989).

One litre of bloodstream form trypanosome culture medium HMI-9 contains:

730ml IMDM, 100ml HIFCS,

100ml Serum plus (or horse serum),

20ml 50 *x* H.T. supplement, 10mg sodium pyruvate,

182mg L-Cysteine, 14µl β-mercaptoethanol,

28.2mg Bathocuprione sulfonate, 50ml distilled water.

Fresh HMI-9 medium was normally prepared monthly and stored at 4^{o}C.

HMI-9 was sterilized with a 0.22 micron filter and prewarmed to 37^{o}C prior to use.

Procyclic form culture medium.

SDM 79 (Recipe of Brun and Schonenberger, 1979).

7g MEM B-14 powder, 2g Medium 199 TC 45 powder,

8ml MEM amino acids w/o L-Glutamine, 6ml MEM non-essential amino acids,

1g D-Glucose, 8g HEPES, 5g MOPS,

2g NaHCO, 100mg Sodium pyruvate,

200mg L-alanine, 100mg L-argenine, 300mg L-glutamine, 70mg L-methionine,

80mg L-phenylalanine, 600mg L-proline, 60mg L-serine, 160mg L-taurine,

350mg L-threonine, 100mg L-tyrosine, 10mg adenosine, 10mg guanosine,

50mg glucosamine-HCl, 4mg Folic acid, 2mg p-aminobenzoic acid, 0.2mg biotin.

The pH was adjusted to 7.3 with 2N NaOH.

The medium was sterilized with a 0.22 micron filter.

HIFCS, to 10% (v/v), and sterile bovine haemin, to 7.5µg/ml, were added just prior to use.

2.2 Methods

2.2.1 Growth of trypanosomes in rodents

Mouse inoculation was by an intraperitoneal route, with approximately 1×10^6 slender trypanosomes from a frozen stock. These were delivered in 0.3ml of PSG (pH 7.8) through a 25 gauge needle. Rats were also infected by an intraperitoneal route, but were lightly anaesthetized. In each case, the rat was allowed to inhale Fluothane (Zeneca, Macclesfield, UK.) vapors in a dedicated chamber until balance was lost. 1×10^7 trypanosomes, in up to 200µl of citrated blood from a freshly sacrificed mouse, were then delivered directly into the peritoneum using a 23 gauge needle.

Unless otherwise stated, parasitaemias were monitored daily from the third day after infection. Samples were obtained initially by tail-snipping with a sharp surgical scissors and subsequently, by removing the scab formed over this cut. Rats were again lightly anaesthetized during this procedure. Parasitaemia was monitored by tail bleeding at appropriately spaced intervals and assessed microscopically using an improved Neubauer haemocytometer or a rapid scoring technique (Herbert and Lumsden, 1976). The rapid scoring technique allows estimation of the bloodstream parasitaemia from a drop of fresh, infected blood, that has been placed on a microscope slide, under a glass coverslip. Air dried blood smears (Section 2.2.7) were made from the same tail bleeds as used for bloodstream counts. This allowed assays by morphological, immunofluorescence (Section 2.2.9) and NAD diaphorase (Section 2.2.10) criteria to be compared to the parasitaemia at each time point.

To harvest trypanosomes from infected rodents, exsanguination by cardiac puncture was performed under anaesthetic. Rats and mice were allowed to inhale Fluothane vapors in a dedicated chamber until the “involuntary twitching response” stopped. At this point the rodents are not conscious and are very heavily sedated but their hearts are still beating. Sodium citrate (2% w/v) dissolved in PSG was present as an anticoagulant in the syringes used for cardiac puncture. The final concentration of citrate in infected blood was

approximately 0.4% (w/v). Cervical dislocation of rodents under terminal anesthetic was performed after exsanguination to ensure fatality.

2.2.2 Cryopreservation of bloodstream trypanosomes

Trypanosomes in blood were harvested from a mouse three days post-infection, at a parasitaemia of approximately 8×10^7 trypanosomes per ml. An equal volume of sterile 14% glycerol (v/v in PSG) was added dropwise to infected blood, mixed and injected into 0.63mm (internal diameter) translucent vinyl capillary (Portex), which was cut into 2cm lengths. These "straws" were placed in 1ml screwtop vials (Nunc), cooled slowly to -80^{o}C in a polystyrene container over a 24h period and then transferred to liquid nitrogen for long term storage.

2.2.3 Purification of bloodstream trypanosomes from blood

Bloodstream form trypanosomes were purified from blood cells using a column packed with DE-52 - granular preswollen diethyl aminoethyl cellulose anion exchanger (Whatman) - according to the method described by Lanham (1968). The VSG coats of bloodstream form trypanosomes have a basic character; in mild alkaline conditions they remain effectively uncharged. This is in contrast to proteins on the surface of blood cells which, under such conditions, become anionic. Consequently, at pH 7.8 the anion exchanger DE-52 will bind to blood cells but not to bloodstream form trypanosomes.

DE-52 was suspended as a slurry in PSG. The pH of the slurry was adjusted to 7.8 using 5N hydrochloric acid. A glass column, containing a sintered glass disc of porosity 1, was packed by pouring the slurry into it. The column was then equilibrated with PSG (pH 7.8). Infected blood was poured directly onto the wet column. PSG (pH 7.8) was poured through the column and the eluate containing the trypanosomes collected in 50ml centrifuge tubes. These tubes were centrifuged for 10mins at 1600 *x* g in a benchtop centrifuge, and the supernatant was discarded into a glass beaker containing 0.1% (w/v) sodium hypochlorite (bleach). The trypanosome pellets were then washed by gentle

resuspension in 1ml of PBS (pH 7.4), transferred to microcentrifuge tubes and centrifuged for 5mins at 1600 *x* g. The supernatants were again removed and the trypanosomes pooled, gently resuspending the pellets in a total of 1ml of PBS. Cell counts were performed by appropriately diluting a 2µl aliquot of suspended trypanosomes and counting the trypanosomes in the diluted aliquot with an improved Neubauer haemocytometer. After a final 5mins centrifugation at 1600 *x* g, the supernatant was removed and the pellet was resuspended as required for subsequent procedures (preparation of microscopy, protein and RNA samples, Sections 2.2.8, 2.2.11, 2.2.24, respectively).

2.2.4 Culture of procyclic trypanosomes

Procyclic trypanosomes were maintained by passage every 48-72h in SDM-79 medium (Brun and Schonenberger, 1979) containing 10% (v/v) HIFCS and 7.5µg/ml bovine haemin. Prior to passage, aliquots of cells were diluted appropriately in PBS, and the cell count was made using an improved Neubauer haemocytometer. Passage was to a concentration of 1×10^5 trypanosomes per ml in SDM-79 prewarmed to 27°C. Trypanosomes were harvested during logarithmic growth about 48h after passage and at a concentration of between 10^6 and 10^7 trypanosomes per ml. Once harvested, procyclic trypanosomes were washed twice with PBS and used as described for bloodstream form trypanosomes (Section 2.2.3).

2.2.5 Culture of bloodstream trypanosomes

Detailed discussion of the culture of bloodstream form trypanosomes is found in Chapter 3. Culture of the pleomorphic line GUP 2962 in liquid medium can be initiated at low parasite density. In order to initiate cell culture, a mouse was infected. When the parasitaemia reached no higher than 1×10^7 trypanosomes per ml of blood, 50µl of blood was removed asceptically either by tail bleeding or by cardiac puncture. This infected blood was diluted to 1×10^5 trypanosomes per ml in HMI-9 (Hirumi and Hirumi, 1989) prewarmed to 37°C. 10µl of this suspension was then added to 10ml of prewarmed (37°C) HMI-9 in a 75ml cell culture flask, which was fitted with a 2.2 micron filter cap to allow gaseous exchange. Cells were then cultured at 37°C, in a 5% CO_2 incubator (Forma scientific, Ohio, USA).

During three months of culture adaptation the optimal time between passage of bloodstream cultures varied, becoming progressively shorter. After this period, cells were routinely passaged every 48h by pipetting a small volume into a fresh 50ml flask containing 10ml of prewarmed HMI-9. Cells were always passaged to a concentration of 10^4 trypanosomes per ml.

2.2.6 Bloodstream form to procyclic form differentiation

In order to initiate differentiation, bloodstream forms suspended in HMI-9 at 2×10^6 trypanosomes per ml were incubated at 27°C in the presence of 6mM cis-aconitate (Czichos et al., 1986; Zeigelbauer et al., 1990; Matthews and Gull, 1994). Samples were taken by removing 1ml aliquots, at regular intervals, into microcentrifuge tubes and then centrifuging and washing trypanosomes as described before (Section 2.2.3). Trypanosomes were assessed for the gain of procyclin and the differentially regulated cytoskeleton associated protein, CAP5.5, (Matthews and Gull, 1994) by immunofluorescence microscopy (Section 2.2.9).

2.2.7 Preparation of air dried bloodsmears

Single drops of blood (approximately 10μl) were placed onto individual "twin frosted" microscope slides close to the bottom edge. An unused slide was used as a spreader and the bottom edge of this slide placed onto the drop of blood, so that the drop of blood was drawn across the bottom edge of the spreader. The spreader was held at approximately a 45^{o} angle to the sample slide, and a firm constant pressure applied as the blood was drawn across the sample slide by the spreader. Bloodsmears were air dried for 5mins prior to fixation.

2.2.8 Preparation of "wet fixed" slides for immunofluorescence and in situ hybridization

This procedure is adapted from Nuovo et al. (1994). Slides were prepared by first washing in 2N hydrochloric acid (soak 5mins), rinsing in distilled water for 1min and then in acetone for 1min. Slides were then washed in a suspension of 2% organosilane (v/v) (Aldrich) in acetone, for 1min, and air dried prior to an acetone only wash, for 1min. Finally, the slides were air dried again. Organosilane coated slides are stable at room temperature indefinitely.

Organosilane coated slides were marked using a liquid wax pen (DAKO, High Wycombe, UK) to prevent leakage of sample from the marked area. Samples containing purified trypanosomes, at a concentration of approximately 1×10^{9} trypanosomes per ml (Sections 2.2.3 and 2.2.4), were applied to the marked region of the slide and allowed to settle and attach in a humid chamber for at least 15mins. Excess liquid was removed (leaving a thin layer of moisture) into a Gilson pipette tip, which was jettisoned into a glass beaker containing bleach. Slides so prepared were immediately placed in methanol at -20^{o}C, without allowing time for them to begin to air dry.

2.2.9 Indirect immunofluorescence assay

The methods used were adapted from those described by Sherwin and Reed (1993). Samples were normally fixed in methanol at -20^{o}C for 1-48h. Alternative fixation methods were also used when appropriate, such as fixation with 4% (v/v) E.M. grade formaldehyde (TAAB, Aldermaston, UK) in PBS for 20mins, followed by permeablization for at least 1h in -20^{o}C methanol.

After fixation, samples were rehydrated for 5mins in PBS. Primary antibodies were applied to samples at an appropriate concentration and incubated for 1h, at room temperature, in a humid chamber. Where previously uncharacterized antibodies were used, it was necessary to perform titrations to determine an optimal concentration for the primary antibody. Where double staining was performed with antibodies of two immunoglobulin subtypes, or from two species, primary antibodies were mixed so that both were present at their optimal concentrations. The mixture of antibodies was then applied for the usual 1h incubation.

After incubation, unbound antibodies were rinsed off the slide by washing three times with PBS for 5mins per wash. The appropriate secondary (fluorochrome conjugated) antibodies were diluted according to the manufacturer's (DAKO, Sigma) instructions for immunofluorescence. In the case of double staining immunofluorescence, secondary antibodies were diluted to twice the concentration used in single staining, mixed in equal volumes and applied to the samples. Samples were again incubated for 1h in a humid chamber. Following this, slides were washed three more times in PBS, stained with 1μg/ml DAPI for 30secs, rinsed with distilled water, mounted with Vectashield (Vector Laboratories, Peterborough, UK) and sealed with nail varnish (Boots, UK).

Microscopy was performed using a Leica DMRXA fluorescence microscope, using a *x* 100 oil immersion planar fluorotar objective lens. Resultant images were either captured on Ilford XP-2 film, or using a cooled charge coupled device (CCD) (Photometrics series 200: Munich, Germany) with IPlab spectrum software. Images were processed using Adobe Photoshop 3.0.

2.2.10 NAD diaphorase assay

This cytochemical assay provides a suitable method for discriminating between bloodstream forms of trypanosomes. It was adapted by Vickerman (1965) to *T.brucei* and further modified during the course of these studies. Using this technique, trypanosomes were assessed for the NAD diaphorase (believed to reflect dihydrolipoamide dehydrogenase) activity in the mitochondrion. Air dried blood smears were initially fixed for 5mins, with 2.5% (v/v) E.M. grade glutaraldehyde (TAAB) buffered by PBS (pH 7.4). Slides were then rinsed quickly in distilled water and incubated in a humid chamber for 2h with freshly prepared reaction mixture. The reaction mixture contained 1.3mg/ml of the reduced form of β-nicotinomide adenosine dinucleotide (NADH) disodium salt and 0.3mg/ml nitrotetrazolium blue, dissolved in PBS (pH 7.4). Slides were stained with DAPI, mounted and sealed as for immunofluorescence (Section 2.2.9).

2.2.11 Preparation of protein samples

Purified washed trypanosomes (Sections 2.2.3 and 2.2.4) were resuspended at 2 *x* 10^8 trypanosomes per ml in boiling SDS-PAGE sample buffer and boiled for 5mins. These protein samples were chilled on ice and the protease inhibitor mixture was added. The DNA present in the sample was then sheared by passing the sample repeatedly through a 19 gauge needle. Protein concentration was assayed using the Bradford protein assay kit (Bradford, 1976) (Bio-Rad; Hemel Hempstead, Herts, UK). Once protein concentration had been assayed an equal volume of 0.004% (w/v) bromophenol blue was added to each sample. Samples were aliquotted, and stored at -70^{o}C.

2.2.12 SDS-PAGE (Laemmli, 1970)

Protein samples were separated by SDS-PAGE. Gels consisted of a lower, resolving gel and upper stacking gel. The lower gel mix was normally 7.5% to 15% acrylamide. It was prepared by the mixing of a 30% (v/v) stock of 19:1 acrylamide/bis-acrylamide with 4 *x* lower gel buffer and distilled water to give a 1 *x* solution of lower gel buffer containing the appropriate concentration of acrylamide. The lower gel was polymerized using 0.06% (w/v) ammonium persulphate and 0.1% (v/v) N,N,N',N' tetramethyethylene diamine (TEMED). Prior to polymerization, this lower gel mix was poured between cleaned glass plates. The plates were separated by spacers of 0.4mm thickness and were supported and sealed in a gel casting tray. The stacking gel mix was generally 5% acrylamide. It was made by mixing the acrylamide stock, 4 *x* upper gel buffer stock and distilled water, in the same way as the lower gel mix. The stacking gel was polymerized using 0.06%(w/v) ammonium persulphate and 0.1%(v/v) TEMED. Prior to polymerization, this upper gel mixture was poured onto the already polymerized main gel and a plastic comb for the sample wells inserted.

The apparatus used allowed ten lanes to be loaded. Samples were typically loaded onto gels as three replicates of three lanes each for slender, stumpy and procyclic protein samples and a lane containing protein size markers (Gibco). Loading of the samples was for equal protein concentrations. Gels were electrophoresed using SDS-PAGE gel electrophoresis buffer as an electrolyte, at a constant current of 30mA until the dye front reached the bottom of the gel. Gels were cut so that one set of sample lanes and half of the size markers were Coomassie stained. The other two sets of replicate lanes and half of the size marker lane were electroblotted onto nitrocellulose

2.2.13 Western blotting (Towbin et al., 1979)

Electroblotting of proteins from SDS-PAGE onto nitrocellulose was performed, using fresh blotting buffer in a wet blotting system (Gibco), according to the manufacturers instructions. The gel was overlaid by prewetted nitrocellulose cut to the size of the gel, avoiding air bubbles. Gel and nitrocellulose were then "sandwiched" between two leaves of prewetted, precut 3MM Whatman paper and in turn this was sandwiched between sponges and perspex supports. The final sandwich was oriented so that the nitrocellulose filter was on the anode side of the gel and was then immersed in the blotting tank. Transfer was typically performed overnight at 80mA, constant current.

Protein transfer was confirmed by immersing the blot in improved Ponceau's solution. The size markers were then cut from the blot for use in sizing bands. The remainder of the blot was then washed with distilled water, until the staining was no longer visible, prior to probing with the antibody under investigation.

Probing of the blots was performed using the enhanced chemiluminescence, (ECL) non-radioactive western blotting kit (Amersham; Amersham, Kent, UK) as described in the manufacturers instructions. The blot was first blocked, for 1h, with 1% (w/v) milk powder (Marvel, Stafford, UK) made up in TBS. The primary antibody, diluted in this blocking buffer, was then applied to the blot at the appropriate concentration (typically 1% of the optimal concentration for immunofluorescence). Following incubation, for 1h at room temperature, the blot was washed four times in TBS containing 0.1% (polyoxyethylenesorbitan monolaurate) Tween 20 (Sigma). The secondary antibody, which was conjugated to horse radish peroxidase, was diluted in TBS according to the manufacturer's instructions (Sigma). The diluted secondary antibody was then incubated with the blot, again for 1h at room temperature. The blot was then washed with TBS tween as before.

Equal volumes of solutions I and II, supplied with the ECL, kit were applied evenly across the surface of the blot which was then sealed in clear polythene. The sealed blot was overlaid with x-ray film (X-OMAT, Kodak, Hemel Hempstead, Herts.) in the dark and, after exposure, the film was developed. Exposure times varied from a few seconds to more than an hour depending on the intensity of the chemiluminescent signal.

2.2.14 Agarose gel electrophoresis of DNA

Agarose gels were prepared by suspending 0.8-2% (w/v) agarose in 1 *x* TAE. The agarose was dissolved by heating in a microwave oven. After the agarose had melted, ethidium bromide was added to a final concentration of 1µg/ml. The gels were cast in gel tanks and had a well forming comb placed in position. After the gel had set, the comb was removed and the tank filled with 1 *x* TAE.

DNA samples were added to 0.2 volumes loading buffer and loaded into the wells. The gels were run at 100V for analytical gels (250mm *x* 150mm) or 4V/cm for preparative gels (140mm *x* 110mm) under normal conditions. Analytical gels were occasionally run overnight at 20V.

2.2.15 Restriction enzyme digestion of DNA

All restriction enzymes were supplied by Boehringer-Mannheim and digestions were performed under the manufacturer's recommended conditions. An analytical digestion typically contained 0.5-2µg DNA and 2µl 10 *x* reaction buffer. Sterile distilled water was added to a final volume of 19µl followed by 1µl restriction enzyme (1-10U). The mixture was incubated at the recommended temperature for 1h. For larger scale digestions, such as those needed for preparative gels, the volumes were scaled up and the enzyme added in aliquots over several hours ensuring that the glycerol concentration remained less then 5% (v/v).

2.2.16 Polymerase chain reaction

Reactions were normally carried out in a final volume of 100µl under 40µl of sterile mineral oil. Reaction mixtures used standard buffer conditions according to the manufacturers instructions, 1mM dATP, dCTP, dGTP and dTTP, and 2.5U of Taq polymerase (Boehringer Mannheim) as previously described in Sanchez et al. (1994). Reactions were done in the Omnigene thermocycler (Hybaid, Teddington, Middlesex, UK) and although the cycle used varied with the experiment, typically denaturation was at 94°C, annealing was at 42°C and elongation was at 72°C.

Following the reaction, 8µl of the reaction mixture was normally mixed with 2µl of loading dye and loaded directly onto a gel. Care was taken to ensure that no oil was taken up with the reaction mix. Alternatively, where a purified product was required, QIAquick columns (Quiagen, UK) were employed according to the manufacturers instructions.

2.2.17 Differential display of amplified cDNA fragments

This protocol was modified from one used for differential display of mammalian cDNA by Sokolov and Prokop (1994), (Matthews and Gull, in preparation). The templates used were first strand cDNAs, prepared from Poly $(A)^+$ selected mRNAs of slender, stumpy and procyclic form populations, which were generously provided by Dr. K. Matthews.

A mixture of three primers (prepared by the oligosynthesis service, School of Biological Sciences, University of Manchester) was used. One primer sequence was part of the 5' spliced leader (5'SL) sequence of all nuclear encoded mRNAs. The other two were sequences selected from several possible primers in the paper of Sokolov and Prokop (1994) and which had been designed by them to favour the selection of open reading frames.

The polymerase chain reaction was conducted as described in Section 2.2.16, with optimal cycling conditions of 94^{o}C denaturation for 1min, 38^{o}C annealing for 2mins and

72^{o}C elongation for 3mins repeated for 30 cycles. Approximately 100ng of each template was used per reaction.

The PCR products were compared by loading each reaction onto adjacent lanes and using agarose gel electrophoresis (Section 2.2.14) to resolve the PCR products into bands. Where life-cycle specific bands were observed, these bands were excised from the gel and cloned.

Primer sequences:

(5'SL) 5'-CAGTTTCTGTAC-3'

BS52 5'-CAAGCGAGGT-3'

BS54 5'-AACGCGCAAC-3'

2.2.18 Construction of a T-vector

Blunt-ended cloning of PCR products into a linearized vector can be difficult, since the template independent terminal transferase activity of Taq polymerase often results in the addition of an overhanging adenosine at the 3'end of the PCR product. The method of Marchuk et al. (1991) was used to create linearized pBluescript II (pBSK^{+}, Stratagene) plasmid with a 3'thymidine overhang, which facilitates the cloning of PCR generated DNA fragments.

The pBSK^{+} plasmid was digested with EcoRV (as described in Section 2.2.15), to give a blunt ended linear molecule. It was then incubated with Taq polymerase, under standard buffer conditions at 70^{o}C in the presence of 2mM dTTP for 2h, resulting in the addition of one or more thymidine residues at the end of each fragment.

2.2.19 Preparation of competent *Escherichia coli*

XLA-1 Blue (Stratagene) from a commercially obtained glycerol stock was streaked out onto LB agar containing 50µg/ml tetracyclin. A 10ml culture of LB (also containing 50µg/ml tetracyclin) was inoculated with a single colony, picked with a sterile toothpick, and was incubated overnight at 37^{o}C in a shaking incubator.

500ml of LB were inoculated with 1ml of the overnight culture and incubated for 2-3h in a shaking incubator at 37^{o}C, until reaching a culture density of OD_{600} = 0.2. The cells were pelleted by centrifugation at 4^{o}C for 10mins at 4000 *x* g, then resuspended in 20ml of ice-cold sterile 50mM $CaCl_2$ and allowed to stand on ice for 30mins before repelleting. The pellet was finally resuspended in 4ml of ice cold 50mM $CaCl_2$ with 15% (v/v) glycerol, aliquotted into 250µl units and stored at -70^{o}C.

2.2.20 Purification and cloning of DNA fragments from agarose gels

Although a wide range of methods is available, the method of choice was normally to employ the US Bioclean MP kit according to the manufacturers instructions (USB, from Amersham, UK). The desired band of DNA was excised under longwave ultraviolet (UV) transillumination using a new scalpel blade. The band was then weighed in an eppendorf tube and dissolved in 2.5 volumes of 6M NaI at 55^{o}C for 10mins. 3-5 µl of “glass-milk” (glass milk is a suspension of powdered silica which adheres to DNA, particularly at “high salt” concentration) was added to the solution and allowed to adsorb the DNA for 5mins on ice.

A pellet was formed by centrifuging the glass milk/adsorbed DNA suspension in a microfuge for 10secs at 11600 *x* g. The pellet was washed by resuspension in ethanol diluted rinse buffer (100 ml of a solution supplied with the kit mixed with 400ml of ethanol), followed by centrifugation for 10secs at 11600 *x* g. This washing procedure was repeated three times. 50µl of dH_20 was then added to the washed pellet and incubated at 55^oC for 10mins. The eluted DNA was recovered as the supernatant following a brief, final centrifugation. This supernatant was transferred into a fresh tube, leaving all the glass-milk pellet behind.

2.2.21 Ligation and cloning of DNA fragments

DNA ligation reactions were normally conducted under standard buffer conditions, in a final volume of 10µl. Typically, the reaction mixture contained 1U of T4 DNA ligase, 20ng of vector and up to 500ng of insert DNA. The reaction mixture was then incubated overnight at 12-14^oC.

A 2µl aliquot of the ligated plasmid was then added to 100µl of defrosted, competent *E. coli.* The mixture was incubated on ice for 1h, before heat shocking the bacteria at 42^oC for 90secs. SOC medium was added to the bacteria, to a 1ml final volume and incubated at 37^oC for 1h. The mixture was then poured onto and spread over a petri-dish of LB agar containing 100µg/ml ampicillin, 1µM IPTG and 50 µg/ml X-GAL. Colonies were grown overnight at 37^oC. White recombinant colonies were picked against a background of blue colonies, containing plasmid which lacked an insert. The white colonies picked were screened for the correct insertion by agarose gel electrophoresis (Section 2.2.14) of restriction digestions (Section 2.2.15) of small scale plasmid preparations (Section 2.2.22).

2.2.22 Small-scale plasmid preparation (Birnboim and Doly, 1979).

E.coli strains carrying plasmid DNA were grown overnight in a shaking incubator, at 37°C, in 2ml LB medium supplemented with 50µg/ml ampicillin. A 1.5ml aliquot was removed from this overnight culture and the cells harvested by centrifuging at 11600 *x* g, in a microfuge, for 5mins. The medium was removed by aspiration and the pellet resuspended in 100µl ice-cold solution I by vortexing. An aliquot of 200µl of freshly prepared solution II was added, the tube inverted several times to mix the solutions and then incubated on ice for 5mins. Then, 150µl of solution III was added and the solutions mixed by inversion, before incubating on ice for 5mins. The tube was then centrifuged in a microfuge for 5mins and the supernatant transferred to a fresh tube before extraction with phenol/chloroform and recovery by ethanol precipitation. The final pellet was resuspended in 40µl of sterile distilled water (pH 7.4) containing 20µg/ml RNase A.

2.2.23 Large-scale plasmid preparation

Plasmid DNA was purified from appropriately transformed cultures of *E.coli*, using the same three solutions as for small scale preparations (Section 2.2.22). Cells were harvested from a 250ml overnight culture by centrifugation in a J2-21 (Beckman, High Wycombe, UK) centrifuge, using a JA10 rotor (Beckman), at 8000 *x* gfor 15mins. The pellet was resuspended in 100ml STE. The cells were then pelleted as before, resuspended in 4ml of solution I and transferred to a 40ml centrifuge tube (Oakridge type from Dupont, Hertfordshire, UK).

Cell lysis was achieved by addition of 8ml fresh solution II and incubation at room temperature for 10mins. Precipitation followed the mixing of 6ml of solution III with the lysed cells. This mixture was incubated on ice for 10mins and centrifuged at 27000 *x* g for 15mins. The supernatant was incubated with 0.6 volumes of isopropanol, in a clean Oakridge tube at room temperature for 10mins. The precipitate was harvested by centrifugation at 27000 *x* gfor 15mins and the resulting pellet washed in 70% ethanol (v/v) and dried under vacuum. The pellet was dissolved in exactly 4.1ml dH_20 and 4.40g CsCl was added and mixed. Finally, 150µl ethidium bromide (10mg/ml) was added to give a solution of density 1.6g/ml. The solution was loaded into small sealable tubes and precisely balanced (to 0.03g) and centrifuged overnight at 100,000 *x* gat 20^{o}C, in a VTi65 rotor (Beckman), under vacuum.

The plasmid band, visualised using longwave UV light if necessary, was removed by piercing the top of the centrifuge tube and drawing off the band in a 1ml volume into a syringe. This solution was then extracted four times with equal volumes of water saturated butanol. The solution was then made up to 4ml with distilled water. 12ml of ethanol were then added and the mixture was incubated on ice to precipitate the DNA. After centrifugation, the pellet was washed in 70% ethanol, dried and then resuspended in 500µl dH_20.

2.2.24 Preparation of total RNA from trypanosomes

This protocol was adapted from the published methods of Chomzynski and Sacchi (1986). In microcentrifuge tubes, pellets containing 1 *x* 10^8 purified trypanosomes (Sections 2.2.3 and 2.2.4) were dissolved in 0.5ml of denaturing solution. 50µl of 2M sodium acetate, 500µl of phenol (water saturated, pH 5.0) and 100µl of chloroform isoamyl alcohol (49:1) were sequentially added to the samples, and the mixture vortexed after each addition. The samples were allowed to settle on ice for 15mins and were then microcentrifuged at 11600 *x* g, for 20mins, at 4^{o}C. The RNA-containing aqueous phase was then transferred into a new microcentrifuge tube, mixed with an equal volume of

isopropanol and precipitated at -20^{o}C for 1h. Following centrifugation as before, the supernatant was removed and the pellet redissolved in 300µl of denaturing solution. This mixture was heated to 65^{o}C for 5mins and cooled on ice. RNA was then precipitated with 750µl of absolute ethanol and incubated on ice for 15mins. Following a final centrifugation, the supernatant was removed and the pellet air dried in a sterile cabinet. This total RNA was then resuspended in diethypyrocarbonate (DEPC) treated water and either used immediately or stored at -70^{o}C.

2.2.25 Generation of riboprobes

Riboprobes were generated for use in both northern blotting and in situ hybridization. Cloned fragments, inserted into pBluescript II, were amplified by PCR with M13 forward and reverse sequencing primers. PCR was conducted essentially as described in Section 2.2.16. Typically, however, denaturation was at 94^{o}C for 1min, annealing was at 42^{o}C for 2min and extending was at 72^{o}C for between 2 and five minutes. However the exact program depended on the length of the insertion amplified. Amplification was for between 25 and 35 cycles. The purified products were used for the construction of sense and antisense riboprobes according to the instructions of the Boehringer Mannheim in situ hybridization manual.

Approximately 1µg of PCR generated template was mixed with 2µl NTP labelling mixture (kit component containing nucleotides and digoxygenin (DIG) labelled dUTP), 2µl transcription buffer (kit component giving appropriate buffer conditions and containing RNase inhibitor), 4U of the RNA polymerase required (T3 or T7) and DEPC-treated water to a final volume of 20µl. This reaction was then incubated for 2h at 37^{o}C and then stopped by adding 2µl of 200mM EDTA (pH 8.0). The probe was then precipitated with 2µl of 4M LiCl and 3 volumes of ethanol at -70^{o}C for 30mins, before centrifuging in a microfuge at 11600 *x* gfor 10mins, discarding the supernatant, rinsing the pellet in 70% ethanol, air drying the pellet in a sterile cabinet and resuspending it in 100µl of DEPC-treated water.

An estimate of the yield was made by agarose gel electrophoresis (Section 2.2.14) of one tenth of the final reaction. Before use, the probe was heated to 75^{o}C for 5mins.

2.2.26 Northern blotting

Samples containing total RNA were separated by electrophoresis through an agarose gel containing 2.2M formaldehyde and using MOPS buffer as the electrolyte. 20µl samples contained 2µl 5 *x* MOPS buffer, 3.5µl formaldehyde, 10µl deionized formamide and 2µl RNA loading buffer. Samples were heated to 65^{o}C for 10mins and then loaded onto the formaldehyde gel. Gels were electrophoresed at up to 150 Volts, until the bromophenol blue dye front reached the end of the gel. Gels were stained with 100µg/ml ethidium bromide for 10mins, then washed with distilled water for approximately 45mins. After photographing, the gel was washed for another hour to remove further ethidium bromide and equilibrated in 10 *x* SSC for half an hour.

The gel was placed on prewetted 3MM Whatman paper supported on a bridge above a reservoir of 10 *x* SSC, with the Whatman paper trailing into the SSC and acting as a wick. Nylon membrane (Hybond N, Amersham) cut to the shape of the gel was placed on top of the gel. On top of this membrane were then placed layers, first of 3MM Whatman paper (also cut to the approximate shape of the gel), and then layers of tissue paper. Finally, an evenly distributed weight of about half a kilogram was placed on top and the transfer left for up to 16h. Once the blot was transferred, it was cut and marked with a soft pencil for orientation. RNA was then crosslinked to the dry membrane using a UV crosslinker (Stratagene). Care was taken in handling the membrane, to minimize the risk posed by skin borne RNases.

Hybridization to the riboprobes was performed in a hybridization oven (Hybaid, Teddington, UK). Filters were first prehybridized for 1h in prehybridization solution at 68^{o}C and then hybridized overnight, at the same temperature, with approximately 100ng/ml of riboprobe in fresh prehybridization solution. After hybridization, membranes were washed four times at 68^{o}C. The first two washes were for 5mins with wash solution 1 (2 *x*

SSC, 0.1% SDS) and the second two washes were for 15mins each with wash solution 2 (0.1 *x* SSC, 0.1% SDS).

The membranes were equilibrated for 30secs in Buffer I. They were then blocked for 1h with Buffer 2. Anti-DIG antibody diluted in Buffer II (1:10,000, as recommended by the manufacturer) was added to the blot and incubated for 1h at room temperature. Excess antibody was then removed by washing three times in buffer I, containing 0.3% Tween 20. The blot was then equilibrated in Buffer III for 2mins, before applying CSPD (fluorescent substrate from the kit diluted 1 in 100 in Buffer III) evenly across the surface of the blot. The blot was then sealed in clear polythene and overlaid with x-ray film as for a western blot (Section 2.2.13).

2.2.27 In situ hybridization

This protocol is essentially the same as that published by Ersfeld et al. (1996). Due to the lack of constitutively expressed mRNAs that could be used as controls for northern blotting, putative differentially expressed mRNAs were assessed for differential expression at the level of the single cell, by in situ hybridization.

Samples settled on microscope slides (Section 2.2.8) were fixed in 4% (v/v) E.M. grade formaldehyde (TAAB) in PBS for 10mins at room temperature. Slides were washed in PBS three times, for 10mins per wash and then acetylated with 0.25% (v/v) acetic anhydride in 100mM ethanolamine (pH 8.0) for 10mins at room temperature. Slides were then washed for 5mins in 2 *x* SSC, permeablized in 200mM hydrochloric acid and washed twice in PBS for 5mins per wash. The samples were then prehybridized under a layer of hybridization solution without riboprobe, in a humid chamber, for approximately 90mins, at 37^{o}C.

During prehybridization riboprobes (as generated in Section 2.2.25) were prepared for in situ hybridization. For probes longer than 600bp, alkaline hydrolysis in sodium carbonate buffer (pH 10.4) was first employed. The length of incubation at room temperature was calculated in seconds by the formula:

(Original riboprobe length (Kb) - 0.25)/(Original riboprobe length (Kb) x 0.025)

2-5ng of each riboprobe was coprecipitated with 10µg of yeast tRNA and 10µg of herring sperm DNA using three volumes of ethanol. The precipitate was resuspended in 10µl of hybridization solution in a microcentrifuge tube. This riboprobe solution was then heated to 85^{o}C, cooled on ice and centrifuged at maximum speed in a microcentrifuge for 5mins. The supernatant was then applied to the sample and a coverslip was sealed on top. Hybridization was for 6-12h at 60^{o}C.

The coverslip was then removed and the sample washed for 10mins per wash. The first wash was at 60^{o}C using 2 *x* SSC in 50% formamide (v/v). This was followed by 2 *x* SSC in the absence of formamide, then 0.2 *x* SSC and finally 4 *x* SSC for 10mins at room temperature. The samples were then blocked for 30mins with 1% blocking reagent (w/v) made up in 100mM maleic acid, 150mM NaCl (pH 7.4).

Primary antibody (sheep anti-digoxygenin Fab fragments, Boehringer Mannheim) was then applied to the sample and a normal immunofluorescence protocol applied (Section 2.2.9).

2.2.28 The dideoxy chain termination method for DNA Sequencing

DNA sequencing was performed using the USB Sequenase Kit, essentially as described in the manufacturers instructions. The kit contained the modified DNA polymerase “Sequenase”, annealing buffer, labelling mix, termination mixes and stop solution. The glass plates were cleaned with absolute ethanol and allowed to dry. The smaller plate was then treated with silane solution:- 2% (v/v) dimethyl-dichlorosilane in 1,1,1, trichloroethane was wiped onto the plate and allowed to dry for 10mins. This was repeated and the plate was washed with distilled water and allowed to dry. The larger plate was then washed with absolute ethanol and allowed to dry. Gel spacers were attached to the long edges of the larger plate and the smaller plate taped on top using electrical tape.

The gel mix was prepared by mixing 33.6g urea, 8ml 50% stock acrylamide gel and 16ml 5 *x* TBE. This was made up to 80ml with distilled water and the urea dissolved by stirring for 30mins. When the urea had dissolved, 400µl of freshly prepared 10% (w/v) ammonium persulphate and 40µl TEMED were added to the gel mix, the gel poured and a shark’s tooth comb inserted into the top of the gel, flat edge down. When the gel had set it was placed in the running tank and 500ml 1 *x* TBE added to the top and bottom reservoirs. The well forming comb was removed and the wells washed out using a syringe containing 1 *x* TBE. The gel was prewarmed to between 40^{o}C and 45^{o}C, by running at 45W constant power.

Double stranded template DNA (2.5µg in a total volume of 16µl) was incubated with 4µl 1M NaOH at room temperature for 5mins, to denature the DNA. Ammonium acetate (2µl of 5M adjusted to pH 4.6 with acetic acid) was mixed in to the solution and then 50µl of absolute ethanol added. The solution was mixed and stored on ice for 15mins. Denatured plasmid DNA was recovered by centrifugation in a microfuge for 15mins and the ethanol removed. The DNA pellet was washed in 70% ethanol and air dried.

The sequencing primer oligonucleotide (1pmol/µl) was annealed to the template DNA by the addition of 2µl of 5 *x* Sequenase reaction buffer, 1µl primer oligonucleotide and 7µl distilled water. The contents were mixed and heated for 2mins in a boiling water

bath. The water bath was allowed to cool to less than 35°C, before placing the tube on ice. The labelling reaction was prepared by combining 1µl 0.1M dithiothreitol (DTT), 2µl 1 *x* labelling mix and 0.5µl (5µCi) radio-labelled dATP (α-^{35}S with specific activity 1300Ci/mmol: supplied by Amersham) with the template-primer mixture prepared earlier. The reaction was begun by the addition of 2µl Sequenase version 2.0 diluted 8 *x* with ice-cold enzyme dilution buffer. The tube was incubated at room temperature for 2-5mins.

Four tubes were labelled A, C, G, T and to each was added 2.5µl of the ddATP, ddCTP, ddGTP and ddTTP termination mixes respectively. These tubes were prewarmed at 37^{o}C for at least 5mins. 3.5µl of the completed labelling reaction mixture was added to each tube, the solutions were then mixed and incubated at 37°C for 5mins. After this time, 4µl of stop solution was added to each tube and mixed thoroughly. The samples were stored on ice until ready to load onto the sequencing gel or stored at -20^{o}C for up to 3 days before use.

The samples were boiled for 2mins and cooled rapidly on ice. 2µl of sample were loaded into each well of the gel, the remaining sample being stored on ice. The gel was run at 45W constant power until the bromophenol blue had reached the end of the gel when, if a second loading was required, the wells were washed out and the samples boiled as before. The second loading was run until the bromophenol blue had run to the end of the gel.

When the gel had run, the apparatus was dismantled, the glass plates carefully separated and the gel fixed in 10% (v/v) glacial acetic acid, 10% methanol for 20mins. The gel was supported on a sheet of 3MM Whatman paper and dried on a gel drier at 60°C until dry (approximately 45mins) before overlaying with X-ray film and placing in the dark, overnight, at room temperature to expose the film.

2.2.29 Preparation of trypanosome cytoskeletons and transmission electron microscopy

The electron micrographs shown in Chapter 3 were prepared by Dr T. Sherwin (University of Manchester) and were prepared using published methods (Sherwin and Gull, 1989).

Briefly, purified trypanosomes (prepared as described in Section 2.2.3) were washed in PBS and settled onto freshly charged, carbon and formvar coated grids. These were transferred into 1% (v/v) Nonidet P-40 in PEME for 1-5 minutes and then transferred to 2.5% (v/v) E.M. grade glutaraldehyde in PEME for 20 seconds. Grids were then negatively stained in 3.5-7.0g/litre Goldthioglucose in double-distilled water.

Chapter 3

Growth, division and development of bloodstream form trypanosomes

3.1 Introduction

3.1.1 Glossary

Akinetoplastid

Refers to trypanosome lines which are apparently devoid of all kinetoplast DNA.

Anterior

Defined by the direction in which trypanosomes swim. The anterior end is marked by the narrow end of the cell and the free flagellum.

Cytokinesis

The point (in the cell cycle) at which the “mother” cell is cleaved into two daughter cells.

(Cell) division

The process by which two cells are produced from one cell. Used here particularly to refer to the portion of the cell cycle between the maturation of the probasal body and completion of cytokinesis.

Dividing-forms

Used here to refer to cell types which have begun to replicate single copy structures and hence are assumed to be committed to the production of two daughter cells.

Dyskinetoplastid

Refers to trypanosomes which apparently lack a kinetoplast when assessed microscopically.

Diaphorase positive

Used here to refer to bloodstream form trypanosomes in which, as a result of the diaphorase assay, a deposit of formazan is visible microscopically along the line of the mitochondrion.

Growth

Net increase in population resulting from proliferation, death and stasis.

Growth rate

The differential increase of population number with time.

Growth curve

A plotted description of the differential change in a population's numbers with time.

Infection

The presence of viable pathogen in a host.

Inoculation

Transfer of (typically pathogenic) organisms to fresh growth conditions, for either in vivo or in vitro culture.

Mean doubling time

The mean time taken for a growing population to double in number under defined conditions.

Monomorphic

The inability of a trypanosome line or strain to produce stumpy forms in a specified host.

Near-cytokinesis forms

Dividing-forms which have replicated and segregated their major organelles, have aligned for cleavage into daughter cells and have established a cleavage plane.

Non-Proliferative

Refers to cells which are not progressing through the cell cycle.

Parasitaemia

The presence of parasites detected in a host during the course of an infection, often given quantitatively. In the case of *T.brucei* , expressed as trypanosomes per ml of blood.

Parasite clearance

Removal of parasites from an infection by lysis and phagocytosis.

Posterior

Defined relative to the anterior end. The posterior end of the trypanosome is the blunt end which encloses the kinetoplast/basal body complex.

Post-mitotic forms

Dividing-forms which have completed nuclear replication and segregation.

Pleomorphic

The ability of a trypanosome line or strain to produce stumpy, intermediate and slender forms (in vivo).

Primary parasitaemia

The period from which parasites first become detectable until the infection becomes subpatent.

Proliferation

Increase in numbers resulting from cell division.

Proliferative

Describes cells which are progressing normally through the cell cycle.

Replication

The process by which a copy of a molecule, structure or organelle is made.

Reproduction

The process by which a new individual is formed.

3.1.2 Maintenance of trypanosome parasitaemias

The African trypanosome is able to maintain a chronic parasitaemia in the bloodstream of a wide range of mammalian hosts. The mechanisms by which this is achieved are necessarily complex (Barry and Turner, 1991). Complexity stems from the balance of trypanosome proliferation which must be great enough to achieve transmission and to avoid clearance, yet, not so great as to cause the death of the host. This balance must also be able to compensate for parasite clearance by the immune system and for the emergence of new VSG types by switching. The parasitaemia is a function of the rate of parasite proliferation and the rate of parasite clearance. The rate of proliferation is affected not only by the duration of the cell cycle but also by the rate of differentiation to the non-proliferative stumpy form.

Even in the absence of an immune response, differentiation to the stumpy form will limit trypanosome growth to a sublethal level in mice for up to 72h (Balber, 1972). In comparison monomorphic lines, which do not differentiate to the stumpy form in mice, invariably reach a lethal parasitaemia even in immunocompetent murine hosts. This is regardless of their mean doubling time which may be longer than that of pleomorphic strains (Herbert and Parratt, 1979; Turner et al., 1995).

In immunocompromised hosts (in which lymphocytes have been severely depleted) the level at which the primary parasitaemic peak is reached is host specific, showing strain variation even within species (Seed and Sechelski, 1988). This observation implies that host factors, that are not solely lymphocyte derived (such as antibody), interact with trypanosomes affecting differentiation to the stumpy form. The potential importance of the host in mediating the slender-to-stumpy differentiation is reinforced by the observation that strains which are monomorphic in rodents are pleomorphic when introduced into other mammals (Black et al., 1983).

Slender forms are more susceptible to complement mediated lysis than stumpy forms (McClintock et al., 1993) and so are cleared preferentially by the immune response. This may be due to the increased level of endocytosis at the flagellar pocket of the stumpy form, which removes attached antibody from the stumpy form surface and hence increases the serum concentration of antibody necessary for lysis to be achieved (Langreth and Balber, 1975; Russo et al., 1993). Stumpy forms, however, have also been associated with an increase in the immune response to trypanosomes (Sendashonga and Black, 1982).

Parasite clearance in an immunocompetent host almost exclusively reflects the onset of a humoral immune response. The effectiveness of this response to any one VAT is apparently host specific (reviewed in Barry and Turner, 1991). It may have a strong genetic component and seems to vary markedly even between related mouse strains (Seed and Sechelski, 1995). Animals mounting an effective and rapid immune response limit the parasitaemia markedly; growth curves obtained from such animals are often of the classically undulating sort, normally prolonged and sometimes self-curing. Other animals, however, apparently mount a slower response which is often less effective at ameliorating the parasitaemia. The parasitaemia of these animals is limited by the differentiation to the stumpy form during the delay, until the immune response is able to act (reviewed in Black et al., 1985).

In establishing a system for the growth and differentiation of pleomorphic bloodstream form trypanosomes, a system with a high degree of reproducibility was sought. It was required that this system be permissive of a high but sublethal parasitaemia and give a nearly complete differentiation from the slender to the stumpy form, so that relatively homogenous populations of each life cycle stage could be obtained.

3.1.3 Trypanosome morphology during bloodstream form differentiation and cell division

The bloodstream form trypanosomes present during an infection of *T.brucei* are described as pleomorphic, the observed variation in morphology arising from the asynchrony of the population with respect to both cell cycle position and progress through the slender-to-stumpy differentiation. The morphological changes, resulting from both differentiation and from progress through the cell-cycle, are likely to be mediated at the level of the trypanosome cytoskeleton. The cytoskeleton of the African trypanosome is composed of a subpellicular array of parallel microtubules which display uniform polarity, with the plus end of the microtubules located towards the posterior end of the cell. Subpellicular microtubules are helically arranged along the entire length of the cell and are tightly adposed to the overlying cell membrane. The presumed structural simplicity of a cell type in which microfilaments and intermediate filaments have never been convincingly documented, making the trypanosome an attractive system in which to look at cellular morphogenesis (reviewed Seebeck et al., 1988).

The analysis of morphological change during trypanosome division and differentiation has been aided by well defined cytological markers. These markers describe division in a proliferative life cycle stage - the procyclic form (Sherwin and Gull, 1989a; Woodward and Gull, 1990). Furthermore, recent work has looked at cytological change during the stumpy-to-procyclic differentiation (Matthews et al., 1995). Understanding of the morphological changes associated with the slender-to-stumpy form differentiation and with slender form cell cycle has, however, progressed little since they were defined at the level of the light microscope by the meticulous drawings of Lady Mary Bruce (Bruce et al., 1912, 1914a) and Muriel Robertson (1912ab) and by the detailed measurements made in

early biometric studies (Bruce, 1910, 1912, 1913ab, 1914a; Robertson, 1912b; Fairburn and Culwick 1946, 1949; Hoare 1956).

The forms observed by Bruce and Robertson were later reinterpreted by Hoare (1972) in the light of recent, detailed analysis of the life cycle (reviewed Vickerman, 1985). Vickerman also developed NAD diaphorase as a cytochemical marker of mitochondrial activation during the slender-to-stumpy differentiation (Steinert, 1964; Vickerman, 1965). It is clear, however, that as recently as 1979 considerable confusion in the field still existed as to the possible significance of many unusual cell types, present in the bloodstream, during an infection (reviewed in Omerod, 1979). In initiating this project a strong observational base was sought to facilitate studies aimed at bridging the gap from microscopic and ultrastructural observation of morphogenesis, to the physical and biochemical mechanisms responsible.

3.1.4 In vitro culture and differentiation of bloodstream forms

In 1896 Bruce sent a dog infected with *T.brucei* from East Africa to Britain, in order that research on African trypanosomiasis might be continued outside Africa. Thereafter, it was necessary to maintain bloodstream forms by syringe passage in laboratory animals. It was soon apparent that there would be problems with maintaining stocks in this way since the trypanosomes increased in virulence during multiple syringe passages, losing the ability to differentiate to the stumpy form and becoming monomorphic (Bruce, 1914bc). Monomorphic lines clearly behave differently to wild-type strains. In addition to the loss of intermediate and stumpy forms, they are cyclically transmitted through tsetse-flies very poorly, if at all (Wijers and Willet, 1960; Ashcroft, 1960). In vitro, monomorphic bloodstream form populations differentiate to procyclic form populations with dramatically reduced efficiency and delayed kinetics (Ziegelbauer et al., 1990; Matthews and Gull 1994a). It is possible that many of

the commonly used monomorphic strains have multiple genetic lesions relating to cell cycle and parasite density perception and may contain other lesions that are less apparent. It is known that the rate of VAT switching is considerably lower in some monomorphic strains than in wild type trypanosomes (Turner, 1997).

Monomorphic strains are, nevertheless, in widespread use in trypanosome basic research since they grow rapidly to high parasitaemia in a highly reproducible fashion. Such growth characteristics lend them to in vitro culture. In contrast, pleomorphic bloodstream forms had, until recently, proved refractory to culture. Culture of the bloodstream form in vitro, in a defined axenic culture, is highly desirable for the accessibility and definition it lends to experiments: in particular, those experiments aimed at manipulating the parasite. Studies with drugs, reverse genetic approaches and mutational screens are enormously facilitated by an in vitro system. It is also no longer considered ethically acceptable to continue to use large numbers of laboratory animals solely for the purpose of raising parasites if a good alternative can be found.

Prolonged culture of bloodstream forms was first reported in the presence of mammalian feeder cells (Hirumi et al., 1977). It has also been possible to develop cell free systems (Baltz et al., 1985; Hirumi and Hirumi, 1989; reviewed in Hirumi and Hirumi, 1994), although only monomorphic strains adapted readily to these methods initially. The construction of transgenic, bloodstream form trypanosome lines was greatly facilitated by plate based cloning procedures (Carruthers and Cross, 1992) and by adaptation of systems for genetic transformation of trypanosomes (Ten Asbroek et al., 1990; Lee and Van der Ploeg., 1990) to the bloodstream form (Carruthers et al., 1993). It was clear at the outset of this project that the first step in enabling the use of these new transgenic technologies, in the study of the slender-to-stumpy differentiation, must be the establishment of an in vitro culture system for pleomorphic lines of trypanosomes. Although a system for the culture and differentiation of pleomorphic *T. brucei*

brucei on solid media has recently been developed (Vassella and Boshart, 1996), this system was not available at the outset of the project and was developed contemporaneously.

It has been reported that at high culture densities monomorphic trypanosomes undergo morphological changes and mitochondrial activation at least superficially akin to the slender-to-stumpy differentiation (Hamm et al., 1990). However, this could also be interpreted as a response to nutrient deprivation. In the light of this report it was necessary to apply a rigorous standard to the definition of the stumpy form.

Various criteria exist with which to define a stumpy form. Traditional morphological criteria, which are used to discriminate between slender and stumpy forms in vivo, could be applied to any in vitro changes. However, morphology does not provide a direct link to cell viability. For instance, it may be that as slender forms are killed they can adopt a stumpy morphology but are no longer viable. Alternatively, it may be that cultured bloodstream forms, which do not undergo the usual morphological changes of the slender-to-stumpy differentiation, do remain viable, do arrest in G_1, do activate their mitochondria, are able to transform rapidly to procyclic forms and are even able to initiate tsetse infections efficiently.

Stumpy forms could be defined by mitochondrial activation. This could be assessed by the cytochemical assay for diaphorase (Vickerman, 1965) and by increased mitochondrial size and complexity at the level of the electron microscope (Brown et al., 1972). However, in organisms such as yeast stress, especially by starvation, is known to lead to mitochondrial activation. Consequently, assays for mitochondrial activation do not necessarily point towards the formation of a stumpy form, but could indicate that cells are dying.

It is possible to assay for the increased residence in G_1 of the cell cycle of stumpy populations. This has been done by FACS or microscopically at the level

of the single cell. Like mitochondrial activation, however, this arrest in G_1 may also result from stress and starvation. Residence in G_0 could be assessed by loss of infectivity or ability to establish cultures. However, such assays are difficult to interpret unambiguously especially when dying forms may also be unable to establish infections or cultures. Ideally, preadaptation (transmission potential) would be assessed by ability to establish tsetse infections, however, such experiments are likely to prove difficult to conduct and to interpret and were not feasible at the University of Manchester.

Consideration of these difficulties led to the adoption of the following definition. Stumpy forms should show evidence of mitochondrial activation, morphological change and of arrest in G_1 of the cell cycle. In addition, stumpy forms should (as a population) be able to initiate rapid and synchronous re-entry into the cell cycle accompanied by the acquisition of procyclic form markers in response to reduced temperatures (27^oC) and the presence of cis-aconitate (Czichos et al., 1986; Ziegelbauer et al., 1990; Matthews and Gull, 1994a). This definition was chosen since it was clear that cells which failed to differentiate at all did not represent the viable stumpy life cycle stage, perhaps being dead or dying. Moreover, if the rapid synchrony criterion was not met, apparently stumpy forms could in fact be forms following a nutrient deprivation pathway to mitochondrial development and arrested cell division.

3.2 Results

3.2.1 Trypanosome growth in rodents

Figure 3.1

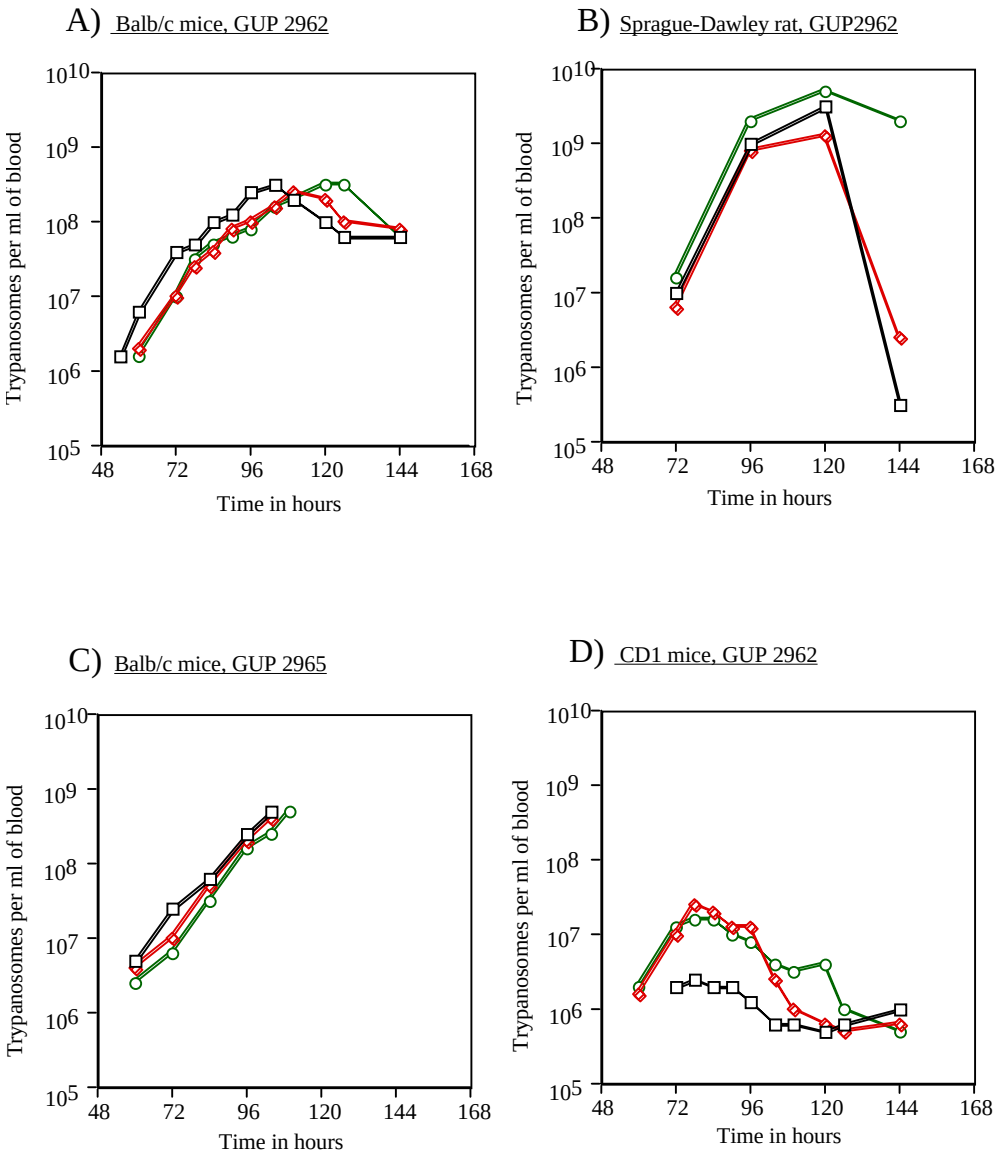


Fig. 3.1 Growth of pleomorphic (GUP 2962) and monomorphic (GUP 2965) lines of *T.b.rhodesiense* in immunocompetent inbred lines of mice and rats. The data is presented in triplicate sets. The inoculations of Balb/c mice and CD1 mice with GUP 2962 were done in parallel as a single experiment. Inoculation of Balb/c mice with GUP 2965 was performed as a single but separate experiment. Infection of Sprague-Dawley rats are the results of three separate experiments.

In developing a model system for the growth and differentiation of bloodstream forms, two widely available inbred strains of mice were initially tested; CD1 and Balb/c. Immunocompetent adult female ex-breeders were selected because of their large size and comparative docility. Representative growth curves are shown in Figures 3.1A and 3.1D (and see also Fig. 3.2A for a second triplicate set of Balb/c infections). The prepatent period of infection with the pleomorphic line GUP 2962 varied by up to 12h in mice regardless of whether mice were Balb/c or CD1. In comparing the growth curves of GUP 2962, in CD1 and Balb/c mice, prepatent periods and initial growth appeared similar. However, it was clear that by 80h the immune response was active in clearing the slender forms from the CD1 mouse, with the parasitaemia dipping long before the peak reached by the Balb/c mice.

In order to raise larger numbers of parasites it was sometimes necessary to use rats. Immunocompetent Sprague-Dawley rats have been used previously as a model host for raising *T.b.rhodesiense* (Matthews and Gull, 1994a). Adult female ex-breeders were chosen for their large size and docility. These rats were tolerant of a very high primary parasitaemia, but dramatically ablated infections with GUP 2962 once the population had become predominantly stumpy forms. Immune clearance by Sprague-Dawley rats normally occurred approximately 120h after infection (Fig. 3.1B). Variation in growth curves obtained during Sprague-Dawley infections were, however, greater than with mice: immune clearance occasionally occurring before differentiation was complete.

The monomorphic line GUP 2965 was also tested in mice (Fig. 3.1C) and rats. Extremely high parasitaemias were reached and in neither host was there an obvious reduction in growth rate or evidence of morphological differentiation to stumpy forms.

From the growth curves it was clear that CD1 would not be a suitable choice for host in a model system. It was therefore eliminated from further consideration. The differentiation of trypanosomes in Balb/c mice, which gave the most consistent growth curves, was then assessed using the diaphorase assay (Fig. 3.2, 3.3 and further characterized in Chapter 4). The diaphorase assay (Steinert, 1964; Vickerman, 1965) is a cytochemical assay of differentiation which exploits the activation of mitochondrial NADH dehydrogenase activity during the slender-to-stumpy differentiation. The NADH dehydrogenase activity reduces nitrotetrazolium blue, producing formazan deposits which delineate the mitochondrion of intermediate and stumpy forms. In Figure 3.2, forms which show this characteristic staining have been characterized as being “diaphorase positive.”

At each time point shown in Figure 3.2, tail bleeds were taken from each of the three mice. This allowed the level of parasitaemia to be established using the rapid scoring technique and bloodsmears to be prepared. Diaphorase assay was conducted on the bloodsmears and assessed using phase-contrast microscopy. 1000 cells were assessed, for each mouse at each time point, even though at low parasite densities this typically involved the examination of more than one slide.

Initially, at low parasitaemia, less than 10% of trypanosomes showed staining by diaphorase assay. It is worth noting that at very low parasitaemia there were at least a few diaphorase positive forms with clearly stumpy morphology. During logarithmic growth the proportion of cells showing staining with diaphorase increased steadily. By the time the peak of the parasitaemia was reached, over 75% of forms showed evidence of formazan deposits which delineated the mitochondrion. After the peak of the parasitaemia in the Balb/c mice, approximately five days post-infection, more than 95% of forms showed extensive mitochondrial staining using the diaphorase assay (Fig. 3.2D).

Figure 3.2

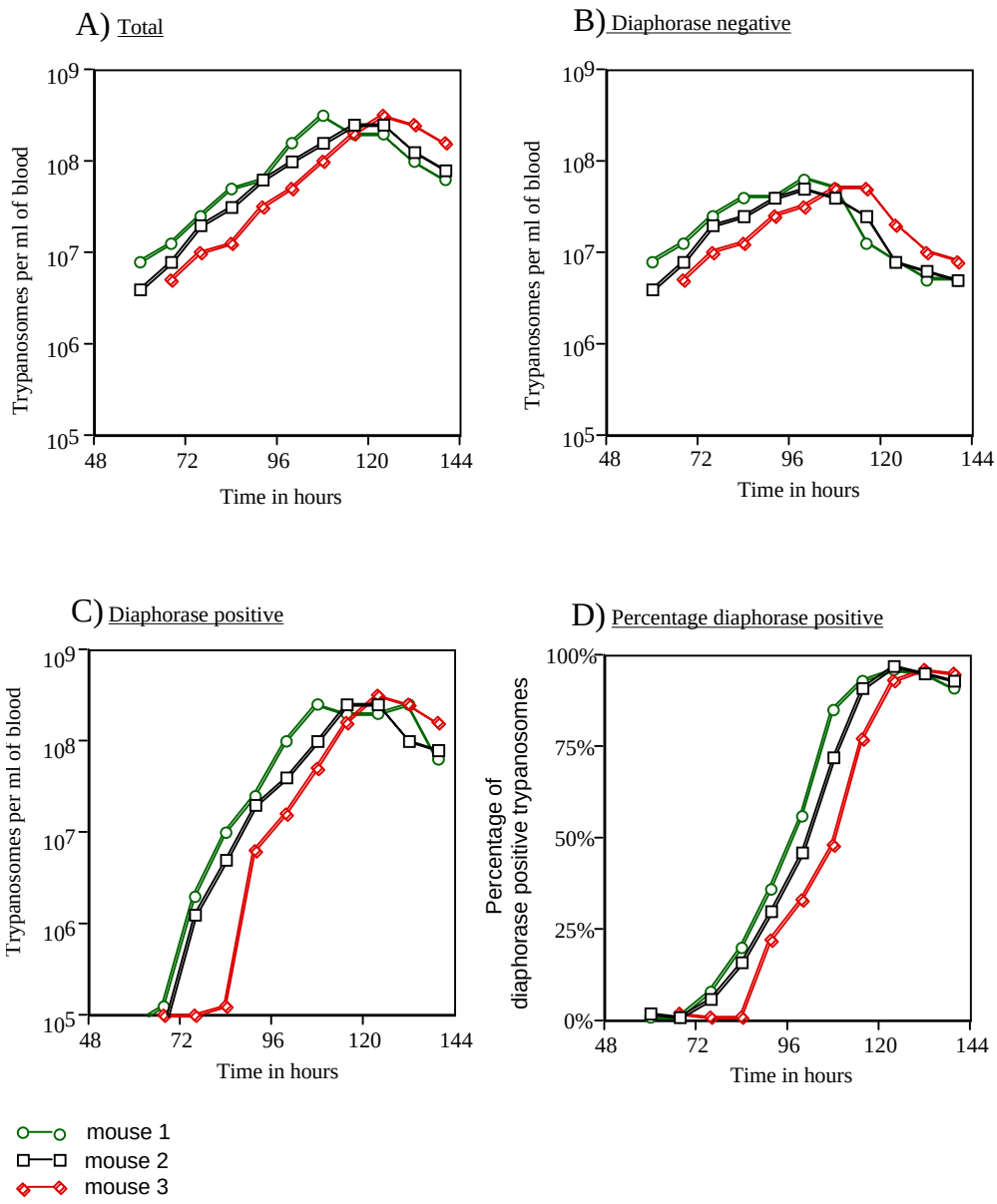


Fig. 3.2 Acquisition of diaphorase activity as a marker of the slender-to-stumpy differentiation during the primary parasitaemia of GUP 2962 in a Balb/c mouse. This single experiment was performed in triplicate. At each 8 hour time point, bloodsmears were prepared which then were assayed cytochemically for diaphorase activity. Where a characteristic deposit of formazan along the mitochondrion was observed, cells were scored as “diaphorase positive”. 1000 cells were assayed at each time point for each mouse.

3.2.2 Bloodstream form trypanosome cytology

Using the murine system established above, the greatest degree of heterogeneity, in the cell types composing bloodstream trypanosome populations, was seen approximately 96h after infection (Fig. 3.2). At this time point much of the trypanosome population was clearly in flux, differentiating from slender to stumpy forms. 10,000 cells were characterized 96h post-inoculation from both mouse and rat infections. The mouse parasitaemia was approximately $2x10^8$ trypanosomes per ml of blood, the rat parasitaemia was approximately $3x10^9$ trypanosomes per ml of blood. The diaphorase assay was performed on bloodstream smears which were then examined by phase-contrast microscopy. Examples of the commonly observed cell types are shown in Figure 3.3A and the incidence of each cell type within these populations is listed in Table 3.1. From Table 3.1 it can be inferred that the rat population is more differentiated. The cell types observed in both mouse and rat infections were the same.

The upper panel (Fig.3.3A) shows cells which do not stain positively by diaphorase assay. These include forms with a slender morphology and either a single nucleus and a single kinetoplast (1k1n, Fig. 3.3A1), two kinetoplasts and one nucleus (2k1n, Fig. 3.3A2), or two kinetoplasts and two nuclei (2k2n, Fig. 3.3A3). These three cell types seem likely to encompass the forms which make up the normal cell cycle of bloodstream trypanosomes. Table 3.1 shows these to be present in significant numbers in both mouse and rat populations. The relatively low numbers of 2k1n and 2k2n diaphorase negative forms (dividing-forms) may reflect the large proportion of differentiating and differentiated cells within this population. Figure 3.3A3i represents a subset of 2k2n diaphorase negative forms (near-cytokinesis forms) in which a cleavage plane is generally apparent and in which the kinetoplasts and nuclei are aligned for cleavage.

The other cell type which is relatively common in these populations possesses one nucleus and one kinetoplast (1k1n) and is diaphorase positive (Fig.

3.3A9). This cell type appears to encompass the vast majority of stumpy and intermediate forms. Cells with a stumpy morphology generally show a stronger degree of staining than cells of intermediate morphology. While cells that stain positive for diaphorase and possess two kinetoplasts and one or two nuclei (Figs 3.3A10 and 3.3A11) do exist, they are present in very small numbers (approximately 1 in 2,000 cells for each cell type).

Dyskinetoplasty occurs naturally in both rat and mouse infections but less than one percent of cells are dyskinetoplastids. The cell types observed (Figs 3.3A4, 3.3A5, 3.3A12, 3.3A13), which show dyskinetoplastids with one or two nuclei, are consistent with dyskinetoplastids forming a proliferative subpopulation. These cell types are also consistent with dyskinetoplastids undergoing mitochondrial biogenesis as part of the slender-to-stumpy differentiation, in spite of the apparent lack of a mitochondrial genome. Since diaphorase activity is thought to be mediated by dihydrolipoamide dehydrogenase, and this enzyme is believed to be nuclear encoded, the acquisition of diaphorase activity by dyskinetoplastid forms implies successful import of at least one protein by the mitochondrion of dyskinetoplastid forms.

There are a significant number of bloodstream form trypanosomes which have one kinetoplast and two nuclei (1k2n, Fig. 3.3A6 and 3.3A14). Some of these forms are diaphorase positive, some are not. The incidence of these forms raises the question of whether some bloodstream forms can divide by segregating first the nucleus, then the kinetoplast. This would be in sharp contrast to procyclic form division (Sherwin and Gull, 1989a). Morphological observation of 1k2n forms shows no evidence of duplication of other single copy structures such as the flagellum, or the mitochondrion in diaphorase positive forms. Instead, it seems most likely that these represent forms which have either 1) failed to segregate their basal body/kinetoplast complex but have continued through the cell cycle

and completed mitosis, 2) given rise to a cytoplast with a kinetoplast but which lack a nucleus, or 3) resulted from fragmentation of a single nucleus.

The presence of another cell type present in significant numbers - the zoid- favours the second explanation. Zoids (Robinson et al., 1995) are trypanosome cytoplasts which lack a nucleus. Zoids are believed to arise by aberrant cytokinesis, the sister cell of each zoid having a kinetoplast and twice the normal nuclear DNA content (Robinson et al., 1995). Both slender zoids (Fig. 3.3A7) and stumpy zoids (3.3A15) are present in the bloodstream (Matthews and Gull, 1994a).

Rare cell types, apparently resulting from abnormal organellar segregation, were sometimes observed. These cells contained more than two nuclei and two kinetoplasts, but the exact number of each varied from cell to cell. Some of these cells showed staining with the diaphorase assay (Fig. 3.3A16) some did not. Very rarely, zoids were seen which lacked a kinetoplast (Fig. 3.3A8) or had two. In total these unusual and varied forms, which appeared to be the product of dramatically defective attempts at cell division, made up less than 0.05% of the population and so their incidence is not shown in Table 3.1.

In populations taken from Balb/c mice at 120h post-infection, approximately 1.2% (122 of 1000 trypanosomes counted) were stumpy by morphology and positive by diaphorase, but had a nuclear structure (as seen by DAPI staining, Fig. 3.3B) suggestive of a nuclear degradation. Such forms were not observed at earlier time-points during the parasitaemia, which suggested that this reproducible observation was not an artifact arising from glutaraldehyde fixation.

	Negative for mitochondrial staining by diaphorase assay							Positive for mitochondrial staining by diaphorase assay						
Fig. 3.3 label	1	2	3	6	4	5	7	9	10	11	12	13	14	15
DAPI	1k1n	2k1n	2k2n	0k1n	0k2n	1k2n	1k0n	1k1n	2k1n	2k2n	0k1n	0k2n	1k2n	1k0n
96 hours rat	58.6%	10.2%	3.4%	0.3%	0.2%	1.4%	0.5%	24.4%	<.1%	<.1%	0.3%	<0.1%	0.7%	.1%
96 hours mouse	46.2%	9.5%	1.3%	0.1%	0.1%	0.1%	0.4%	41.0%	<.1%	<.1%	0.6%	0.1%	0.4%	0.2%

Table 3.1. Bloodstream smears of mouse and rat infections were taken 96h post-infection. In each case 10,000 trypanosomes were assayed individually by the diaphorase assay using phase-contrast microscopy for staining localized to the trypanosome mitochondrion. Each of the cells was also assessed by DAPI fluorescence for the number and position of kinetoplasts (1k or 2k) and nuclei (1n or 2n). A representative cell of each type is shown in Figure 3.3A. The mouse population appears to be more differentiated at this time point, however the same cell types are seen in both infections. 1-3 and 9 represent the commonly occurring cell types; 1-3 are the types seen during the normal cell cycle of the slender form, 9 representing cell cycle arrested intermediate and stumpy forms, which have acquired diaphorase activity.

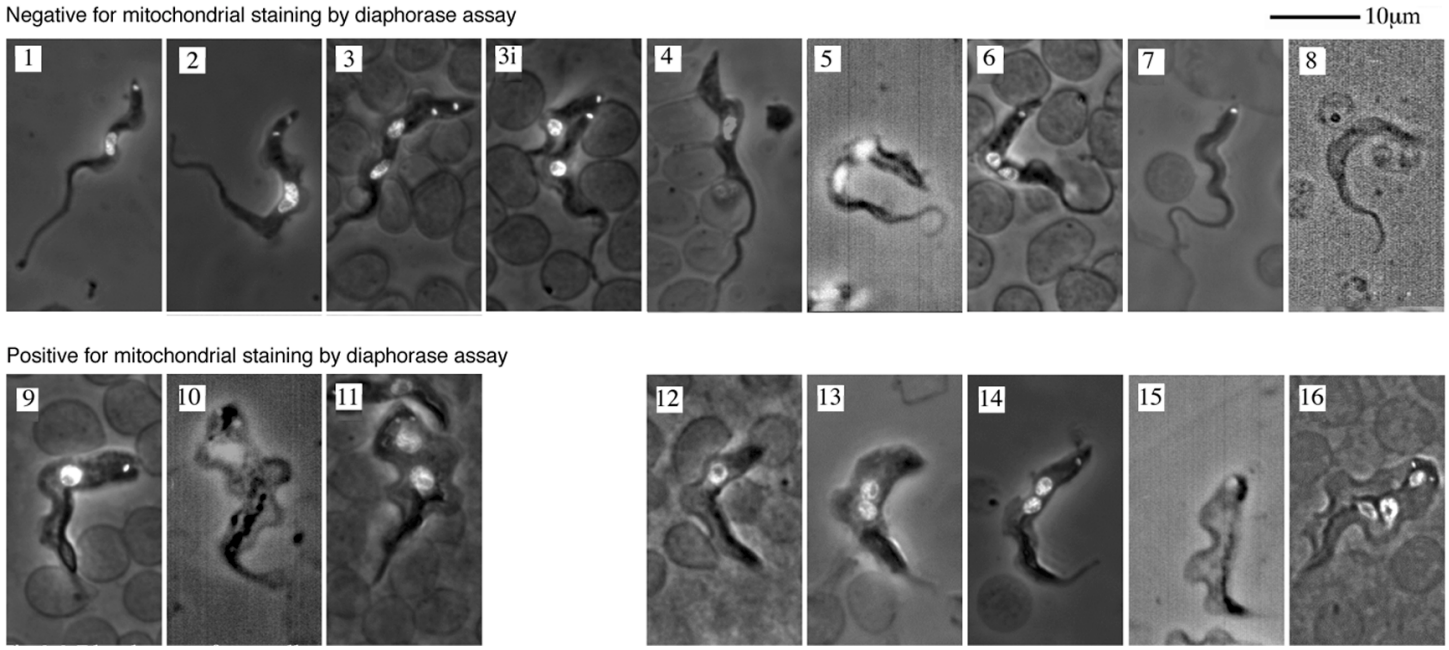


Figure 3.3A

Fig. 3.3 Bloodstream form cell types

Fig. 3.3A 10,000 trypanosomes from murine infection taken 96h post-infection were assessed using the diaphorase assay and DAPI fluorescence of nuclei and kinetoplasts. 1-8 Show cell types which do not show mitochondrial staining by diaphorase, when viewed by phase-contrast microscopy. 9-16 show distinct staining of the mitochondrion by diaphorase assay. Phase-contrast is combined with DAPI fluorescence to show the number and position of nuclei and kinetoplasts. Table 3.1 shows the abundance of each cell type in this infection and in an equivalent rat infection. 1-3 and 9 show the usual cell types seen and represent over 96% of these populations. 1-3 show the usual slender form morphologies during the cell-cycle, 3i shows a form close to cytokinesis. Form 9 is representative of most intermediate and stumpy forms. Forms 8 and 16 are very rare forms representing less than 0.1% of the population and are not shown in Table 3.1

Figure 3.3B

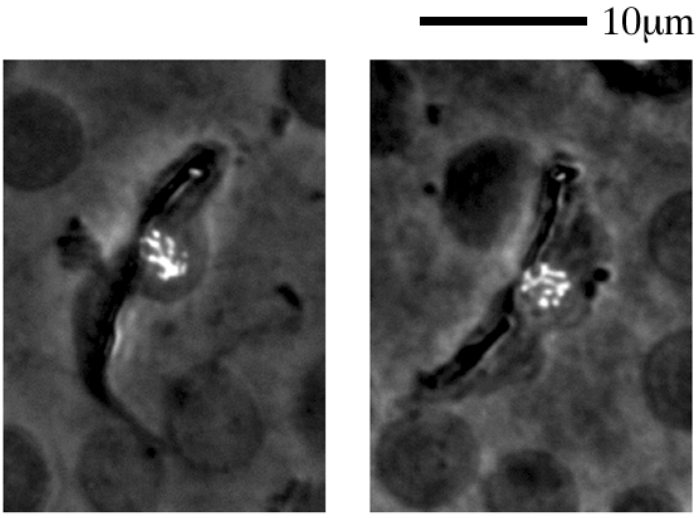


Fig. 3.3 Bloodstream form cell types.
Fig. 3.3B Possible breakdown of stumpy form nuclear structure. Characteristic mosaic staining of the nucleus by DAPI staining; represents 1.2% of forms seen in a sample taken 120h post-infection from a mouse. All such forms were stumpy in morphology and positive by diaphorase assay.

3.2.3 Slender form division

Although procyclic form cell division and the procyclic form cell cycle has been characterized in considerable detail (Sherwin and Gull, 1989a; Woodward and Gull, 1990; Robinson et al., 1995), this is not the case for the proliferative bloodstream form, which is responsible for disease pathogenesis, and which is necessarily different in some respects.

1. The slender form cell cycle is variable in length between strains (as determined in vivo: Herbert and Parratt, 1979; Turner et al., 1995), but is generally shorter than the procyclic cell cycle, as determined during in vitro culture (Sherwin et al., 1989a).

2. The slender form is narrower than the procyclic form and this may limit the scope for repositioning of some of the organelles.

3. An immediately apparent difference, revealed by DAPI fluorescence, is that the posterior kinetoplast never occupies a posterior nuclear position as it does in the procyclic form.
4. Another difference is the mitochondrion. There is little published work on division and segregation of this organelle in either the slender or procyclic form. The structural simplicity and defined position of the tubular, slender mitochondrion is very different to the activated mitochondrion, which forms a cage-like structure in the procyclic form. This difference means that there is a substantially different spatial relationship between organelles in bloodstream and procyclic forms. This difference may be reflected in different divisional mechanisms required in regenerating the spatial relationships of each life cycle stage with fidelity, following each cell cycle.

In order to begin to investigate the mechanisms by which bloodstream trypanosomes replicate, it was first necessary to establish the relative order of events which occur during the course of the cell cycle. The approach taken was to triangulate the cell cycle with markers.
1. Probasal body maturation and duplication provided early markers of commitment to division.
2. Outgrowth of flagellar axoneme and paraflagellar rod provided a marker for the whole subsequent period until cytokinesis.
3. Mitochondrial, kinetoplast and nuclear segregation provided useful markers of organellar segregation during the cell cycle.

Probasal body maturation and duplication

As a 3-dimensional cell, the trypanosome is elongate and approximately ovoid in shape but lacks obvious planes of symmetry. One dimension is characterized; the anterior and posterior ends are clearly defined. The anterior end, the end towards which trypanosomes swim, is marked by the free flagellum

and is the narrow end of the cell. The posterior end, defined relative to the anterior end, is the blunt end of the cell which is marked by the basal body/kinetoplast complex and flagellar pocket. The other two dimensions have not been defined, however there are “landmarks” available with which to define them. First, procyclic form trypanosomes have been shown to have a helical twist, which is the same for all procyclic forms. On scanning electron micrographs the flagellum exits the flagellar pocket near to the posterior end and then follows a left-hand helical path towards the anterior end of the trypanosome (Sherwin and Gull, 1989a). Second, the flagellar pocket is a single structure on the surface of the trypanosome which is clearly defined. By orienting the flagellar pocket so that it lies on the trypanosome’s upper surface, perpendicular to the substrate on which the trypanosome rests, a vertical polarity can be defined.

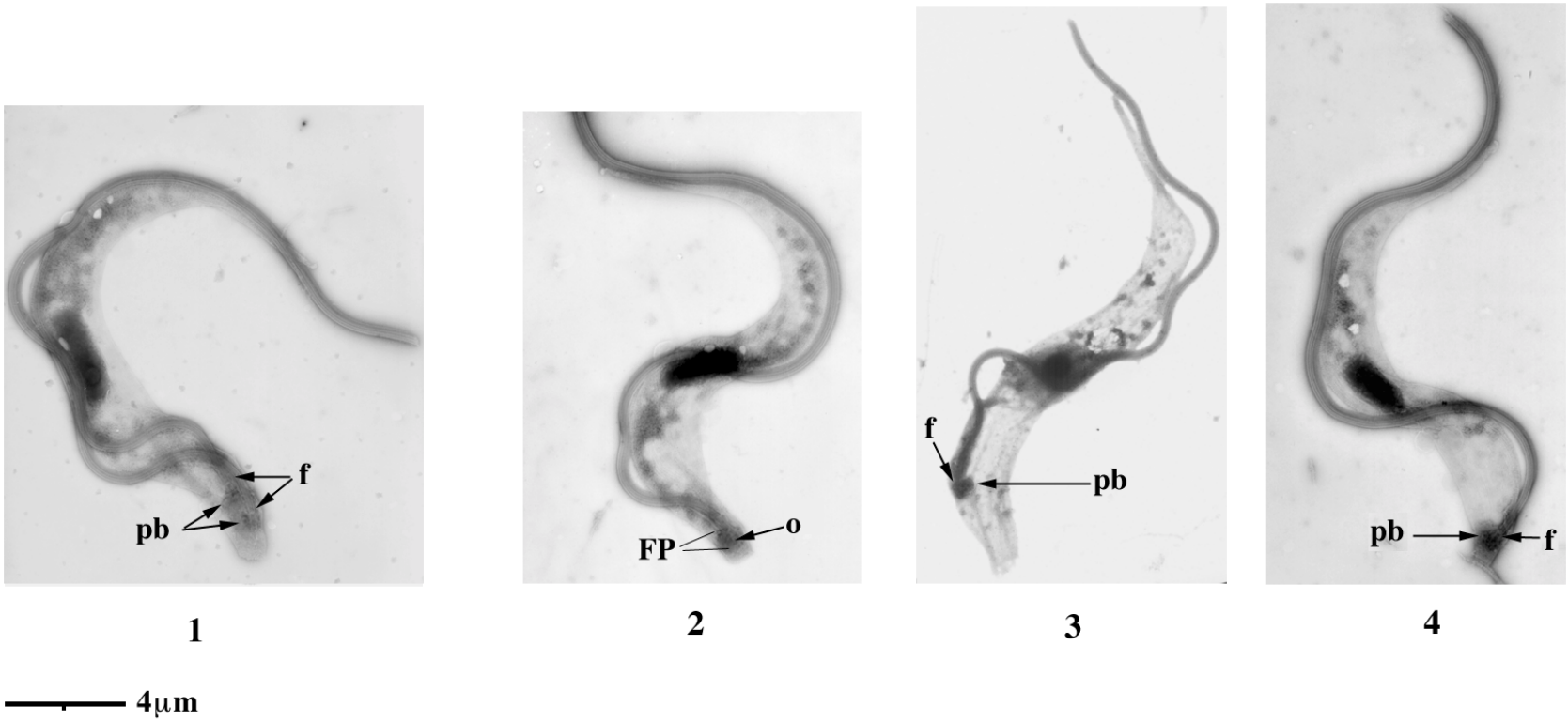
In order to examine trypanosome cell division, it was important to ensure that the left handed helix was maintained in the bloodstream form. Published scanning electron micrographs of 14 bloodstream forms were examined (Carr et al., 1971; Gorenflot et al., 1980; Ito et al., 1981; Abolorin et al., 1983). All 14 showed a left handed twist and this indicated that the left handed helix was likely to be maintained in bloodstream forms. That this was so, was further supported by evidence from some of the micrographs of whole mount cytoskeletons, where the twist was clearly visible (3.4A1). In dividing-forms, both flagella followed parallel left-handed helical paths (3.4A1). It was felt that the unidirectional twist of the trypanosome would allow for handedness rules to be established once a vertical polarity was established.

Examination of electron micrographs of 64 whole cell cytoskeleton mounts of bloodstream trypanosomes (gifts from Dr. T. Sherwin and Dr. K. Matthews, University of Manchester) was then undertaken. Micrographs were oriented with the anterior end uppermost. Examination of the micrographs also made it clear that if trypanosomes were viewed in an orientation which looked

onto the flagellar pocket, then the flagellum obscured the probasal body (Fig. 3.4A2). If the trypanosome was tilted, as was generally the case, then the probasal body would appear either to the left or to the right of the basal body subtending the flagellum, depending upon the direction of the tilt. Specifically, if the posterior end of the trypanosome was tilted left, then the basal body would lie to the right of the flagellum (Fig. 3.4A3); if it was tilted right then the basal body would lie to the left of the flagellum (Fig. 3.4A4). Of the 64 cytoskeletons, the posterior end was tilted to the right in 52 cases (80%), perhaps because of the initial left handed twisting at the posterior end.

Fig. 3.4A Orientation of whole cell cytoskeletons:
f - flagellum, FP - flagellar pocket, pb - probasal body.
3.4A1 It appears that all slender forms, like procyclic forms, have a left-handed twist. The reason for this can be seen in dividing-forms, where it can be seen that the new flagellum curls around the dividing-form with the same left-handed twist towards the anterior end as the old flagellum.
3.4A2-4. As an asymmetric three dimensional object, the trypanosome dimensions can be described as anterior and posterior, above and below, right and left. In addition to anterior and posterior which are well defined, above can be defined by the flagellar pocket, left and right by the direction of the twist. Trypanosomes viewed as cytoskeletons on EM grids are normally tilted.
3.4A2 Shows a cytoskeleton where the view at the posterior end is directly onto the flagellar pocket. From this view the probasal body is obscured.
3.4A3 Shows a cytoskeleton where the posterior end is tilted to the left, which is a rare orientation. The probasal body is visible to the right of the mature basal body.
3.4A4 Shows a cytoskeleton where the posterior end is tilted to the right, which is the most frequent orientation. The probasal body is visible to the left of the mature basal body.

Figure 3.4A



3.4B Basal body maturation, elongation and duplication in bloodstream form trypanosomes.

Electron micrographs of the posterior end of trypanosome cytoskeletons can be subdivided into three sequential stages, with regard to the number and positioning of their probasal bodies and flagella. In the illustration, the new flagellum and its probasal body are drawn in black. pb - probasal body, f - flagellum, o.f. - old flagellum, n.f. - new flagellum.

Stage 1: Single probasal body and single flagellum.

Stage 2: An old and a new flagellum, no probasal bodies visible. Maturation and elongation of the probasal body is the first detectable cytoskeletal change which shows commitment to division.

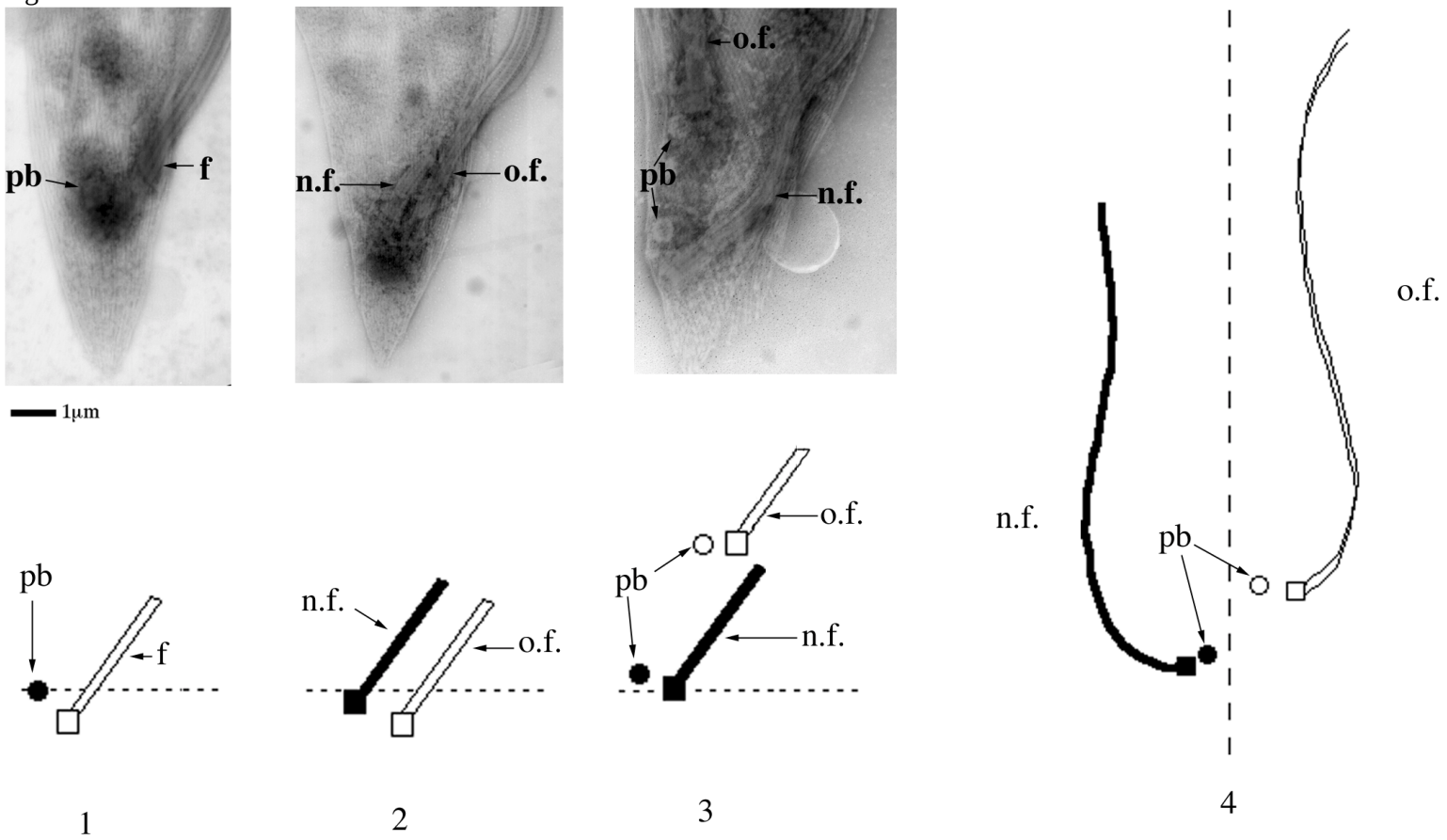
Stage 3: Old and new flagella both associated with probasal bodies. Probasal bodies are formed close to the onset of new flagellar growth, prior to the exit of the new flagellum from the flagellar pocket. The new flagellum assumes a position posterior to the old flagellum.

Upper panel shows representative electron micrographs all oriented with right hand tilts (as in Fig. 3.4A4)

To aid interpretation the events are also illustrated below. Care should be taken in interpreting stage 3. The new flagellum is actually positioned to the left of the old flagellum so that in this picture which is tilted to the right, the new flagellum is actually passing above the old flagellum. The cell is effectively folded along its cleavage plane.

To aid interpretation 3.4B4 effectively unfolds a stage 3 cell, so that it can be seen from the perspective of the cleavage plane. In this theoretical cell the left hand daughter is effectively tilted left so the probasal body lies to the right. The right hand daughter cell is effectively tilted right so that the probasal body lies to the left.

Figure 3.4B



Taking into account orientation, electron micrographs of whole cell cytoskeleton mounts were examined and a basic order of events established. Figure 3.4B shows micrographs of probasal body maturation, elongation and duplication. All three micrographs (upper panel) have a rightward tilt to simplify interpretation. A figurative representation (lower panel) is also provided for the sake of clarity. In the interphase cell, the probasal body is positioned underneath the basal body subtending the flagellum (Fig. 3.4B1 and Fig.3.4A). The probasal body then matures and forms a new basal body which elongates to form the new flagellum (Fig. 3.4B2). The new flagellum moves to a position which is posterior to the old basal body and at this point two new probasal bodies become apparent (Fig. 3.4B3).

The right tilt must be taken into account in interpreting Fig.3.4B3, in which the new flagellum appears to pass to the right of the old flagellum. If it were possible to look down the cleavage plain, the new flagellum would actually lie to the left of the old flagellum but, in tilting the cell to the right the cell is effectively folded. In Figure 3.4B4 a figurative illustration of cytokinesis, looking down the cleavage plane, is provided to illustrate this idea. The figure is theoretical since the electron micrographs analyzed did not contain any cells in this orientation. At cytokinesis the base of the new flagellum is left and posterior to the base of the old flagellum. There is also likely to have been some degree of rotation of the basal bodies relative to one another. Looking down the plane of cleavage, the left hand cell, inheriting the new flagellum, is effectively tilted left. Conversely, the right hand cell, inheriting the old flagellum, is effectively tilted right. Consequently the probasal bodies, of both daughter cells, face inwards towards the plane of cleavage. The final result is that the daughter cell receiving the old flagellum segregates anterior and to the right of the daughter cell receiving the new flagellum, which therefore segregates posterior and to the left.

The old and the new flagellum follow parallel left-handed helical paths around the cell. Since this is the case, the posterior movement of the new flagellum

body may be enough to ensure that it will partition to the left of the old flagellum. New probasal bodies can be formed in the same orientation, with respect to the new and the old flagellum, that they are found in G_1 cells. In this way the basal bodies are matured and segregated, new probasal bodies are formed, and spatial relationships are maintained, without the need to resort to extensive repositioning mechanisms. Segregation of basal bodies can be achieved simply by the insertion of additional subpellicular microtubules (as described for the procyclic form in Sherwin and Gull, 1989b and Robinson et al., 1995) in the region between the basal bodies. In addition to providing directional movement for the segregation of the basal bodies, these microtubules may form the basis for the completion of each daughter cell's subpellicular corset.

Outgrowth of the new PFR

Outgrowth of the new PFR had previously proved useful for studying division of the procyclic form (Sherwin and Gull, 1989a; Robinson et al., 1995). Probasal body maturation and elongation seem to be early markers of commitment to cell division in slender forms; the outgrowth of the new flagellum then continues, presumably until it reaches full length. It was likely, therefore, that outgrowth of the new flagellum would be able to provide a good marker for much of the latter part of the cell cycle.

The paraflagellar rod (PFR) is associated with the flagellar axoneme once it exits the flagellar pocket. Defined monoclonal antibodies to the PFR are available (Robinson et al., 1995; Kohl and Gull, 1997) making it possible to highlight the PFR by immunofluorescence and facilitating the assay of PFR length. PFR length is essentially the length of the flagellar axoneme outside of the flagellar pocket. In the absence of a fixed time-point to assess whether the rate of flagellar growth was linear during the cell cycle, the PFR of the new flagellum of one hundred cells was measured to determine whether the distribution of lengths was approximately

constant. The pleomorphic population from which these cells was drawn was taken on day 3 post-infection, at a parasitaemia of approximately 3 x 10^7 trypanosomes per ml of blood when over 90% of forms had a slender morphology. Only dividing-forms which showed staining of two paraflagellar rods, by immunofluorescence with a monoclonal antibody recognizing both PFRA and PFRC proteins, were selected.

Immunofluorescence from each cell was photographed with a CCD camera and imaged with IPlab software. Corresponding phase-contrast/DAPI images were also captured. Both paraflagellar rods were then measured by tracing each PFR on a captured image: the IPlab facility "measure" then gives a value for length. For each cell each PFR measurement was then recorded. In the course of these measurements it was clear that, in every case, the anterior PFR was longer than the posterior PFR.

The new and old PFR lengths obtained from each cell were paired on a spread sheet and the pairs were sorted according to the length of the new PFR. The lengths were then plotted as a graph (Fig. 3.5). In Figure 3.5 it can be seen that, when only the new PFR length is considered (black bars), an even slope is obtained. The slope is from close to 1μm which was the shortest length measured, to approximately 27μm which was the longest length measured. If the new PFR had reached full length well before cytokinesis the slope might be expected to plateau as a maximum length was reached. Moreover, if growth of the PFR was non-linear this might be expected to appear as an uneven slope. The uniformity of the slope formed by the black bars in Figure 3.5 implies that growth of the PFR occurs at a relatively constant rate until cytokinesis. By estimating the bloodstream form trypanosome cell cycle as 6h, the rate of PFR growth can be estimated as 4.5μm per hour.

In bloodstream forms, the maximum length reached by the new PFR is considerably shorter than the maximum old flagellar PFR. The implication of this is that outgrowth of the new flagellum does not cease at cytokinesis during proliferative growth, but continues at least into G_1 of the next cell cycle. The

minimum old PFR lengths of dividing-forms are actually shorter than the maximum length of the new PFR - 27μm. This striking heterogeneity raises the question of whether the variability in the old PFR length of dividing-forms can be explained in terms of the slender-to-stumpy differentiation.

Figure 3.5

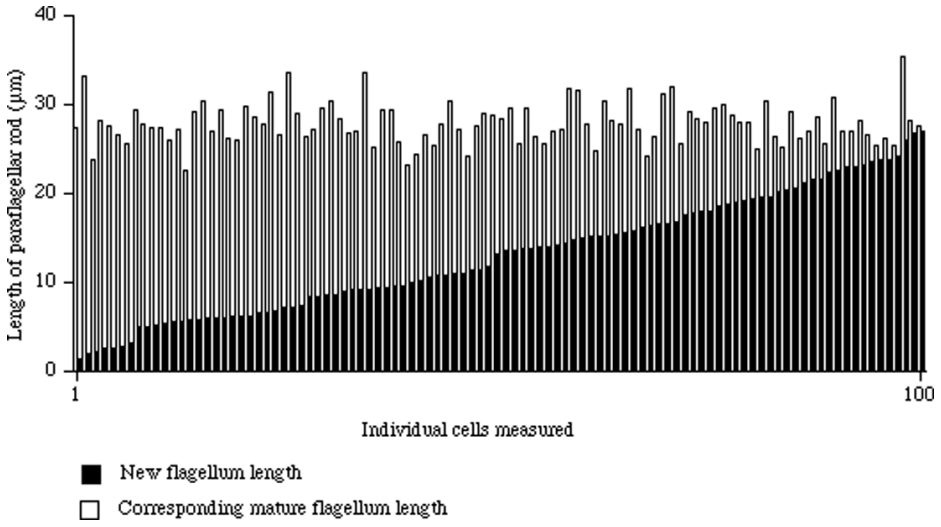


Fig. 3.5. Cells with two paraflagellar rods (PFRs). The new PFRs show an even distribution of lengths, which implies linear growth. The old PFR of each cell was also measured and is shown to the left of the new PFR for each cell, however, no relationship between the length of the old PFR and the length of the new PFR is apparent.

Kinetoplast and nuclear segregation

The apparent linearity of new PFR outgrowth deduced from Figure 3.5A, was complemented by data indicating that outgrowth of the new PFR is constant for at least most of its growth in procyclic forms (Bastin et al., unpublished observation). It seemed reasonable, therefore, to use outgrowth of the PFR as a marker with which to fix the relative order of other events such as organelle segregation during the cell cycle. The 100 dividing-forms which had been imaged and measured for Figure 3.5 were categorized according to the number of their kinetoplasts and nuclei and the distance between their kinetoplasts (measured with IPlab software in the same way as for flagellar length). Five categories, or phases, were derived and these are described in Figure 3.6.

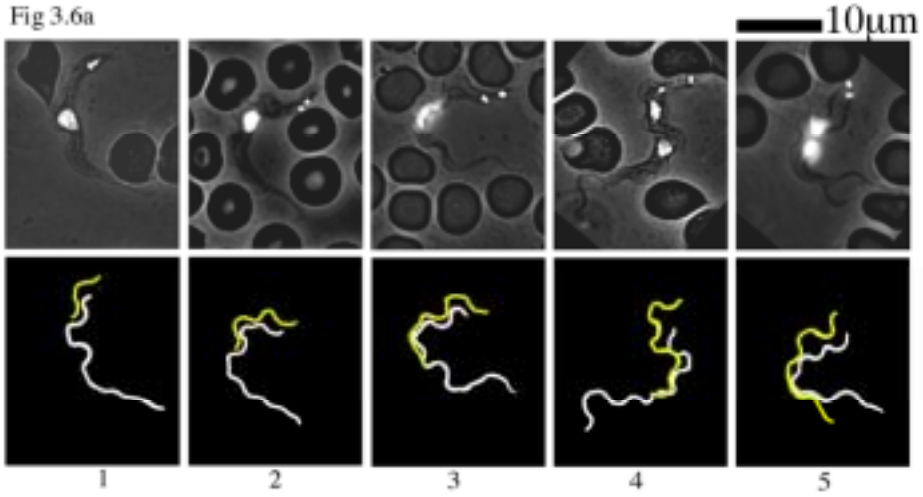
Phase 1 trypanosomes have a single nucleus and either one kinetoplast, a dumbbell shaped kinetoplast (as shown in Fig. 3.6A1), or two kinetoplasts less than 1.5μm apart.

Phase 2 encompasses trypanosomes which are generally premitotic but in which the kinetoplasts are clearly distinct and positioned between 1.5μm and 2.5μm apart (Fig. 3.6A2).

Phase 3 is defined by forms which have kinetoplasts over 2.5 μm apart and a nucleus that is not fully segregated, however in most of these cases the nucleus appeared to be undergoing mitosis as in Figure 3.6A3.

Phase 4 consists of post-mitotic forms with 2 kinetoplasts separated by more than 2.5μm and with two discrete nuclei.

Phase 5 forms were relatively rare (6 from 100 cells). These near-cytokinesis forms had kinetoplasts separated by less than 2.5μm and two nuclei.



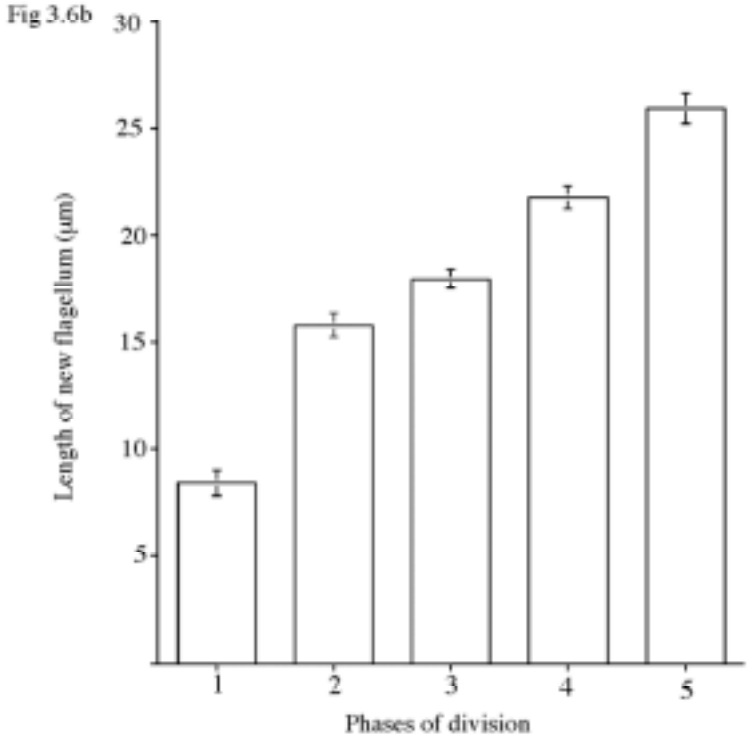


Fig. 3.6 Outgrowth of the new PFR is a marker of the bloodstream form cell cycle. Interkinetoplast distance (id) and number of nuclei were used to define 5 phases of division: 1) 1N id<1.5µm; 2) 1N id =1.5-2.5µm; 3) 1N (often mitotic), id>2.5µm; 4) 2N id>2.5µm; 5) 2N id <2.5µm. Fig. 3.6A shows phase contrast images with DAPI fluorescence of the nuclei and kinetoplasts at each phase (above) with the corresponding image (below) showing immunofluorescence of the PFR, which was used as a marker of flagellar length. The new PFR (yellow) was pseudocoloured with adobe photoshop to aid discrimination from the old PFR (white). Figure 3.6B shows the mean new PFR length at each phase of division. Error bars show one standard error. The significance of the differences between the means was then tested by a one way analysis of variance.

One way analysis of variance

Phase	1	2	3	4	5	Total
n	52	11	15	16	6	100
SX	439	173	270	348	156	1386
SX^2	4536	2751	4886	7636	4044	23856
mean	8.43	15.75	17.98	21.76	25.94	13.86

	Between phases	Within phases	Total
SS	3702	944	4646
MS	925.5	9.94	
df	4	95	99

K = 5
F_{crit} (P<0.01) = 3.51
F_{obt} = 93.14

Ad hoc comparisons between groups by Fisher's protected t-test

t_{crit} (P<0.05) = 2.00
t_{crit} (P<0.01) = 2.66

t_{1-2} = 7.0
t_{2-3} = 1.8
t_{3-4} = 3.3
t_{4-5} = 1.5

It seemed intuitively obvious from the data presented in Figure 3.6 that segregation of the kinetoplast and nuclear genome could be correlated with outgrowth of the new PFR. In order to test the significance of the five proposed phases of division, however, an analysis of variance was also conducted. Although the individual formulas by which F and t values were obtained are not shown, they can be obtained from most basic statistics books (e.g. Heinman, 1992). The breakdown of the statistics used is shown above. The F value obtained (F_{obt}) was clearly higher than the critical value of F (F_{crit}) for establishing significance . This indicated that PFR outgrowth did indeed show a correlation with kinetoplast and nuclear segregation.

With respect to the significance of differences in the PFR length between phases, results were mixed. It was not possible to demonstrate significant differences in the mean PFR lengths between phases 2 and 3 or between phases 4 and 5. In the first case this is probably because the difference between the means of phases 2 and 3 is small. In the second case significance could probably not be demonstrated, primarily because of the small number of cells making up phase 5. All other differences between the five populations were significant. The t values obtained for each of the sequential phases are given. Although significance was not shown in every case, it remains likely that these 5 sequential phases do accurately describe cell division in bloodstream form trypanosomes. It is difficult to imagine how the trypanosome can go from phase 2 to phase 4 without progressing through phase 3. Further, the clear definition of a cleavage plane in cells which appear to be in phase 5 seems to indicate that it is does represent the form adopted by bloodstream form trypanosomes just prior to cytokinesis . Indeed, significance might be established for the remaining groups by expanding the study approximately five-fold, so that the smallest group was larger than n = 30. Finding, individually capturing (photographically) and measuring the new PFR of at least 400 more cells would, however, represent a considerable investment.

This study establishes the order in which bloodstream form trypanosomes segregate their nuclear and mitochondrial genomes. It determines that kinetoplast segregation occurs prior to nuclear segregation. It also establishes that kinetoplasts never occupy an anterior nuclear position in bloodstream dividing-forms as they do in procyclic dividing-forms (Sherwin and Gull, 1989a). From this study it was possible to categorize dividing-forms using only DAPI staining into four readily recognizable, sequential stages. Unfortunately, however, it is not readily possible to use DAPI staining to discriminate some of the phase 1 cells where the kinetoplasts are in too close a proximity to distinguish reliably. The four stages are:-

1) Pre-mitotic 2k1n (incorporating some of phase 1 and phase 2).

2) Mitotic 2k1n (Phase 3).

3) Post-mitotic 2k2n (Phase 4).

4) Near-cytokinesis 2k2n (Phase 5).

Mitochondrial segregation.

The single slender mitochondrion has a narrow acristate tubular structure which runs along the length of the non-undulating side of the cell, close to the subpellicular microtubules. The order of segregation of the kinetoplast and nucleus having been established, attention was turned to the segregation of this large single copy organelle. The bloodstream form is the natural choice to study the segregation of the mitochondrion in trypanosomes because the structure is smaller, simpler and more discrete than in the procyclic form. The trypanosome alternative oxidase (TAO) is an enzyme active in the slender form mitochondrial matrix, the presence of which can be detected with monoclonal antibodies (gift of Prof. G. Hill, Meharry Medical College, Nashville, Tennessee, USA). Immunofluorescence with this antibody clearly delineates the mitochondrion of slender forms (See Chapter 4 for more detailed characterization of TAO expression). In Figure 3.7A TAO was used to follow mitochondrial segregation by immunofluorescence and reveal the way in which it appears to “unwind” to form a branching network.

An attempt was made to follow the segregation of the mitochondrion in the temporal order of the four phases defined by DAPI. It was immediately clear that the segregation was structurally complex. It was also clear that the process took place primarily during mitosis and that equating captured images with the four phases defined by DAPI did not permit an ideal description of mitochondrial segregation. Nevertheless, images were first sorted into the four phases defined by DAPI. Images were then subdivided in each category (particularly mitosis) and in this way a working hypothesis of how mitochondrial segregation occurred was constructed (shown figuratively in Figure 3.7B and described as follows).

- A point of bright staining is present which appears to be localized at the kinetoplast. It can be seen to divide and separate along the single mitochondrial tube as the kinetoplasts segregate in premitotic forms (3.7A1-2).
- The majority of branching occurs during mitosis (3.7A3-6).
- Segregation appears to proceed initially by the formation of two loops (3.7A3).
- The loops expand during mitosis (3.7A3-5).
- The posterior loop fuses to the posterior kinetoplast (3.7A6).
- In post-mitotic forms the posterior loop then fuses to the anterior loop (3.7A7).
- In near-cytokinesis forms, the resultant newly formed mitochondrion is then detached from the original mitochondrion (3.7A8).

Although the data shown in Figure 3.7a was combined with many other images captured (often as multiple slices using a 'semi-confocal' technique) during the construction of the working hypothesis (Fig. 3.7b), the study presented here remains a preliminary study. It is clear that it will be substantially improved by using double immunofluorescence with PFR antibody and TAO antibody, which will allow outgrowth of the new PFR to be used as a potentially more amenable temporal marker. This future study should also make use of confocal microscopy, which will make the spatial arrangement of the segregating mitochondrion easier to interpret in a three dimensional context.

Figure 3.7

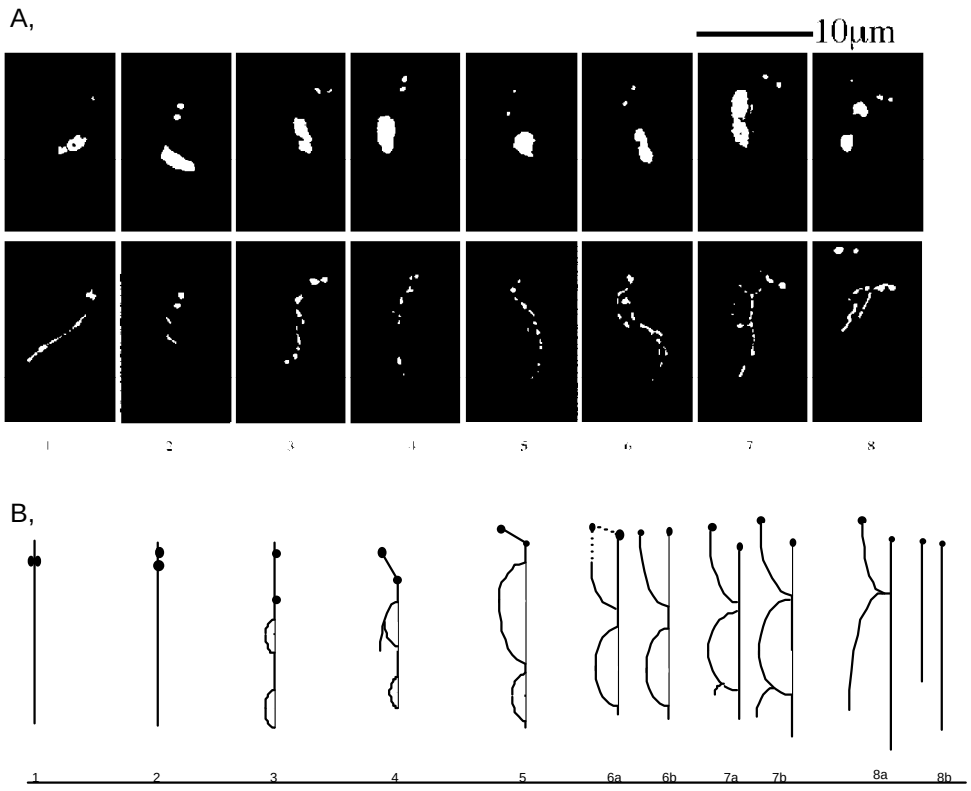


Fig. 3.7 Mitochondrial segregation.
Fig. 3.7A Shows phase-contrast /DAPI images (upper panel) of eight dividing-forms. One representative cell for each stage in mitochondrial segregation is shown. Immunofluorescence for the mitochondrial marker TAO (lower panel) reveals the pattern of mitochondrial localization.
Fig. 3.7B Uses these images and others to compile a figurative illustration of the proposed temporal order of events during mitochondrial segregation:- the kinetoplast region is separated first (1-2); two "loops" arise from the mitochondrion (3-4); the posterior loop increases in size (4-6); the posterior loop becomes subtended by the posterior kinetoplast region (6a); the connection between the kinetoplasts is lost (6b); and the anterior loop is expanded (6-7). A new mitochondrion is then formed by extending the anterior loop to the anterior of the cell (7); by joining the lateral processes from each of the original loops (7); and by loss of the connections between the mitochondria from the original anterior loop (8).

3.2.4 In vitro culture of pleomorphic bloodstream forms

An axenic culture medium, HMI-9, for monomorphic bloodstream form trypanosomes had already been developed when this project was begun (Hirumi and Hirumi, 1989). However, the methods by which monomorphic forms were adapted to culture had not been applied successfully to the culture of pleomorphic bloodstream forms. Hirumi recommends the addition of approximately 10^7 trypanosomes in 50µl of blood to 5ml of sterile prewarmed HMI-9 in a culture flask and then passaging daily with an equal volume of fresh HMI-9. Using this regime monomorphic cells initially increase in culture density for up to three days, reaching up to $5x10^6$ trypanosomes/ml before the culture dies almost completely. By continuing to passage daily, however, the remaining cells begin to recover and are then considered "culture adapted".

This regime, however, failed to produce "culture adapted" pleomorphic lines. Under these conditions GUP 2962 reaches a maximum density on the first day, of approximately 10^6 trypanosomes/ml, before the culture dies and cannot be recovered. Microscopic examination of these abortive cultures, at peak density, showed cellular morphologies akin to those described by Hamm and coworkers (Hamm et al., 1990), for monomorphic lines cultured in vitro at high density.

It had been proposed that in vivo the slender-to-stumpy differentiation occurs as a function of parasite density (Seed and Sechelski, 1989). It had also been proposed that this differentiation is mediated by a parasite derived growth inhibitor to which pleomorphic lines were more sensitive than monomorphic lines (Hamm et al., 1990). It seemed reasonable that if this was the case then such a factor might accumulate preferentially in vitro but be labile in blood. This would prevent cultured cells from reaching a high density in vitro.

It was therefore decided to attempt to initiate pleomorphic culture at low culture densities (10^2-10^3 trypanosomes per ml). This stratagem proved successful and after 48h trypanosomes were readily detectable in culture. Trypanosomes were

passaged every two days to a concentration of 10^3 trypanosomes per ml. Continuous culture of pleomorphic trypanosomes was sustained for a period of six months. Following every month of culture, frozen stocks were prepared and stored in liquid nitrogen. It is worth noting that cultures of monomorphic lines can be initiated much more effectively at low density since the period after culture death, when such cultures often fail to be regenerated, is completely avoided. During this period of culture adaptation many aberrant morphologies were commonly seen; in particular, cells remaining joined at the posterior end apparently unable to complete cytokinesis. After six months of culture and passage, however, these forms represented less than 0.5% of the population.

Figure 3.8 shows growth curves obtained from replicate flasks after 4 months of continuous in vitro culture. 10^3 trypanosomes suspended in approximately 10μl of culture medium were used as an inoculum. The inoculating trypanosomes were taken from a logarithmically growing culture of bloodstream forms, with a trypanosome density of 10^5 trypanosomes per ml of culture medium. Figure 3.8 shows that logarithmic growth apparently begins immediately and continues for 48h to an approximate density of $8x10^5$ trypanosomes per ml. Growth then slows reaching a peak of $3x10^6$ trypanosomes per ml at 72h. Parasite numbers then fall rapidly and by 120h motile trypanosomes are not detectable. During logarithmic growth the mean doubling time of the culture was less than 6 hours. Figure 3.8 also shows the densities at which one of the flasks was sampled for further analysis in Sections 3.2.5 and 3.2.7.

Figure 3.8

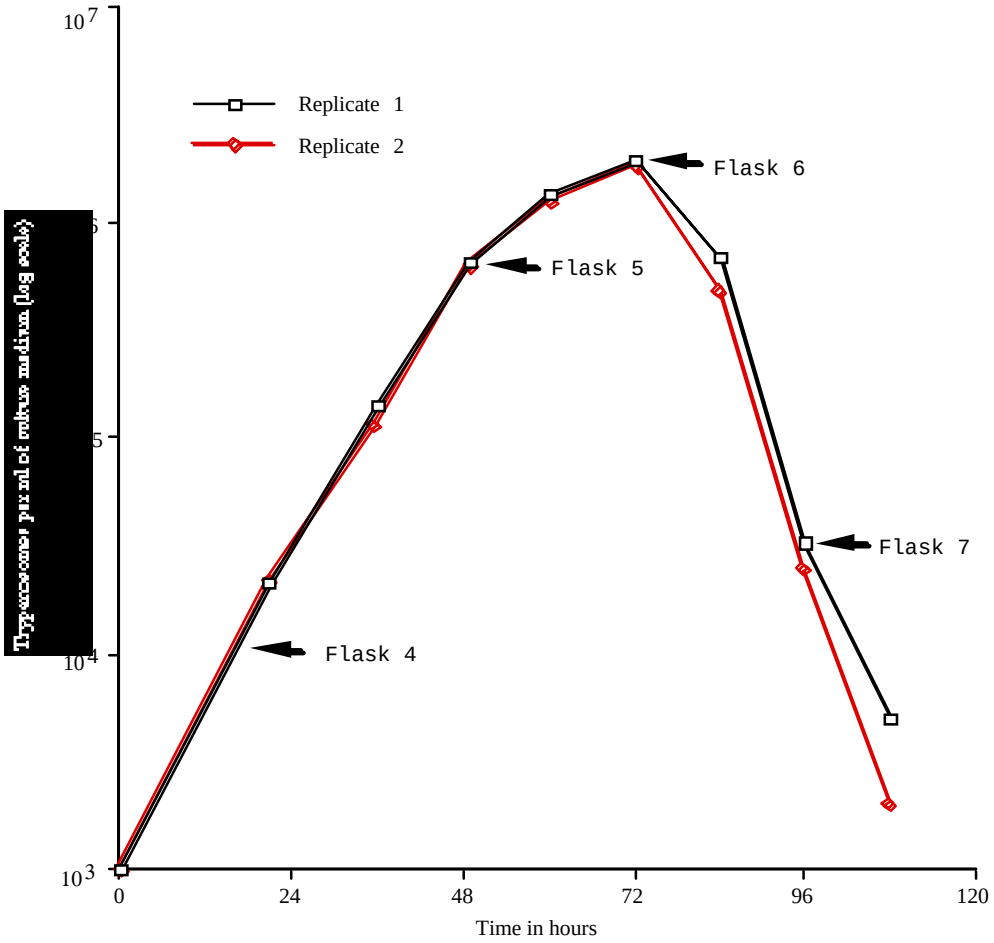


Fig.3.8 Growth curves of pleomorphic *T.b.rhodesiense* (GUP 2962) after 4 months of in vitro culture (passage 55) in duplicate flasks containing HMI-9 medium. Trypanosomes were initially passaged to 1000 cells per ml at 0h. Arrows indicate the cell densities at which trypanosomes were harvested for the subsequent assessments of competence to differentiate to the stumpy form (see Sections 3.2.5 and 3.2.7).

3.2.5 Observation of cytological change during axenic culture of pleomorphic bloodstream form trypanosomes

The slender-to-stumpy differentiation has been proposed to occur as a function of parasite density (Seed and Sechelski, 1989). It has also been observed that at high culture density monomorphic lines appear to undergo differentiation to stumpy-like forms (Hamm et al., 1990). Consequently, once culture had been established, evidence was sought of the slender-to-stumpy differentiation by pleomorphic lines in vitro.

Assay of morphological change and mitochondrial activation by microscopy was undertaken after four months of continuous in vitro culture. Figure 3.8 shows the growth curves obtained from duplicate flasks, seeded at a density of 10^3 trypanosomes per ml and then left unpassaged until no motile trypanosomes were apparent by microscopy. Trypanosomes were harvested at four time-points which are indicated on the growth curve (Fig. 3.8); during logarithmic growth, non-logarithmic growth, at the peak parasite density and during cell death. Harvested trypanosomes from each time-point were prepared for a double immunofluorescence with anti-tubulin antibody (TAT: Woods et al., 1989a), to emphasize the cytoskeleton and morphology, and with antibody to dihydrolipoamide dehydrogenase (Gift of Prof. L. Krauth-Siegel at the University of Heidelberg, Germany), a stumpy enriched marker of mitochondrial activation which is diagnostic for stumpy forms (see Chapter 4 for detailed analysis). Morphological transition and mitochondrial activation occurred during growth of trypanosomes in culture (Fig. 3.9). At culture densities of less than 10^5 trypanosomes/ml (flask 4) cells had a morphology typical of some of the slender morphologies seen in the bloodstream and were uniformly small and highly motile. Analysis by immunofluorescence for dihydrolipoamide dehydrogenase (DHLADH) showed no mitochondrial staining and staining for diaphorase activity (not shown) was likewise negative.

At cell densities of between 10^5 trypanosomes/ml and 10^6 trypanosomes/ml (flask 5) the trypanosomes retained a slender morphology and were apparently slightly larger and less motile. Mitochondrial expression of DHLADH was not detectable by immunofluorescence, while staining by diaphorase assay revealed some granular staining of some of the cells, although not necessarily at the mitochondrion (not shown). This staining and general appearance of the cells was very similar to the characteristics of monomorphic trypanosomes taken from high parasitaemias in vivo.

At cell densities above 10^6 trypanosomes/ml (flask 6), trypanosome morphology appeared largely akin to intermediate forms seen in vivo. Some cells showed mitochondrial immunofluorescence staining with DHLADH. Diaphorase staining was stronger, remaining largely granular, but only some cells showed a mitochondrial localization of stain (not shown). At these densities some cells had begun to lyse but living cells could not be described as truly stumpy. As cells died, and the cell density was reduced again to below 10^6 trypanosomes per ml (flask 7) a few cells which showed mitochondrial localization of dihydrolipoamide dehydrogenase by immunofluorescence and possessed a convincingly stumpy morphology were observed amongst the cellular debris.

Figure 3.9

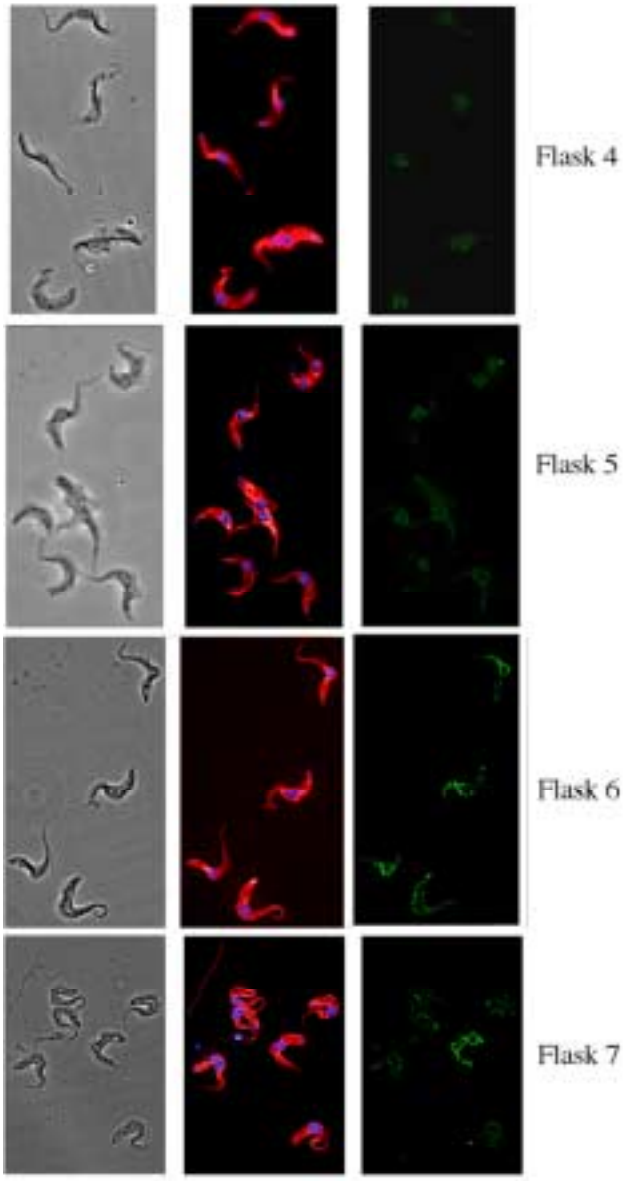


Fig. 3.9 Morphological change and acquisition of dihydrolipoamide dehydrogenase (DHLADH) occurs during in vitro growth in HMI-9. Phase contrast reveals cellular morphology (left panel); the kinetoplast, nucleus and cytoskeleton are visualized by DAPI fluorescence -blue- and immunofluorescence for alpha-tubulin -red- (middle panel). Acquisition of DHLADH is also shown by immunofluorescence double labelling (right panel).
These trypanosomes were taken from flasks at the trypanosome densities shown in Fig. 3.9.

3.2.6 Maintenance of pleomorphism

In developing a system for the in vitro culture of pleomorphic trypanosomes it was essential that they remain infective and able to differentiate in vivo in order that putative genes associated with the slender-to-stumpy differentiation, such as those developed in Chapter 4, could be exploited using transfection technology. To this end, following every month of continuous in vitro culture, the cultured trypanosomes were inoculated into mice to ensure the lines remained pleomorphic.

The growth curves obtained from mice infected with 10^6 trypanosomes harvested from in vitro culture, and mice infected with 10^6 trypanosomes from frozen stocks of GUP 2962, remained very similar during the six month period tested (Fig. 3.10A). The infections remained pleomorphic. Moreover, the slender, intermediate and stumpy forms present in each infection were morphologically indistinguishable from those in infections obtained from the GUP 2962 frozen stock. The only difference observed was that, after six months of culture, infection of mice produced infections in which only stumpy forms ever stained for diaphorase. This was in contrast to GUP 2962 and prior infections with culture forms which gave parasitaemias, as previously described, in which both intermediate and stumpy forms stained with diaphorase (Fig. 3.10B).

Figure 3.10

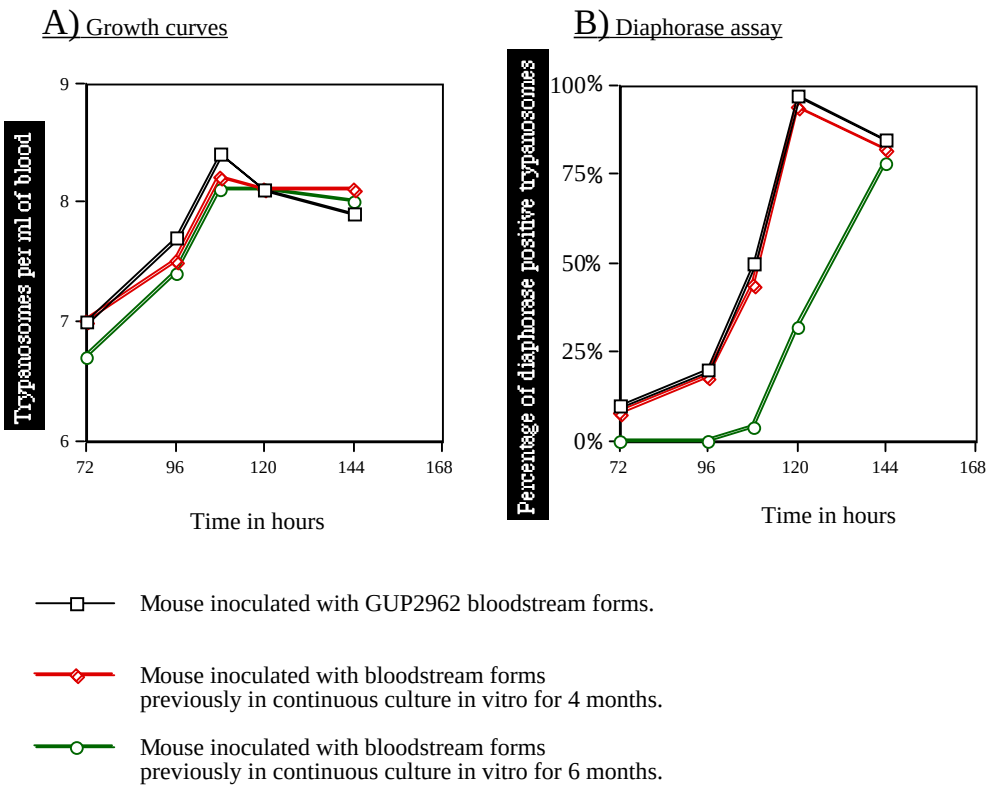


Fig. 3.10 Acquisition of diaphorase activity is delayed in infections with forms from long term cultures.

3.2.7 Competence to differentiate to the procyclic form

When mice were infected with trypanosomes maintained in continuous in vitro culture for up to four months, the infections were clearly pleomorphic and stumpy populations were obtained which were indistinguishable from other GUP 2962 infections by cytology (Section 3.2.6). Regardless of the cytology, however, it was important to determine whether prolonged culture had compromised the ability of these forms to undergo a primary facet of the stumpy form's phenotype, the ability to differentiate to the procyclic form.

Cytological evaluation of the cultured bloodstream forms, later than 72h through the growth curve, was ambiguous with respect to the status of many of these

culture forms as stumpy or intermediate forms (Section 3.2.5). Consequently, the need for a functional assay was even more important in the evaluation of these culture forms as potential stumpy forms.

At 27^{o}C in the presence of cis-aconitate, stumpy forms differentiate rapidly and synchronously to the procyclic form and re-enter the cell cycle, expressing procyclic form markers in a well established temporal order (Matthews and Gull, 1994ab, 1997 and reviewed in Pays et al., 1997). Under these conditions slender and monomorphic populations can also differentiate to the procyclic form, but they do so less efficiently, more slowly and with non-synchronous kinetics. It was therefore possible to use in vitro differentiation to the procyclic form as a functional assay for stumpy populations, by determining whether the cultured bloodstream forms were capable of rapid and synchronous differentiation to the procyclic form.

In order to test whether the cultured trypanosomes could produce viable stumpy forms either during in vitro culture, or following passage in mice, seven flasks were prepared. Each of the seven flasks contained approximately 5 x 10^6 bloodstream form trypanosomes suspended in 5ml of HMI-9. In each case trypanosomes were concentrated by centrifugation and resuspended to 10^6 trypanosomes per ml in fresh HMI-9 before proceeding. Specifically:

Flask 1 contained trypanosomes from a GUP 2962 parasitaemia taken from a mouse three days post-infection and was composed of over 90% forms which were slender in morphology and showed no mitochondrial immunofluorescence when assayed for DHLADH. This was included as a control slender population.

Flask 2 contained trypanosomes from a GUP 2962 parasitaemia taken from a mouse five days post-infection and was composed of over 95% intermediate and stumpy morphologies which showed mitochondrial immunofluorescence when assayed for DHLADH. This was included as a control stumpy population

Flask 3 was taken from a mouse inoculated with trypanosomes that had been in continuous culture for over four months. The trypanosomes were taken five days post-infection when the parasitaemia appeared to be composed of over 95% intermediate and stumpy morphologies which showed mitochondrial immunofluorescence when assayed for DHLADH.

The remaining four flasks were harvested from in vitro culture. These harvests, corresponding to the points marked on the growth curve (Fig. 3.8), were the same populations as were assessed for cytological change (Fig. 3.9) at each growth curve time-point (Section 3.2.5).

Flask 4 was of culture forms concentrated from a cell density of 10^4 trypanosomes/ml.

Flask 5 was of culture forms taken before the peak of the growth curve at approximately 10^6 trypanosomes per ml.

Flask 6 was of culture forms taken at the peak density of approximately $4x10^6$ trypanosomes/ml.

Flask 7 was from cultures that had begun to die but where the viable count remained approximately $5x10^4$ trypanosomes per ml.

The seven flasks were placed at 27^{o}C and cis-aconitate was added to a final concentration of 6mM in order to initiate differentiation to the procyclic form. Culture forms were heterogeneous with respect to VSG type, so loss of VSG was not assayed. Acquisition of procyclic specific markers was therefore used to assess differentiation. Expression of two antigens was assessed by immunofluorescence; the procyclic coat protein PARP and cytoskeleton associated protein CAP 5.5 which is expressed in procyclic but not bloodstream forms (Matthews and Gull, 1994a).

At time-points of 4h and 12h, 1ml of each flask was centrifuged gently and resuspended in 50μl of HMI-9. For each flask, five samples were then prepared by smearing 10μl onto a microscope slide, air drying and fixing in methanol at -20^{o}C. It is known that PARP expression is acquired within 4h by stumpy forms and that CAP5.5 expression is acquired more slowly but within 12h (Matthews and Gull, 1994). Consequently, at 4h slides were assayed solely for the acquisition of PARP, at 12h for the acquisition of PARP and for CAP 5.5.

Table 3.2 gives the results of this study. For each sample 1000 cells were assessed. The results obtained with the slender and stumpy populations of GUP 2962 (flasks 1 and 2) are consistent with those published elsewhere (Matthews and Gull, 1994). Both the control stumpy population (Flask 2) and the stumpy population from the infection initiated with culture forms (Flask 3) underwent highly efficient, rapid and synchronous differentiation to a procyclic population. Both populations showed comparable kinetics with regard to acquisition of the PARP and CAP 5.5. This result confirms that infections of mice, with trypanosomes harvested after 4 months of continuous in vitro culture, remain pleomorphic. Such infections continue to produce forms which are truly stumpy by the criteria of morphology, cell cycle arrest, mitochondrial activation and competence to differentiate rapidly and synchronously to the procyclic form.

The forms derived from culture were unable to differentiate to the procyclic form rapidly, synchronously or with high efficiency. Flasks 4, 5, and 6 were unable

to differentiate even as well as the control flask of slender forms (Flask 1). Flask 7, obtained when cell numbers were falling, showed some increase in differentiation to the procyclic form by the remaining cells. This may indicate that a degree of slender-to-stumpy differentiation was underway along with considerable cell death. Nevertheless, the results indicate that the "stumpy-like" and "intermediate-like" forms seen in culture, close to the peak culture density, were predominantly unable to differentiate to the procyclic form. These forms could not, therefore, be regarded as viable stumpy forms.

The most surprising result was that at low culture density (Flask 4) there was initially no differentiation to the procyclic form at all. In fact 10,000 cells were counted at the first time-point and no single cell was seen to express procyclin after incubation at 27^{o}C at 4h. Even at 24h (Table 3.2) acquisition of PARP expression did not exceed 1%.

Table 3.2

	4h PARP	12h PARP	12h CAP5.5	24h PARP
Flask 1	2.8%	4.8%	3.1%	nd
Flask 2	95.2%	96.8%	95.3%	nd
Flask 3	94.3%	95.5%	94.9%	nd
Flask 4	0%	1.2%	0.4%	0.6%
Flask 5	0.8%	4.1%	3.3%	nd
Flask 6	0.4%	3.7%	3.2%	nd
Flask 7	6.3%	7.2%	6.7%	nd

Table 3.2. Acquisition of procyclic stage specific markers by bloodstream form trypanosomes.
Flask 1 contains predominantly slender form trypanosomes from a pleomorphic infection of GUP 2962 raised in a Balb/c mouse and harvested 96h post-infection.
Flask 2 contains predominantly stumpy form trypanosomes from a pleomorphic infection of GUP 2962 raised in a Balb/c mouse and harvested 120h post-infection.
Flask 3 contains predominantly stumpy form trypanosomes from a pleomorphic infection with trypanosomes taken from continuous in vitro culture and passage after 4 months, raised in a Balb/c mouse and harvested 120h post-infection.
Flasks 4-7 contain cells directly from cell culture after 4 months harvested at different points of the growth curve as indicated in Fig. 3.8.
Cells were transferred to 27^{o}C in the presence of cis-aconitate. The efficiency of differentiation to the procyclic form of each population was determined by immunofluorescence assay for the acquisition of procyclic form specific markers the coat protein PARP and the cytoskeleton associated protein CAP 5.5.

3.3 Discussion

3.3.1 Rodent models of trypanosomiasis.

Balb/c mice have been used for raising trypanosome parasitaemias in other studies (Seed and Sechelski, 1989; Matthews and Gull 1994a, 1997; Matthews et al., 1995) and GUP 2962 is a well documented pleomorphic cell line (Barry et al., 1985; Turner et al., 1995; Matthews and Gull, 1994, 1997; Matthews et al., 1995.) The immune response of Balb/c mice and Sprague-Dawley rats to GUTat 7.2 is unable to limit the parasitaemia before it is limited by differentiation to the stumpy form and, in this way, the model is akin to immunosuppressed murine models (Balber, 1972; Luckins, 1972; Seed and Sechelski, 1988). After the fourth day of infection, however, the immune response of these animals begins to take effect (Fig. 3.1A, B).

The result of the slow onset of the immune response in these animals is that the primary parasitaemic peaks are high, over 10^8 trypanosomes per ml for Balb/c and 10^9 for Sprague-Dawley, and that these parasitaemias are maintained while the population becomes up to 90% stumpy by morphology. Although this situation is not analogous to the course of disease in man or bovine host, following the bite of a tsetse fly, it is ideal for studying the mechanisms and temporal pattern of the slender-to-stumpy differentiation and provides relatively homogenous populations of cell types for molecular analysis.

The size and history of the inoculum are critical parameters. If the inoculum size is small the founder population of trypanosomes that initiate the infection will also be small. A small founder population can be affected by variations, e.g. in the exact site of inoculation and random variations between and within hosts, to a much greater extent than larger founder populations. Consequently a large inoculum size is desirable for reproducibility. Large numbers however lead to population effects both within the inoculum and at the site of inoculation. This could mean that the slender-to-stumpy differentiation is occurring even before the inoculum reaches the bloodstream.

This potential for “early” differentiation is also the reason that inoculum history is important. If trypanosomes are drawn close to the peak of an infection, they may appear slender but may have already made an irrevocable decision to become stumpy. This would lead to stumpy forms being seen in the bloodstream early in an infection even at very low density, especially if the original inoculum is a large one. Indeed this may be the explanation for the stumpy forms observed at very low density both in these studies (Section 3.2.1) and elsewhere (Turner et al., 1995).

In the system used here 10^6 trypanosomes from a frozen stock were inoculated into a Balb/c mouse, since this gave a highly reproducible infection. The frozen stock was prepared from an infection which had been harvested during logarithmic growth in order to minimize population effects. Nevertheless, there is a strong argument for the use of smaller inoculums drawn earlier during infections.

3.3.2 Incidence and implications of different bloodstream trypanosome forms

Commonly observed cell types

In the bloodstream of a mammalian host, African trypanosomes must undergo two distinct processes both of which require profound structural rearrangements: division and the slender-to-stumpy differentiation. These processes may not necessarily be mutually exclusive, since differentiation may have commenced prior to exit from the cell cycle. Arising out of these processes are the commonly observed forms shown in Figure 3.3A. The diaphorase negative 1k1n, 2k1n and 2k2n were the forms seen to be associated with the normal proliferative cell cycle of the slender form (Fig. 3.6). DAPI staining of diaphorase positive cells normally reveals a 1k1n configuration which encompasses both intermediate and stumpy morphologies.

Uncommon cell types

The four commonly observed cell types encompass the expected bloodstream forms of the African trypanosome. However, up to 4% of the population may be composed of other morphologies in rodent infections, many of which are clearly not viable. It is unlikely that these represent cryptic life cycle stages or sexual forms as has previously been proposed (reviewed in Omerod, 1979). Rather, the existence of these forms calls attention to the division of the bloodstream form being error prone in comparison with, for instance, tissue division in metazoan cells. That this is the case is supported by their increased incidence when introduced to non-mammalian hosts (Wendelstadt and Felmar, 1910; Lun and Vickerman, 1991) or before adapting to axenic culture (Vassella and Boshart, 1996).

It is possible that the high rate of genetic recombination required for antigenic variation (see Chapter 1 and Turner, 1997), contributes to a high error rate during cell division by adversely affecting the expression of important division associated genes. Much of the reason for a high error rate can be ascribed, however,

to the absence of cell cycle checkpoints (Robinson et al., 1995; Ogbadoyi, 1997) which have been shown to be present in cells evolutionarily closer to the metazoan lineage (reviewed by Woolard and Nurse, 1995). The existence of naturally occurring zoids exemplifies this lack of cell cycle checkpoints (Fig. 3.3A7, 15). One reason for the lack of checkpoints may be the absence of dual function MTOC able to control trypanosome mitosis and the formation of a new trypanosome cytoskeleton (discussed further in Section 3.3.3).

Zoids and one kinetoplast/two nuclei forms

Although the presence of 1k2n (Fig. 3.3A6, 14) forms raised the question of whether nuclear replication could precede prior to kinetoplast replication. The rarity of the 1k2n cell type, and the absence of evidence of duplication of any other single copy structures in this cell type indicated that this was unlikely to be the case. Rather, the incidence of trypanosome cytoplasts - 1k0n forms or zoids - may explain the incidence of the 1k2n forms.

In procyclic forms, zoids have been shown to be formed when correct segregation of the trypanosome nucleus is prevented by drug treatment (Robinson et al., 1995). Trypanosomes apparently lack the machinery to check for correct nuclear segregation prior to cytokinesis and so occasionally produce cells which have twice the diploid nuclear DNA content and presumed sibling forms, zoids, containing only a kinetoplast. The appearance of zoids “joined” to binucleate cells during in vitro culture, apparently documenting zoid biogenesis, supports this idea (see figures in Vassella and Boshart, 1996).

2k1n forms may also be formed in another way. Trypanosomes failing to replicate or segregate their basal bodies are unable to undergo cytokinesis. Since other checkpoints seem also to be missing, such cells apparently continue to replicate their nuclear DNA and may even go on to segregate their nuclei (Ogbadoyi, 1997). It is also possible that 2k1n forms arise by fragmentation of the nucleus. Although this is not ruled out, I do not favour this idea since the nuclei appear to be of identical size and staining to those in corresponding, commonly observed cell types.

Dyskinetoplastid forms

There is an incidence of naturally occurring dyskinetoplasty in bloodstream trypanosome populations (Fig. 3.3A4, 5, 12, 13). Viable dyskinetoplastid lines can be generated by drug treatment (Stuart, 1970). The naturally occurring dyskinetoplastid forms may also form a proliferative subpopulation. This is consistent with the presence of 0k2n forms (Fig. 3.3A5) in bloodstream trypanosome populations. Dyskinetoplastid forms possess a mitochondrion (Stuart, 1971; see also Fig. 3.3A12) but may lack the capacity to synthesize the kinetoplastid encoded metabolic enzymes associated with oxidative phosphorylation. This is apparently acceptable in bloodstream forms where oxidative phosphorylation is suppressed. Dyskinetoplastid forms are not, however, viable upon differentiation to the procyclic form.

Dyskinetoplastids are likely to arise when kinetoplast replication or segregation is compromised. Under these conditions the existing kinetoplast may remain attached to the basal body of the old flagellum. The basal body of the new flagellum would then segregate as usual, but without a kinetoplast, permitting the cell to complete division. It is interesting that the mitochondrion is maintained in dyskinetoplastid trypanosomes, since it implies that mitochondrial segregation occurs as normal during division even in the absence of a kinetoplast; the

mitochondrion of the dyskinetoplastid form segregating with the basal body and flagellum of the daughter cell rather than with the kinetoplast. This observation is suggestive of “hard-wiring” between the mitochondrion, flagellum and cytoskeleton even in the absence of a kinetoplast.

Dyskinetoplastid stumpy forms occur both in drug-induced lines and in naturally occurring populations. It has been shown that most dyskinetoplastids do contain at least some kDNA (Stuart, 1971) although it is not clear whether any kDNA is transcribed in such forms. An akinetoplastid line has been produced that is apparently devoid of all kinetoplast DNA (Stuart and Gelvin, 1980) but which also possesses a mitochondrion. This is not surprising since the vast majority of mitochondrial proteins are nuclear encoded. This presumably includes any structural proteins of the mitochondrion, since all of the kinetoplast encoded proteins appear to be related to oxidative phosphorylation. It has recently been shown, by the uptake of rhodamine dyes like mitotracker, that the mitochondria of dyskinetoplastid bloodstream forms have a reduced electron potential (Dr. E. Vassella and Dr. M. Boshart, unpublished observation), perhaps because of inactivation of the mitochondrial ATPase (Williams et al., 1991; Williams, 1994 for ATPase details). This reduced electron potential may have implications for mitochondrial import.

It is shown here that naturally occurring dyskinetoplastid stumpy forms show staining with the diaphorase assay (Fig. 3.3A12), whereas, Vickerman has observed previously that in his hands this was not the case (Vickerman, 1965). Vickerman’s observation is surprising, however, since the enzyme thought to mediate diaphorase activity, dihydrolipoamide dehydrogenase (DHLADH), is nuclear encoded (Else et al., 1993). It may be that DHLADH import is partially compromised in dyskinetoplastids due to the reduced electron potential and that differences in the sensitivity to the original assay, which has been modified here to reduce toxicity, explain the discrepancy between Vickerman’s initial observation and that presented here.

3.3.3 Slender form division

Microtubule organizing centres (MTOCs) have a pivotal role in the control of metazoan cell division. In metazoans, MTOCs (such as centrosomes) have a dual role and organize both the microtubule component of the cytoplasmic cytoskeleton and the nucleation of spindle microtubules. Generally, metazoan MTOCs replicate early in the cell cycle and are then segregated prior to mitosis. Mitosis is then mediated by spindle microtubles nucleated at the MTOC. The dual role of the metazoan MTOC may facilitate the establishment of some cell cycle checkpoints. These checkpoints can ensure that only after correct nuclear segregation is achieved can a plane of cleavage be defined, and an actin based annulus be derived, enabling cytokinesis to proceed.

Division in *T.brucei* is very different. Trypanosomes lack centrosomes, and the MTOCs of the mitotic and subpellicular arrays are ill defined. The basal body and probasal body, however, are present as a well defined MTOC and subtend an axoneme with the classical 9+2 microtubule configuration. Basal body replication is analogous to centriolar replication and basal body segregation occurs in a microtubule dependent manner.

There is no obvious linkage between the basal body and the nuclear genome, although ‘hardwiring’ to the kinetoplast and its associated region of the mitochondrion has been established (Robinson and Gull, 1991; Robinson et al., 1995). Further, hardwiring of the basal body to four biochemically and structurally distinct microtubules of the subpellicular array is also well established (Anderson and Ellis, 1965). The interaction of the basal body with this unique quartet of microtubules may be indicative of a direct interaction in the organization of the subpellicular array and a role in its segregation, although the mechanisms involved have not yet been documented. There is evidence of the direct interaction of basal bodies, flagellum, mitochondrion and the subpellicular array, and of the independence of these structures from direct nuclear interactions. This evidence

comes from the formation of zoids that contain all of these structures except for a nucleus. It may be speculated that trypanosome division is error prone (Table 3.1) relative to the division of most metazoan cells, in part, because of the trypanosome's lack of interdependence between the microtubule organization of its subpellicular cytoskeleton and those microtubules involved in mitosis.

Although division and the cell cycle of the *T.brucei* procyclic form is well characterized (Sherwin et al., 1989a; Woodward and Gull, 1990), this is not the case for the bloodstream forms. In the preliminary characterization of bloodstream cell types (Fig. 3.3), bloodstream form division appeared to be different to procyclic form division. In particular, the spatial relationships of organelles were apparently different:

1) Bloodstream forms did not show a 2k2n cell type where the posterior kinetoplast was positioned posterior to the anterior nucleus, an arrangement seen as part of the procyclic form cell cycle (Sherwin and Gull, 1989a).

2) The tubular slender form mitochondrion (Fig. 3.7A1) is narrow and linear and has a discrete cellular location, running the length of the non-undulating side of the cell. This is in contrast to the procyclic mitochondrion which forms a cage-like, cristate network, apparently throughout the procyclic cell, confined within the subpellicular microtubule array (Vickerman, 1965, 1985; Brown et al., 1972).

In light of the likely additional complexity of mitochondrial segregation alone, it is not surprising that the bloodstream form has a cell cycle which is apparently shorter than the procyclic cell cycle time of 8.5h in vitro (Sherwin and Gull, 1989a). Here the mean doubling time of bloodstream forms in vitro was less than 6 hours during logarithmic growth. This implies that the mean cell cycle time of bloodstream forms in culture must also be less than 6 hours.

The project sought to relate morphogenesis and mitochondrial biogenesis to cell cycle arrest at the level of the single cell, during the slender-to-stumpy differentiation. It was necessary, therefore, to possess markers of cell cycle position

which were shown to be applicable to the bloodstream form. The approach taken to derive such markers was to triangulate division with studies of :

1. Maturation and segregation and duplication of basal bodies.
2. Outgrowth of the paraflagellar rod.
3. Segregation of the mitochondrion.

1. Maturation, segregation and duplication of basal bodies

The longitudinal polarity of the trypanosome is defined by the trypanosome's direction of movement. The anterior end is the end towards which the trypanosome swims and is marked by the narrow end of the cell body and the free flagellum. The posterior, defined relative to the anterior end, is the blunt end containing the kinetoplast, basal body complex. From published scanning electron micrographs (Carr et al., 1971; Gorenflot et al., 1980; Ito et al., 1981; Abolorin et al., 1983) the bloodstream form trypanosome, like the procyclic form (established in Sherwin and Gull, 1989a), seems to have a uniform, left-handed helical twist. The flagellar pocket, a single copy structure present on the trypanosome surface, was used in conjunction with the uniform helical twist of the trypanosome to establish up/down and left/right directions to the trypanosome (Fig. 3.4A). The direction of the flagellum as it exits the flagellar pocket provided a useful marker of orientation.

By observing the basal bodies and probasal bodies of whole cell cytoskeleton mounts, three stages of the cell cycle were apparent (Figure 3.4B).

1) Most cytoskeletons had only one basal body and one flagellum. Looking down onto the flagellar pocket of these cytoskeletons, the basal body was positioned underneath the emergent flagellum so that it was not visible.

2) Some cytoskeletons had only a very short new flagellum and an old flagellum, neither flagellum having yet developed probasal bodies. In these cytoskeletons, the basal bodies of both flagella lay in approximately the same lateral plane.

3) In the remaining cytoskeletons the basal body of the new flagellum was positioned posterior and apparently to the left of the basal body of the old flagellum. The probasal bodies which were apparent in these forms seemed to be formed beneath the flagella (when looking down onto the appropriate flagellar pocket).

Cleavage between the two flagella results in the regeneration of two cells with the stage 1 configuration, the cell inheriting the new flagellum partitioning to the left and posterior of the cell inheriting the old flagellum.

These conclusions are consistent with published electron micrographs of the procyclic form cell cycle (Sherwin and Gull, 1989a) and with more recent data on basal body positioning in the procyclic form (Miss A. Ploubidou, unpublished data).

2. Outgrowth of the paraflagellar rod

In procyclic forms kinetoplasts segregate prior to mitosis. This has been demonstrated using both BrdU analysis (Woodward and Gull, 1990) and outgrowth of the PFR (Robinson et al., 1995) as temporal markers. It was felt that it would be difficult to use BrdU for studies of bloodstream forms in vivo since it would involve dosage of the rodent host. The length of the PFR is an accessible marker for immunofluorescence microscopy due to the availability of monoclonal antibodies for some of its constituent proteins. PFR is acquired by the new flagellum fairly early in the cell cycle, prior to kinetoplast segregation, and continues until cytokinesis. Analysis of the lengths of the new PFR in 100 dividing-forms, which were defined by the possession of two flagella, indicated that outgrowth of the PFR occurred at an approximately linear rate until cytokinesis (Fig. 3.5).

In the same 100 dividing-forms, the length of the PFR was related to the number of nuclei and kinetoplasts in a cell, and to the distance between the kinetoplasts (Fig. 3.6). As a result, the relative order of five phases in the cell cycle were defined. By DAPI fluorescence alone, four distinct stages could be discriminated. This allowed DAPI fluorescence to be used as a marker of the

bloodstream form cell cycle both for mitochondrial segregation (Fig. 3.7) and in later studies (Chapter 5). Although segregation of the kinetoplast and the nucleus followed the same basic order as in the procyclic form, slender form division showed differences to procyclic form division (procyclic form division described in Sherwin and Gull, 1989a; Woodward and Gull, 1990 and Robinson et al., 1995).

During division, in the procyclic form, longitudinal growth of the trypanosome is predominantly at the posterior end (Sherwin et al., 1987). The new basal body segregates from the old basal body as new microtubules are inserted between them. In this way the distance between the posterior tip of the procyclic cell and the new basal body is maintained (Sherwin et al., 1987; Sherwin and Gull, 1989b; Robinson et al., 1995). When the nucleus undergoes mitosis this takes place in a longitudinal plane. The anterior lobe which becomes the anterior daughter nucleus, segregates into the gap left between the basal bodies. The posterior daughter nucleus maintains an approximately constant distance from the posterior end of the cell, throughout. In this way, the approximate distances of the new basal body from the posterior nucleus and of the old basal body from the anterior nucleus, are maintained throughout the cell cycle (Robinson et al., 1995). Finally, in order for cytokinesis to occur, a tangential plane of cleavage is established.

In the bloodstream form, longitudinal growth seems also to occur primarily at the posterior end (Matthews et al., 1995) and lengthening is considerable throughout the cell cycle. The nucleus never comes to occupy the region between the segregated basal bodies. There are several possibilities why this may be the case: there may be less longitudinal displacement during basal body segregation or during nuclear segregation by mitosis. It may be that the distance by which the basal bodies are segregated is the same in procyclic forms as in slender forms but that, since the slender forms are longer, the mitotic spindle never crosses the anterior basal body. Alternatively, the distance between daughter nuclei and basal bodies may not be maintained in the same way in bloodstream forms as in procyclic forms. If so, this

would be an interesting result since it could indicate that a level of cross-talk between the basal bodies and mitotic machinery, present in the procyclic forms, was absent in the bloodstream form. The result of the slender form's segregation of organelles is that the dividing-form lays down a plane of cleavage which, although still tangential to some extent, separates the daughter cells in an equatorial configuration along a more longitudinal axis than is the case during cytokinesis of the procyclic form.

Another apparent difference between bloodstream and procyclic forms was in flagellar length outwith of division. Unlike the procyclic form, the length of the flagellum in slender forms is highly heterogeneous (see Chapter 4). It seems that growth of the slender form flagellum continues outside of division and it is possible that this may even continue for several cell cycles. Heterogeneity in slender form flagellar length corresponds with heterogeneity in the length of the slender form shown in other biometric studies (Fairburn and Culwick 1946, Hoare 1956). In a previous study which used PFR length as a marker for cell division in procyclic forms, the ratio of new PFR length to old PFR length was used (Sherwin et al., 1989a). However, in slender forms no relationship between the lengths of the new and old PFRs could be established (Fig. 3.5), presumably because of the heterogeneity in the old PFR lengths.

3. Segregation of the mitochondrion

Analysis of the segregation of the mitochondrion was enabled by the use of an immunofluorescence marker of the slender form mitochondrion: the trypanosome alternative oxidase antibody (further characterized in Chapter 4). The slender form mitochondrion normally has a linear structure. However, the network of mitochondrial processes revealed during the division of this organelle is reminiscent of the structure of the procyclic form mitochondrion (Chapter 4). This may imply a conservation of segregational mechanisms between the two life cycle stages.

Mitochondrial branching does not become apparent until after the kinetoplasts are clearly segregated. Then, in a period close to mitosis, multiple processes arise, appearing initially as two loops. A second, somewhat shorter, parallel mitochondrion is then formed. This seems to occur by the fusion of these loops with each other and with the region of the posterior kinetoplast, and by concomitant fission of this fused mitochondrion from the original, but maintained, linear mitochondrion (Fig. 3.7).

While many studies have focused on kinetoplast division, the replication and segregation of the *T. brucei* mitochondrion itself has been largely ignored since its description at the level of the electron microscope by Vickerman (1966). Recently, however, segregation of the *Leishmania sp.* mitochondrion has been investigated (Simpson and Kretzner, 1997) using rhodamine 123. In *Leishmania sp.* segregation appears to occur by the formation of a circularized mitochondrion which is then cleaved in two. From the studies presented, here segregation of the *T.brucei* mitochondrion appears to be more complex.

Segregation of the mitochondrion is likely to be microtubule mediated. In both plants (Nangaku et al., 1994) and animals (Pereira et al., 1997) kinesin like proteins, which bind to mitochondria, have been identified as plus end directed motors. Such motors would be able to account for the longitudinal extension of the mitochondrion, but not the transverse movement involved in forming the cage-like mitochondrial networks seen in *T.brucei,* which must necessarily involve crossing the parallel microtubules. Lateral mitochondrial expansion may also be involved in the elaboration of the mitochondrion seen during differentiation to the procyclic form. The mechanisms of mitochondrial division are of considerable interest, given the unique nature of the large single kinetoplastid mitochondrion, since they are likely to be peculiar to the kinetoplastidae and may serve as eventual targets for chemotherapy. In further studies of mitochondrial replication and biogenesis, it will be desirable to identify the adaptive motor proteins involved.

The slender form mitochondrion occupies a highly defined position running the entire length of the trypanosome along the non-undulating side of the cell (the undulating side being defined by the trypanosome flagellum).Together with the flagellum, the position of the mitochondrion can be used to conveniently define a vertical polarity to the cell. By assigning the trypanosome's undulating side as upper and the side marked by the mitochondrion as lower, it is possible also to assign a left and right polarity to bloodstream forms. This will facilitate structural studies in the establishment of "handedness" rules since it allows data from immunofluorescence and confocal microscopy studies to be evaluated in parallel with data from electron microscopy.

Summary of slender form division

These studies represent a primary characterization of slender form division. They were successful in establishing a relative order of events. The first detectable event was maturation and elongation of the probasal body, from which outgrowth of the new flagellum occurs at an approximately constant rate, at least until cytokinesis. Soon after elongation of the new flagellum begins, during the period when the basal-bodies are segregated, two new probasal bodies can be visualized by electron microscopy. Since trypanosome basal bodies and kinetoplasts are normally linked (Robinson and Gull, 1991), this accords with segregation of the kinetoplast as seen by DAPI fluorescence. This order of events is the same as that observed for the procyclic form (Sherwin and Gull, 1989a). Mitochondrial branching becomes apparent, by immunofluorescence for TAO, after kinetoplast segregation but before mitosis is complete. Following nuclear segregation, the branched network undergoes fission into two linear mitochondria. This process is apparently completed only just prior to cytokinesis.

3.3.4 In vitro culture of bloodstream form trypanosomes

The successful development of an in vitro culture system for bloodstream form trypanosomes was important primarily to facilitate future study of differentially expressed gene products during the slender-to-stumpy differentiation. The system developed could not be used for direct studies on the mechanism of this differentiation, since differentiation at high parasite density occurred only at a low level and only in cultures which contained predominantly dead and dying cells. The growth curve obtained from in vitro culture differed from the parasitaemia of immunosupressed animals in two important respects; first, growth in vitro only reached a parasite density of $2x10^6$, two to three logs lower than that of rodent models; second, at the peak of growth in vitro no plateau of parasitaemia was observed, rather, rapid cell death ensued.

After prolonged passaging in vitro, parasites retained pleomorphism when inoculated into rodents. This system is different to another system developed concurrently and recently published (Vassella et al., 1996), in so far that it succeeds to culture pleomorphic forms in liquid media (in the absence of agarose or polysaccharide matrix, described as essential in that paper). This is important since it permits large numbers of trypanosomes to be cultured, particularly if used in conjunction with a recently reported protocol using a 1.5 litre bioreactor (Duszenko, 1997). Vassella and colleagues perform their pleomorphic trypanosome culture on low melting point agarose containing plates, similar to the method described by Carruthers and Cross (1992). A plating method was also employed during the project (in collaboration with Miss C. Hertz, data not presented), and this plating method is useful for the cloning of trypanosome lines once selection in liquid medium is complete. GUP 2962 failed to differentiate to a stumpy population, however, even under the conditions used by Dr. Vassella for his *T.brucei* line AnTat 1.1. The differences observed appear to be strain dependent and several other trypanosome

lines tested by Dr. Vassella did not differentiate with the efficiency of AnTat 1.1. (Vassella et al., 1996).

Despite the failure of cultured bloodstream forms to differentiate to stumpy forms, some interesting observations were made during the course of cell culture and subsequent analysis for maintenance of pleomorphism. In our GUP 2962 trypanosomes, diaphorase activity is detectable in all stumpy forms and at least a large proportion of intermediate forms. After six months of passage in vitro, however, although the parasitaemia and morphological transition followed the same kinetics as the usual GUP 2962 infection, diaphorase activity did not become apparent until day 5 post-infection and was restricted solely to stumpy forms. Moreover, at early day five time-points only some stumpy forms were diaphorase positive, although later, all stumpy cells clearly stained for diaphorase. This peculiarity did not, however, affect the ability of the stumpy forms to transform rapidly and synchronously to the procyclic form under appropriate conditions.

This observation may represent a serendipitous uncoupling of the processes of morphogenesis and mitochondrial biogenesis during the slender-to-stumpy differentiation. It may indicate a branching in the temporal mechanism by which each occurs, following initiation of differentiation. The extent of this divergence certainly warrants further investigation.

In vitro culture of pleomorphic bloodstream forms apparently differs from cultures in living hosts. When cultures were harvested during logarithmic growth stumpy forms were never observed. Indeed at low parasite density, cultured bloodstream forms ubiquitously fail to show staining using the diaphorase assay and do not express DHLADH when assayed by immunofluorescence. It has been proposed that stumpy form production may be an intrinsic function of pleomorphic cell growth, regardless of parasite density (Turner et al., 1995). This does not appear to be the case in vitro.

Another observation was that culture forms derived from low density cell culture were almost completely unable to differentiate to the procyclic form, even with delayed kinetics (Table 3.2), and were certainly much less efficient than a slender population, which was over 90% slender by morphology and contained less than 1% stumpy forms. Indeed, published data for monomorphic lines grown in rodents, and transformed under similar conditions (Matthews and Gull, 1994a), show a greater efficiency of differentiation to the procyclic form than logarithmically growing culture forms.

It has previously been proposed that stumpy forms occupy a window of the cell cycle allowing rapid and synchronous differentiation to the procyclic form. Under differentiation conditions, slender and monomorphic forms occupying this window would also be able to differentiate to the procyclic form at once, whilst those approaching this cell cycle window would be able to progress in the cell cycle until reaching it and then differentiate (Matthews and Gull, 1994a,b). This would explain the delayed kinetics of the slender and monomorphic differentiation to the procyclic form.

The data presented here may call this hypothesis into question. Some proliferative slender populations, raised in vitro, clearly contain few or any cells which can start differentiating to the procyclic form immediately and, at best, a very few cells with delayed kinetics. It may be that this, and indeed the previously mentioned lack of diaphorase positive forms and stumpy forms, represents a difference between the in vitro and in vivo environments in which they were grown; a culture specific effect (such as a nutritional deficiency) which actively inhibits differentiation to stumpy and to procyclic forms, by cultured bloodstream forms, from occurring. However, it may also be that different pleomorphic slender populations have different abilities to differentiate (perhaps by way of entering G_0 transiently as previously proposed) to the procyclic form. This difference can be described as a transitional event and may define two slender populations, those

competent to differentiate to the procyclic form, albeit with reduced kinetics compared to the stumpy form, and those which are not competent to do so.

The idea of two slender populations is not new. Omerod (1979) provided structural definition for a subpopulation of slender forms which he called long narrow forms. He defined these as mostly present at low parasitaemia, being small in size and moving with a spirochete-like motion. There is certainly a substantial size and structural heterogeneity in slender population size (Chapter 4). If there is a discrete population of slender forms that occur primarily at low density, it seems reasonable that a much more homogeneous population can be derived by in vitro culture than from rodent infections. Alternatively, morphogenesis may represent a relatively late event in the slender-to-stumpy differentiation, with competence to differentiate to the procyclic form occurring earlier. If competence to differentiate to the procyclic form followed commitment to the production of a stumpy form in the cell cycle prior to production of a stumpy form, then this could explain the asynchronous kinetics of slender-to-procyclic differentiation and would be consistent with the divide then decide model of Matthews and Gull (1994).

Chapter 4

Structural and metabolic markers of the slender-to-stumpy differentiation

4.1. Introduction

The definition of key events during the long slender to short stumpy differentiation can only be established using well defined markers. These markers are most readily derived from cellular and metabolic processes that differ between the slender and stumpy life cycle stages.

4.1.1. Morphology

In investigating the in vivo system for bloodstream form trypanosome differentiation established in the previous Chapter the need for markers was compounded by three factors: the asynchrony of division of bloodstream form populations; the existence of a range of transitional (intermediate) forms which are morphologically heterogeneous; and by the mixture of cell types (slender forms, intermediate and stumpy forms which coexist in all stages of the parasitaemia). Although the incidence of intermediate forms complicates morphological evaluation of individual cells and of populations (Wijers, 1959), the use of morphological evaluation as the method of choice for evaluating the long slender to short stumpy differentiation has remained widespread (Balber, 1972; McLintok et al., 1993; Seed and Sechelski, 1989; Bass and Wang, 1991; Hamm et al., 1990; Vassella and Boshart, 1996).

Subjective differences between laboratories in assessing each morphological cell type, from what is essentially a continuum (Wijers, 1959; Hoare, 1966), may lead to apparent conflict in data. Consequently, many laboratories evaluate morphology by the procedures espoused by either Hoare (1966) or by Wijers (1959) in an attempt to standardize results. The criteria used by both Hoare and Wijers are grounded in the early drawings and biometric studies of Bruce and colleagues (Bruce et al., 1910, 1912, 1913ab, 1914a) which established the proximity of the nucleus to the kinetoplast and the length of the free flagellum as parameters which changed during the slender-to-stumpy differentiation. The approach of Wijers was to observe

bloodstream trypanosomes from a gestalt of criteria, essentially by comparison with the drawings of slender, stumpy and intermediate forms by Lady Bruce (Bruce et al., 1912, 1914a). Trypanosomes failing to meet these criteria were placed into additional categories of long intermediate or short intermediate. Half of the long intermediates were designated as long slender forms, half intermediate forms. Half of the short intermediates were designated intermediate forms and half as short stumpy forms.

An approach such as this is inappropriate for asking questions aimed at the level of the single cell, since there is an acceptance of such a large degree of error as to completely confound any such studies; even at a population level the technique severely limits the scope of questions to be asked about each subpopulation. To the criteria established by Bruce, Hoare added trypanosome length based on biometric studies of Fairburn and Culwick (Fairburn and Culwick, 1946, 1949; Hoare, 1956). The conclusions reached by Fairburn and Culwick, of syngamy between bloodstream forms of opposing charge, appear unlikely in retrospect. These studies did, however, serve both to re-establish trypanosome length as a parameter which differed between the slender and the stumpy form, and also to reaffirm the idea of three discernible populations; slender, intermediate and stumpy. In this chapter cell length has been re-evaluated as a marker for the slender-to-stumpy differentiation. To do this, cell length was bisected into two constituents: flagellar length (from basal body to the anterior tip of the flagellum) and the distance from the posterior tip of the trypanosome to the basal body.

For the studies in this chapter that evaluate markers with respect to cell type, bloodstream form trypanosomes were categorized by drawing on methodologies used by both Wijers (1959) and Hoare (1966). It was felt that bloodstream trypanosomes must be either undifferentiated (slender), differentiated (stumpy) or differentiating (intermediate). All normal trypanosomes assessed were assigned directly to one of these groupings; clearly aberrant forms (see Chapter 3) were recorded but not assigned. Wherever possible assays of morphology were conducted from photographs

of bloodstream form trypanosomes. This was so that a double blind assessment could be established, where each cell was categorized independently by two researchers (the other investigator being Dr. K. Matthews). A consensus opinion was reached for controversial cells of which there were very few.

Three parameters were used to define morphological type. First, the shape and position of the nucleus in the trypanosome; especially with regard to the amount of cytoplasm between the nucleus and the lateral borders, and to nuclear repositioning in a more posterior region of the cell (Ashcroft, 1957). Second, the length of the free flagellum (Bruce, 1914a). Third, the kinetoplast-nuclear distance (Bruce, 1914a). For a cell to be adjudged stumpy it had to possess a characteristic round nucleus by DAPI fluorescence and this had to be separated by visible cytoplasm from both the lateral borders of the cell. A stumpy form had also to possess either no free flagellum or only a very short free flagellum. Finally, if the first two criteria left doubt as to whether the cell was stumpy or intermediate, the position of the kinetoplast relative to the nucleus was employed. Where the kinetoplast was clearly close to the nucleus the cell was adjudged stumpy; where the kinetoplast was relatively further away the cell was adjudged intermediate.

Cells were adjudged slender if there was no cytoplasm visible between an ovoid nucleus and the lateral borders of the cell and, where a long free flagellum was visible. Cells were adjudged intermediate if they did not meet the criteria of either the slender or stumpy form. Typically, intermediate forms included forms where the nucleus appeared to be ovoid but some cytoplasm was visible between the nucleus and lateral borders, or where the nucleus was round, with little or no cytoplasm between the nucleus and the lateral borders. The free flagellum of such forms was of generally intermediate in length. These morphological criteria are relatively easy to use and give consistent results. They are not ideal in so far as they are observed parameters, derived from representative cells in differing populations; as such, they are not linked directly to the functional characteristics or biogenesis of the stumpy form life cycle stage.

4.1.2. Markers of cell cycle arrest

Other criteria have also been used for assessing populations in the past: re-infection assays point to the number of cells which are irreversibly cell cycle arrested or committed to cell cycle arrest, and this has more recently been expressed in terms of re-plating efficiency using in vitro systems (Hamm et al., 1990; Vassella and Boshart, 1996). Alternatively, effectiveness in establishing tsetse midgut infections and hence tsetse mediated transmission to new hosts has been used as a criterion to assess the preadaption of a population (Ashcroft, 1957; Wijers and Willet, 1960). This has been emulated in vitro by evaluating the ability of a population to undergo rapid and synchronous differentiation to the procyclic form at 27^{o}C in the presence of cis-aconitate (Czichos et al., 1986; Zeigelbauer et al., 1990; Matthews and Gull., 1994a).

The proportion of the population occupying G_1 of the cell cycle has also been used to evaluate the differentiation of a population, either by FACS analysis (Shapiro et al., 1984; Vassella et al., 1997), or at the level of the single cell (Matthews and

Gull, 1994a, 1997; Vassella et al., 1997). Single cell analysis has consisted of counting the number of nuclei and kinetoplasts in each cell with the aid of fluorescence microscopy for DNA staining dyes such as DAPI. The loss of the ability to undergo S-phase has also been used as a cell cycle arrest marker for trypanosome populations; arrested stumpy form populations failing to incorporate either tritiated thymidine (Pays et al., 1993) or the modified nucleotide BrdU (Vassella et al., 1997).

The stumpy form is supposed to occupy a G_0 state within G_1 and ideally this state would be assayed by a specific molecular marker (such as a molecule with a role in suppressing division). Unfortunately, no such marker has yet been discovered. There are reports of cell cycle associated molecules such as cyclins (Affrachino et al., 1993; Hua et al., 1997) and CDCs (Mottram and Smith, 1995), but to date, these have proven difficult to use in assessments of cell cycle position (M. Sarkar and K. Matthews, unpublished results).

4.1.3. Mitochondrial biogenesis - nuclear encoded markers

The slender form trypanosome is able to meet its energy requirements solely by the glycolysis of blood glucose, yet it retains a mitochondrion which is substantial in size, possesses an electron potential (Divo et al., 1993) and is active with regard to a terminal (or alternative) oxidase activity akin to that found in plants (Chaudhuri and Hill, 1995, 1996). The electron potential of the slender form mitochondrion is believed to be generated by an ATP-dependent efflux of protons which is mediated by the mitochondrial ATPase (Williams et al., 1991; Nolan and Voorheis, 1992; Williams, 1994). Krebs cycle enzyme activity and oxidative phosphorylation are, however, apparently completely lacking from the slender mitochondrion which is narrow, tubular and achristate (Brown et al., 1972).

In contrast, the stumpy form mitochondrion is larger, more complex, and supports some Krebs cycle enzyme activity. Oxaloacetate and α-ketoglutarate can be utilized as metabolic substrates by the stumpy form but not by the slender form

(Vickerman, 1965; Bowman et al., 1972; Flynn and Bowman, 1973; Bienen et al., 1991, 1993). Biologically, the ability to metabolize such substrates is significant as a preadaptation for survival in the tsetse fly midgut. In the tsetse midgut glucose is not freely available and proline (a five carbon amino acid) is the most abundant available metabolite. Proline enters Krebs cycle as α-ketoglutarate via the oxidative deamination of glutamate. The utilization of oxaloacetate (and, by analogy, other Krebs cycle intermediates) by the stumpy form mitochondrion generates both ATP and an increased electron potential in the stumpy form mitochondrion (Bienen et al., 1993). Since cytochromes are absent from the stumpy form mitochondrion, only site one of the electron transport chain can be utilized: NADH-Q dehydrogenase. This is presumed to mediate transfer of protons to the alternative oxidase in the stumpy form. The presence of NADH-Q dehydrogenase has not yet, however, been directly demonstrated in the stumpy form mitochondrial membrane. The mitochondrial ATP synthase then utilizes the membrane potential generated to drive ADP phosphorylation.

Biochemical assays for enzyme activities, particularly mitochondrial ones, have been used as markers for the slender-to-stumpy differentiation. Since they are lacking in the slender form, α-ketoglutarate dehydrogenase, pyruvate dehydrogenase, proline oxidase and a functional NADH dehydrogenase have all formed the basis of functional assays. Bienen and colleagues have suggested that a change in electron potential facilitates uptake of fluors, such as rhodamine 123, to the stumpy mitochondrion and so these fluors could be used as a marker of differentiation (Bienen et al., 1991).

Vickerman has also applied a cytochemical test, the diaphorase assay for NADH dehydrogenase activity, to *T. brucei* (Vickerman, 1965). This diaphorase assay has been used as a benchmark test for the slender-to-stumpy differentiation ever since (Hamm et al., 1990; Giffen and McCann., 1989; Vassella and Boshart, 1996; Vassella et al., 1997). As a marker of the acquisition of mitochondrial activity during differentiation to the stumpy form, the diaphorase assay is limited only in so far as it measures activity, not protein levels, and cannot be definitely ascribed to the activity of any single enzyme. It has been suggested, though, that lipoamide dehydrogenase is the primary mediator of its activity (Massey, 1960). Unfortunately, the diaphorase assay has not proven readily compatible with immunofluorescence, making its activity difficult to correlate with the acquisition or loss of other markers.

4.1.4. Mitochondrial Biogenesis - kinetoplast encoded markers

In producing a truly akinetoplastid (completely lacking kinetoplast DNA) line, Stuart observed that the line continued to produce morphologically stumpy forms (Stuart and Gelvin 1980; Stuart 1980). This indicated that the slender-to-stumpy differentiation was not dependent on the upregulation of kinetoplast gene expression. Nevertheless, kinetoplast gene expression does vary significantly between the slender and stumpy form, in a transcript specific manner, with several transcripts showing an increased abundance in the stumpy form and a few showing a decrease (Feagin and Stuart, 1985; Feagin et al., 1986). The differences in transcript abundance have been correlated with RNA stability, differential polyadenylation, differences in transcription and differences in editing (reviewed in Priest and Hajduk, 1994). Unfortunately to date these differences in RNA abundance have not produced antibodies to proteins which are also upregulated, largely because many of the stumpy RNAs showing increased abundance are not fully edited.

There is evidence of only one kinetoplast encoded protein in bloodstream forms, one subunit of the f0/f1 ATPase -ATPase 6 (formerly MURF4)- is encoded by

the kinetoplast (Bhat et al., 1990) although it has not been directly shown to be translated and incorporated in the mitochondrion. The transcript is fully edited in slender, stumpy and procyclic forms and its RNA levels appear to be constant. The f0/f1 ATPase itself however does show some degree of regulation- a two-fold increase in activity and protein levels in the stumpy form and three-fold increase in the procyclic form relative to the slender form (Williams et al., 1991). This degree of regulation is not however likely to be sufficient for use as a marker at an immunofluorescence level.

4.1.5. Endocytosis

Stumpy forms show increased activity at an enlarged flagellar pocket where the majority of endocytosis occurs (Langreth and Balber, 1975). This corresponds to an increase in granularity in these cells observed by Ormerod and used by him to assay the slender-to-stumpy differentiation (Ormerod, 1979). Associated with these events is one of the two previously described antigens which has been found to be upregulated in the stumpy form; CB1 is a glycosylated epitope on a lysosomal protein GP57/42. The protein itself is thought to be expressed equally between the slender and stumpy form, although the CB1 epitope is a result of a post-translational modification which has been proposed to be stumpy specific (Brickman and Balber, 1993, 1994). While the pathway associated with increased endocytosis appears to be a promising source for markers, caution must be exercised. It may be that endocytosis increases, at least in part, as a response to the clearance of attached antibody, which has been endocytosed at the flagellar pocket; rather than directly, as part of the developmental program to produce a stumpy form. The use of immunodeficient hosts and in vitro culture systems may be useful in the assessment of the validity of such markers.

4.1.6. Protein kinase activities

Studies on differential phosphorylation between the slender and stumpy form succeeded in demonstrating two phosphoproteins, P37 and P42, which had enhanced phosphorylation in the stumpy form and which were phosphorylated by a serine/threonine kinase (Aboagye-Kwarteng et al., 1991). Subsequently multiple serine/threonine and tyrosine kinase activities were demonstrated to show increased levels in the stumpy form (Parsons et al., 1993). One of these serine/threonine kinases has since been characterized extensively. The protein kinase *nrk* is a serine threonine kinase with homology to the NIMA subfamily of protein kinases, including the multifunctional *nek1*. Regulation of *nrk* is exerted at the level of translation (Gale et al., 1994). The *nrk* protein remains the only protein, showing increased stumpy form expression, for which the level where developmental control is exerted has been determined. The protein *nrk* was not used as a marker during this project since α-nrk antibodies gave a weak generalized immunofluorescence unsuitable as a marker at the single cell level (Dr. M. Parsons, University of Seattle, U.S.A.; personal communication).

4.1.7. Strategies

At a molecular level there remains little definition as to what constitutes a stumpy form and the molecules that mediate cell cycle arrest, morphogenesis and mitochondrial biogenesis remain almost completely undefined. Even the Krebs cycle associated enzymes activated during the course of the differentiation are described in terms of their enzyme activity, rather than the molecules that mediate the activity. To relate the co-ordination of cell cycle arrest with morphogenesis and mitochondrial biogenesis ideally requires markers of each process defined to a molecular level. In initiating work to achieve this long term aim and to begin to define markers of the slender-to-stumpy differentiation three approaches were adopted.

1) A primary screen of existing antibodies raised against mitochondrial antigens and trypanosomal proteins was undertaken by immunofluorescence, to look for detectable increases in expression.

2) A limited number of electron micrographs of whole cell cytoskeletons (kindly provided by Dr. K. Matthews and Dr T. Sherwin) from a pleomorphic population were examined as a basis for establishing morphological parameters which appeared to vary during differentiation.

3) Differential display analysis of three life cycle stages (slender, stumpy and procyclic) was undertaken to derive putative cDNA fragments upregulated in the stumpy form.

4.2. Results

Many approaches, with varying degrees of complexity, have been used for the establishment of stage specific antigens in biological systems. In initiating work on the slender-to-stumpy differentiation three straightforward strategies were adopted.

4.2.1. Antibody screening

In view of the increase in size and activity of the trypanosome mitochondrion during the slender-to-stumpy differentiation antibodies were requested, from laboratories which had published work on mitochondrial proteins, to be used in a primary, immunofluorescence based screen. Polyclonal antibodies were requested in preference to monoclonal antibodies to increase the likelihood of the antibody recognizing conserved epitopes in the evolutionary divergent trypanosome. Where possible, the screen was biased towards molecules which were known to show some degree of evolutionary conservation in structure, and towards species closer to the root of the eukaryotic evolutionary tree, particularly the Kinetoplastida. Other trypanosome antibodies, which were considered potentially stage specific (such as CB1), were also included in the screen.

Screening was performed with individual slides for each antibody tested. Polyclonal antibodies and ascites were tested at concentrations of 1 in 10, 1 in 100 and 1 in 500. Hybridoma supernatants were initially tested neat and diluted subsequently as necessary. Samples were prepared from parasitaemias in either mice or rats depending on the origin of the antibody being tested (for example, a mouse monoclonal antibody would be tested on trypanosomes from a rat parasitaemia to avoid cross reactivity of the secondary antibody with endogenous antibody from the host). Antibodies were initially tested on bloodsmears fixed for one hour in methanol and, on bloodsmears fixed with 4% formaldehyde for 15 minutes and then permeabilized for up to one hour in methanol. Positive and negative controls were

included. The positive control used anti-tubulin (TAT) (Woods et al., 1989a) as the primary antibody. The negative control used PBS only in place of a primary antibody.

Antibodies were selected for further studies by their ability to stain slender and stumpy forms differentially by immunofluorescence. Where a differential staining pattern was observed, but the staining was weak or inconsistent, samples were remade from trypanosomes which were cleaned over a DE-52 column and then adhered to siliconized slides and fixed without being allowed to dry.

The results recorded in Table 4.1 show that the antibodies raised to non-kinetoplastid proteins largely failed to show differential staining between slender and stumpy forms. Indeed few showed increased staining relative to the background observed in "no first antibody controls". Although disappointing, this was not unexpected in view of the huge evolutionary distance between trypanosomes and the organisms (predominantly plants and yeast) from which these antibodies were raised. Mitochondrial hsp70 (mhsp70) and hsp60 were exceptions; they show a high degree of evolutionary conservation and on purified trypanosomes antibody to these proteins was partially effective at delineating the stumpy form mitochondrion. Unfortunately, this staining was weak and had a high background so even these antibodies were not pursued further as potential markers.

Antibodies raised to kinetoplastid proteins appeared to show better specificity for trypanosome homologues. All such antibodies recognized structures in which the target antigen was believed to be localized. Antibodies to α-ketoglutarate dehydrogenase and pyruvate dehydrogenase of *Crithidia fasciculata* components recognized the stumpy form mitochondria in cleaned methanol fixed cells and, it seems likely that the trypanosome homologues of these genes are resident there and differentially expressed. However, the staining was extremely weak and so these proteins were not progressed further as markers. Antibody to leishmanial mhsp70 also recognized the whole stumpy form mitochondrion and the slender form kinetoplast,

but again at low concentrations staining was very weak while at high concentrations a high level of background ruled it out as a marker.

The antibodies which gave a high level immunofluorescence with a low level of background were all raised to trypanosome antigens, either *Trypanosoma cruzi* in the case of mhsp70, hsp60 and dihydrolipoamide dehydrogenase (DHLADH) or *Trypanosoma brucei* in the case of CB1. A number of other antibodies raised to trypanosome structural proteins and kinetoplast associated antigens were also screened. These antibodies gave good immunolocalization for each antigen but failed to differentiate between life cycle forms. Staining by immunofluorescence for the trypanosome alternative oxidase (TAO) clearly delineated the mitochondrion of both the slender and stumpy form and was adopted as a control for further studies. Five antigens (CB1, TAO, hsp60, mhsp70 and DHLADH), were selected from the screen for further characterization.

Antigen	Organism	Antibody	Usual localization	Trypanosome localization	Supplier
NADH-Q DH I	Wheat	polyclonal/rabbit	mitochondrial	background	R.Lever, Cambridge
NADH-Q DH II	Wheat	polyclonal/rabbit	mitochondrial	background	R.Lever, Cambridge
NADH-Q DH III	Wheat	polyclonal/rabbit	mitochondrial	background	R.Lever, Cambridge
NADH-Q DH IV	Wheat	polyclonal/rabbit	mitochondrial	background	R.Lever, Cambridge
NADH-Q DH VI	Wheat	polyclonal/rabbit	mitochondrial	background	R.Lever, Cambridge
F1 ATPase subunit 1	Wheat	polyclonal/rabbit	mitochondrial	nuclear/ procyclic only	R.Lever, Cambridge
F1 ATPase subunit 2	Wheat	polyclonal/rabbit	mitochondrial	background	R.Lever, Cambridge
Pyruvate DH IV	Wheat	polyclonal/rabbit	mitochondrial	background	R.Thompson, Manchester
PCNA	Plant	polyclonal/rabbit	nuclear	background	R.Docherty, Manchester
Cytochrome oxidase II	Budding Yeast	polyclonal/rabbit	mitochondrial	background	A.Schneider, Switzerland
Cytochrome oxidase IV	Budding Yeast	polyclonal/rabbit	mitochondrial	background	A.Schneider, Switzerland
DV2-T	Budding Yeast	polyclonal/rabbit	mitochondrial	background	A.Schneider, Switzerland
SS87T	Budding Yeast	polyclonal/rabbit	mitochondrial	background	A.Schneider, Switzerland
MAS70	Budding Yeast	polyclonal/rabbit	mitochondrial	background	A.Schneider, Switzerland
Cytochrome C I	Budding Yeast	polyclonal/rabbit	mitochondrial	background	A.Schneider, Switzerland
Porin	Budding Yeast	polyclonal/rabbit	mitochondrial	background	A.Schneider, Switzerland
C/C Peroxidase	Budding Yeast	polyclonal/rabbit	mitochondrial	background	A.Schneider, Switzerland
Citrate synthase	Budding Yeast	polyclonal/rabbit	mitochondrial	background	A.Schneider, Switzerland
Hsp60	Budding Yeast	polyclonal/rabbit	mitochondrial	mitochondrial/weak	A.Schneider, Switzerland
Mhsp70	Budding Yeast	polyclonal/rabbit	mitochondrial	mitochondrial/weak	A.Schneider, Switzerland
Pyruvate DH	*Crithidia fasciculata*	polyclonal/rabbit	mitochondrial	mitochondrial/weak	R.Kommunieki, USA
α-ketoglutarate DH	*Crithidia fasciculata*	polyclonal/rabbit	mitochondrial	mitochondrial/weak	R.Kommunieki, USA
mhsp70	*Leishmania major*	polyclonal/rabbit	mitochondrial	mitochondrial/dirty	D.Smith, London
Mtp70	*Trypanosoma cruzi*	polyclonal/mouse	kinetoplast	mitochondrial	D.Engman, USA
Hsp60	*Trypanosoma cruzi*	polyclonal/mouse	mitochondrial	mitochondrial	D.Engman, USA
Dihydrolipoamide DH	*Trypanosoma cruzi*	polyclonal/rabbit	mitochondrial	mitochondrial	L.Krauth-Siegel, Germany
DR3	*Trypanosoma brucei*	polyclonal/rat	kinetoplast	kinetoplast	D.Robinson, Manchester
Trypanothione reductase	*Trypanosoma brucei*	polyclonal/rabbit	cytoplasmic	cytoplasmic	A.Fairlamb, Dundee
Trypanosome alternative oxidase	*Trypanosoma brucei*	monoclonal/mouse/IgM	mitochondrial	mitochondrial	G.Hill, USA
CB1	*Trypanosoma brucei*	monoclonal/mouse/IgM	lysosomal	lysosomal	A.Balber, USA

Table 4.1 Summarizes the results from a primary screen of antibodies, by immunofluorescence assay, for antigens which are potentially regulated during the slender-to-stumpy differentiaion.

Figure 4.1

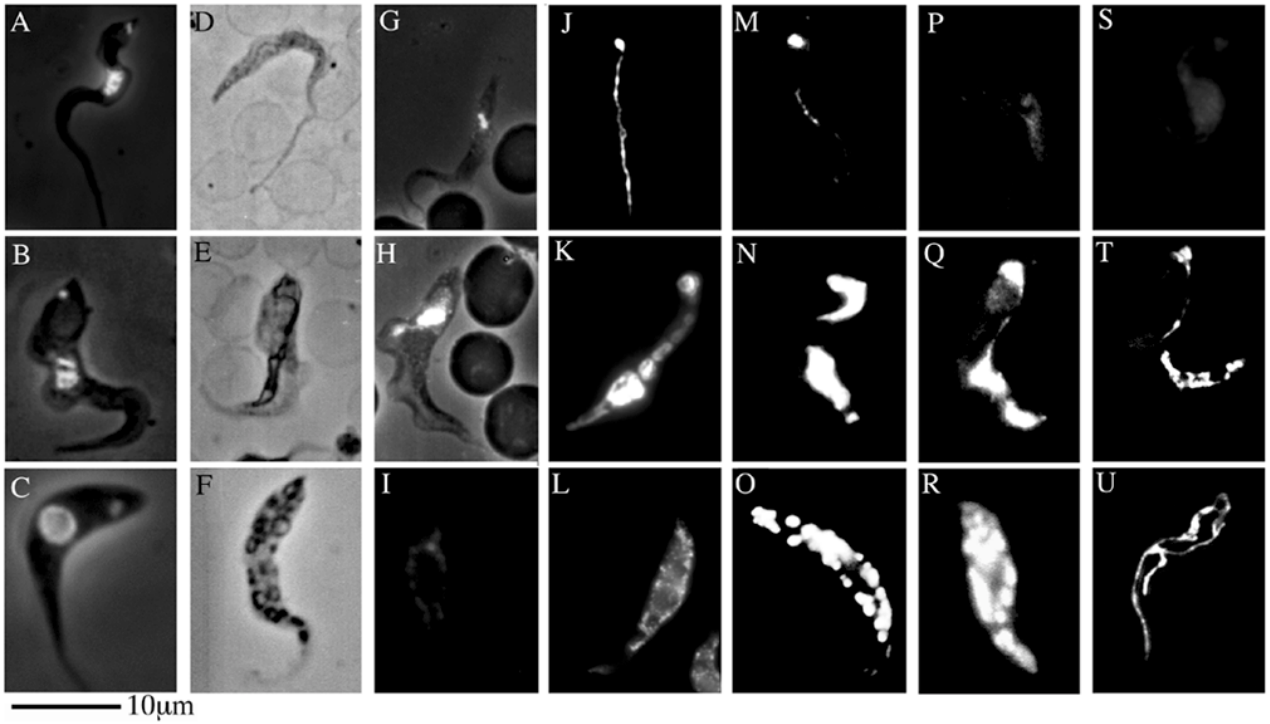


Fig. 4.1 Life cycle stage regulation of established and putative markers for the long slender to short stumpy differentiation. The upper row shows slender form trypanosomes; the middle row shows stumpy form trypanosomes; and the bottom row procyclic trypanosomes. **A, B, C**: Differentiated cell types can be distinguished by morphological criteria such as length to width ratio, cell volume, and length of free flagellum using phase contrast microscopy. This discrimination is further facilitated by fluorescent localization of the nucleus and kinetoplast using DAPI which allows visualization of shape, size and relative positions of these organelles. **D, E, F**: The NAD diaphorase assay is a cytochemical assay used as a benchmark test to discriminate slender from stumpy forms: cell morphology and localization of formazan deposits are visualized by phase contrast microscopy. **G** through to **U**: show the results of immunofluorescence microscopy; **G, H, I** CB1 localizes to the lysosomes and endosomes of some bloodstream forms; **J, K, L** TAO localizes throughout the mitochondrion of all forms tested albeit less strongly in the procyclic form; **M, N, O** mhsp70 stains the mitochondrion of procyclic, stumpy and some slender forms. Staining of other slender forms is restricted to the region of the kinetoplast **P, Q, R** hsp60 specifically stains the mitochondrion of the stumpy and procyclic forms; **S, T, U** DHLADH also stains specifically the mitochondrion of the stumpy and procyclic forms. The DHLADH antibody gave good immunofluorescence with a paraformaldehyde fix preserving mitochondrial integrity, **U,** compared with methanol fixation used for other markers, **L**, **O**, **R**, which disrupted the mitochondrial network giving a distinctive punctate appearance. In **G** and **H**, an immunofluorescence/phase-contrast image is shown to facilitate the localization of staining. **I** shows a fluorescence only image, to highlight the very low level of fluorescence in the procyclic form by immunofluorescence microscopy.

4.2.2. Characterization of putative stage regulated antigens as markers by immunofluorescence assay

A cytological localization was performed, for each putatively stage regulated antigen, in bloodstream and culture form procyclic cell types. Immunofluorescence microscopy was used to determine whether there was a clear difference in localization or expression of any of these putative markers between cell types (Fig. 4.1).

CB1: This antigen is associated with the lysosomal protein gp57/42 of *T. brucei* (Brickman and Balber, 1993, 1994). Since the structure stained was not a tightly defined single organelle, the immunofluorescence localization of CB1 merged with the phase image of the cell, as shown in Figure 4.1G, H. In order to demonstrate the very low level of fluorescence of the procyclic stage the fluorescence-only image is shown (Fig. 4.1I). As can be seen from Figure 4.1G, H, CB1 appears to show increased expression in the stumpy form, although some staining can also be present in slender forms.

Trypanosome alternative oxidase (TAO): This *T. brucei* gene has been cloned and monoclonal antibodies raised to the expressed protein (Chaudhuri and Hill, 1995, 1996). Both slender and stumpy form mitochondria were clearly visualized by the immunolocalization of TAO (Fig. 4.1J, K) which precludes the use of TAO as a stumpy specific marker. The procyclic form mitochondrion showed relatively weak staining with the antibody to TAO compared to that seen for the other mitochondrial markers assessed in the procyclic form (Fig. 4.1L).

Mitochondrial hsp70 (mhsp70): *T. cruzi* and *T. brucei* genes for some mhsp70 proteins have been identified (Engman et al., 1989; Bangs et al., 1993). The polyclonal antibody raised to *T. cruzi* mtp70 was used in these studies. The mhsp70 of *T. brucei* recognized by this antibody, localized to the mitochondrion of intermediate (not shown), stumpy and procyclic trypanosomes (Fig. 4.1N, O). Slender forms were apparently subdivided into two different populations. One population (not shown) showed strong staining of the thread-like mitochondrion with a bright point at the kinetoplast, the other population (as in Fig. 4.1M) showed either staining at the kinetoplast only, or comparatively strong kinetoplast staining, with only very weak staining at the rest of the mitochondrion.

Hsp60: The *T. cruzi* and *T. brucei* genes have been cloned and sequenced (Giambiagi de Marvel et al., 1993; Bringaud et al., 1995) and a polyclonal antibody raised against the *T. cruzi* protein (Sullivan et al., 1994) which, in the absence of an available *T. brucei* antibody, was used in these studies. The hsp60 localization pattern was similar to mhsp70 in the stumpy (Fig. 4.1Q) and procyclic forms (Fig. 4.1R) clearly staining the mitochondrion, but staining of the slender form mitochondria was scarcely detectable (Fig. 4.1P).

Dihydrolipoamide dehydrogenase (DHLADH): The *T. brucei* gene for this nuclear encoded enzyme, which is thought to be responsible for the NAD diaphorase activity, has been isolated and sequenced (Else et al., 1993). The *T. cruzi* protein is also well characterized. A polyclonal antibody raised to this protein (Lohrer and Krauth-Siegel, 1990) was used. A similar staining pattern to the diaphorase assay was obtained when this antibody was used for immunofluorescence, localising to the mitochondrion of the stumpy and procyclic forms (Fig. 4.1T, U), but failing to detect the protein in the slender form (Fig. 4.1S). The use of paraformaldehyde fixation resulted in far superior preservation of mitochondrial structure (Fig. 4.1U). Methanol fixation disrupted the mitochondrial network giving it the distinctive punctate appearance seen with the other mitochondrial markers (Fig. 4.1L, O, R).

4.2.3. Comparison of regulated expression of antigens and cellular morphology

To determine the specificity of these antigens as markers of the slender-to-stumpy differentiation, individual cells were first considered in morphological terms: by size and shape, by nuclear positioning and by the length of free flagellum. Using the criteria discussed in the introduction to this section (Section 4.1.1) cells were categorized from photographs of fields of cells. Cells were categorized as slender, intermediate or stumpy. The expectation was that the intermediate category would show some degree of overlap with the slender and stumpy categories, but that slender and stumpy forms would seldom be confused.

Having been categorized morphologically each cell was subsequently assessed qualitatively by immunofluorescence for the expression of each putative marker. This was done by scoring cells, in photographs of fields of cells which were fluorescence only images. The photographs of fluorescence only images corresponded to those photographs of phase-contrast images used in scoring the cells for morphology. Two investigators, the other being Dr. K. Matthews, assessed each photograph independently and in this way a double blind regime was established. Only after morphology (by phase-contrast) and expression (by immunofluorescence) counts were completed were the results compared. Expression was assessed for 100 slender, 100 stumpy and 100 intermediate forms.

Table 4.2

	Diaphorase assay	DHLADH	Hsp60	mhsp70	CB1	TAO
Slender	0	32	3	36	27	100
Intermediate	94	99	88	99	74	100
Stumpy	100	100	100	100	82	100

Table 4.2 Cell type specificity of putative markers assessed by immunofluorescence.
100 bloodstream trypanosomes of each morphological form (slender, intermediate and stumpy) were assessed for expression of putative markers by diaphorase assay and immunofluorescence assay for DHLADH, hsp60, mhsp70, CB1 and TAO. The samples used were different for each expression assay and were taken from mixed (day 4) populations.

Representative results from one of three independent experiments are summarized in Table 4.2, to give an indication of the potential of each antigen as a cytological marker.

Although CB1 staining was predominant in stumpy forms, there were obvious exceptions and some slender forms also showed clear staining. Moreover, some stumpy forms appeared to completely lack staining for CB1. While there is clearly a skewed distribution of expression weighted towards intermediate and stumpy forms, the observed lack of specificity for any one cell type meant that it was not possible to use CB1 as a marker for the slender-to-stumpy differentiation. TAO was localized to the mitochondrion of all bloodstream form cell types, confirming that TAO expression could not be used as a marker of the slender-to-stumpy differentiation (Table 4.2).

Cytochemical staining by the NAD diaphorase assay and immunofluorescence of DHLADH, hsp60 and mhsp70 localized to the mitochondrion of all stumpy trypanosomes, the vast majority of intermediate forms but only a minority of slender forms. NAD diaphorase activity at the mitochondrion first becomes detectable close to the onset of morphological change (Table 4.2), as is also the case for detection of hsp60 expression by immunofluorescence. By comparison, immunofluorescence shows DHLADH and mhsp70 expression in the mitochondrion of some slender forms, suggesting that a detectable increase in expression of these markers occurs prior to the onset of morphological change. It is notable that immunofluorescence for DHLADH detects mitochondrial localization of this enzyme in a higher proportion of slender forms than the NAD diaphorase stain which is thought to reflect the enzymatic activity of DHLADH.

4.2.4. Stage specific marker expression by western blotting

Use of immunofluorescence assay allows the comparison of protein expression between single cells and can be extremely sensitive; moreover, it may convey considerable specific information with regard to subcellular localization of an antigen. The results obtained from an immunofluorescence assay can, however, be affected by factors such as antibody accessibility, steric hindrance and localized saturation which can give a misleading idea of the amount of protein in individual cells. At a population level the best quantitative indication of protein expression (relative to other populations) is ascertained by western blotting.

Western blotting with the antibodies used for immunofluorescence was conducted to allow a direct comparison of stage specific expression of each of the putative markers (Fig. 4.2). Samples were prepared from predominantly (>90%) slender trypanosomes (taken on day 3 of the primary parasitaemia), from predominantly (>90%) stumpy trypanosomes (taken on day 5), and from procyclic trypanosomes (taken from in vitro culture). Protein concentration of the samples was assessed using the Bradford assay. Samples were then loaded to equal protein concentrations, as sets of replicate lanes, on two SDS-PAGE gels. One set of lanes from each gel was stained using Coomassie Brilliant Blue (Fig. 4.2 A, F) and the other replicate lanes were blotted overnight onto nitrocellulose. Replicate lanes were then probed with each of the antibodies to the putative marker antigens.

Figure 4.2 shows the results from western blotting for each antigen assessed. The differences in expression between populations of the life cycle stages tested, as assessed by western blot, were marginal for most of these markers. CB1 (Fig. 4.2C) expression showed only modest increases in stumpy population samples compared with slender population samples but was highly expressed in both and formed a smear rather than a discrete band, consistent with its recognition of a highly glycosylated molecule. CB1 expression was not detected in procyclic populations (Fig. 4.2C).

Mitochondrial markers might be expected to increase in expression from slender-to-stumpy and then from stumpy to procyclic, consistent with the perceived mitochondrial biogenesis which occurs during the life cycle. This was found not to be the case

with TAO (Fig. 4.2D). There was little difference in the level of expression between slender and stumpy populations, supporting the idea that TAO expression is constant in bloodstream forms. In comparison, the TAO detected was considerably lower in the procyclic sample than in either bloodstream form. Surprisingly, hsp60 (Fig. 4.2H) did not show a reproducible increase in expression between slender and stumpy populations and it is not clear why the immunofluorescence should show such a clear difference in staining for hsp60 between slender and stumpy forms (see Fig. 4.1P, Q). There was, however, a marginal increase in hsp60 expression in the procyclic form.

The mitochondrial markers mhsp70 and DHLADH did show increases in expression between the slender and stumpy and between the stumpy and procyclic form, consistent with expression increasing throughout mitochondrial biogenesis; although, in the case of mhsp70 (Fig. 4.2G) these increases were marginal. DHLADH, however, gave a clear increase in expression in stumpy form populations relative to the slender form populations and also showed another clear increase in the procyclic form populations relative to stumpy form populations (Fig. 4.2B). α-tubulin expression was also assayed as a control for protein integrity using the monoclonal antibody TAT. α-tubulin was found to be less abundant in the stumpy form populations, which are predominantly non-proliferative, than in either the slender or the procyclic form populations (Fig. 4.2E, I) which consist of predominantly proliferative cell types.

Figure 4.2

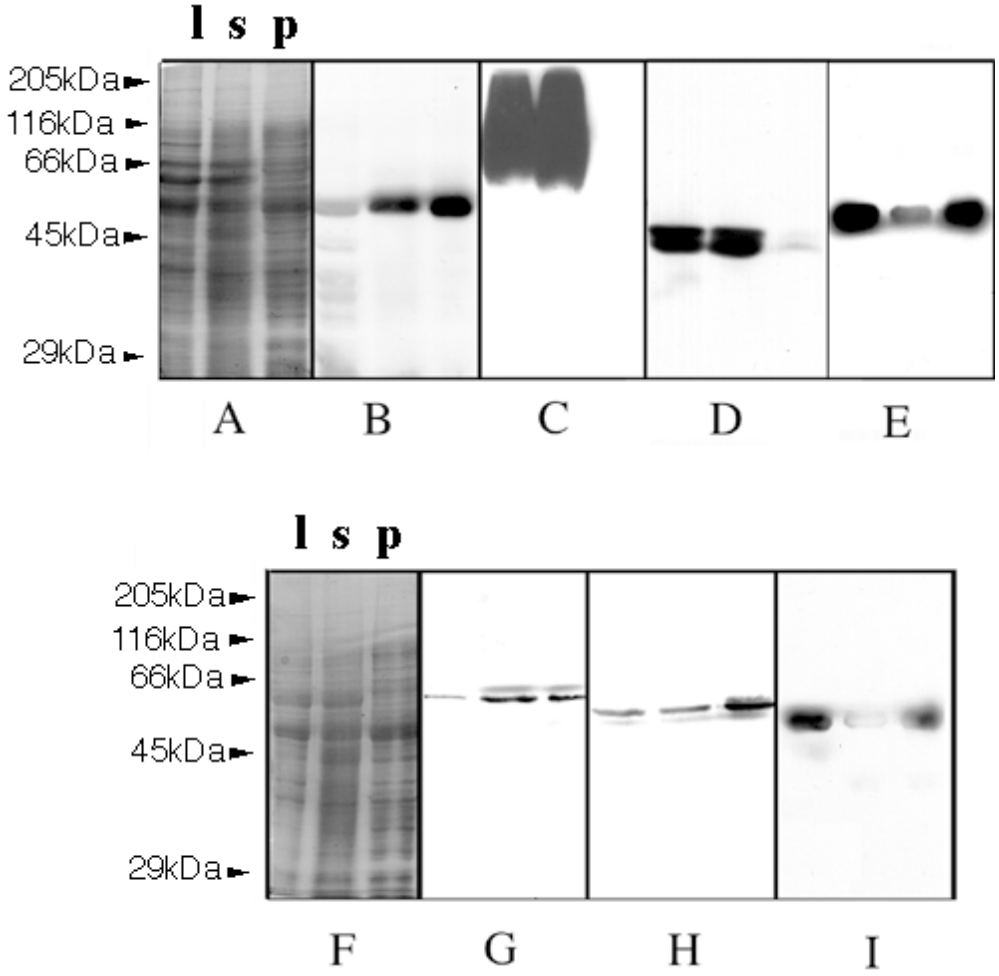


Fig. 4.2 Life cycle stage expression of putative markers of the slender-to-stumpy differentiation.
Gels were loaded by equal protein concentration: duplicate sets of lanes (> 90 % long slender (labelled **l**), > 90% short stumpy (labelled **s**) and procyclic (labelled **p**)) were run in parallel on the same SDS-polyacrylamide gel. One set of lanes was cut off and stained with coomassie, the other sets of lanes were then blotted and the blots probed with antibody to one of the putative markers. In the absence of an obvious loading control coomassie stained gels are shown (**A** and **F**). Anti-α-tubulin was also included as a control for protein integrity. It was shown to be lower in the stumpy form than in either the slender or procyclic form (**E** and **I**). **B** shows the immunoblot of DHLADH, **C** of CB1, **D** of TAO, **G** of mhsp70 and **H** of hsp60.

4.2.5. DHLADH expression increases during the primary parasitaemia

On the basis of immunofluorescence and western blot, DHLADH was selected as the best marker to study the slender-to-stumpy differentiation. DHLADH showed a more pronounced increase in expression than other markers tested by western blotting (Fig. 4.2) and was undetectable in the vast majority of slender form mitochondria by immunofluorescence (Fig. 4.1), compared with the distinctive kinetoplast staining of mhsp70.

The results presented in Table 4.2, using a relatively small number of cells, indicated that DHLADH was expressed by all stumpy forms, almost all intermediate forms and some slender forms in a mixed (Day 4) population. It remained possible, however, that the acquisition of DHLADH expression was not coupled to the morphological differentiation from the slender to the stumpy form. Acquisition of DHLADH might follow a different time-course from morphological transition and this would be reflected by differences between populations during the course of the slender-to-stumpy differentiation. Figures 4.3 and 4.4 demonstrate that the numbers of cells which showed a clear mitochondrial staining for DHLADH increased during the course of the primary parasitaemia, consistent with DHLADH expression being upregulated during differentiation and induced in intermediate and stumpy forms.

Fig. 4.3 shows the results of double-labelling imunofluorescence with the anti-α-tubulin antibody, TAT (left column) and DHLADH (right column). Immunofluorescence for tubulin is useful in highlighting trypanosome morphology since the trypanosome cytoskeleton is strongly stained but not the surrounding erythrocytes which lack microtubules. The samples were prepared as bloodstream smears taken 3 days, 4 days and 5 days post-infection. It can be seen that on day 3 (upper panel) trypanosomes appear predominantly quite slender in morphology and show little detectable staining for DHLADH. There is, however, some weak staining associated with the nuclei of these slender forms. On day 4 (middle panel) a mixture of morphologies is clearly present, staining of the trypanosome mitochondrion is present in only some of the cells and the intensity of this staining is variable. On day 5 (lower panel) the morphologies are predominantly stumpy and all stumpy forms show clear staining of the mitochondrion for DHLADH.

Figure 4.3

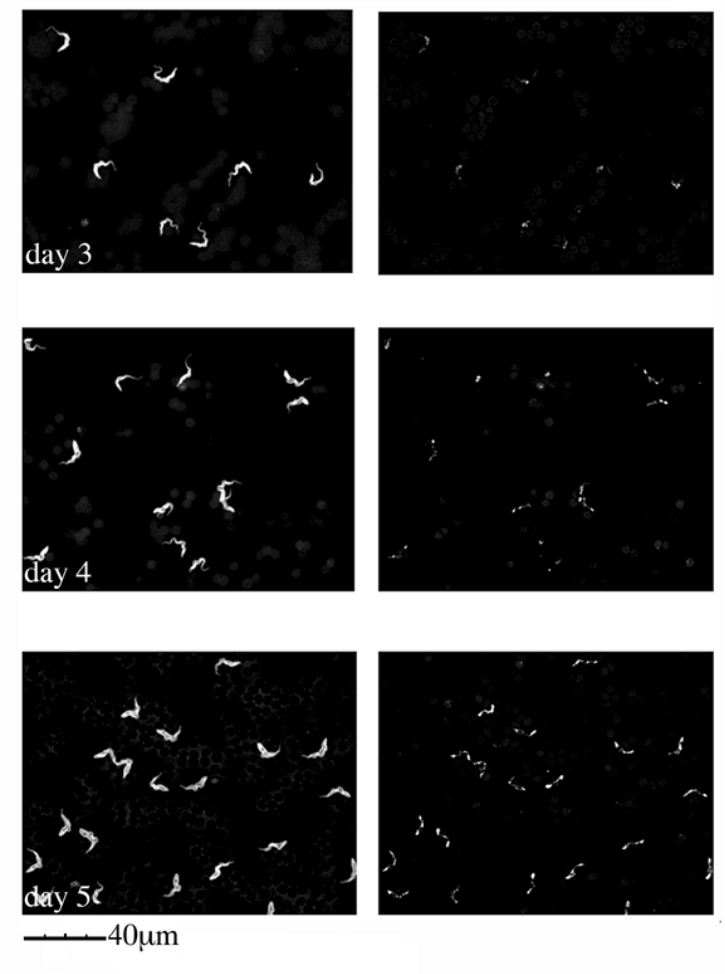


Fig. 4.3 Immunofluorescence demonstrates an increase in DHLADH expression during the course of the primary parasitaemia. Cells were double-labelled with the mouse monoclonal anti-α-tubulin antibody TAT and the rabbit polyclonal antibody to DHLADH. Staining for α-tubulin was visualized in the rhodamine channel and was used to highlight the morphology of the cells. Staining for DHLADH was visualized in the fluorescein channel and increased during the course of the primary parasitaemia. Samples were prepared as blood smears taken from an infected mouse 3 days, 4 days and 5 days after infection.

To confirm that the proportion of trypanosomes expressing DHLADH matched the proportion of intermediate and stumpy forms during an infection, the morphology of the cells in each population and the number of cells which showed staining of the mitochondrion for DHLADH by immunofluorescence was assessed quantitatively. Figure 4.4 compares

morphological change during the slender-to-stumpy differentiation with the acquisition of DHLADH expression as assessed by immunofluorescence. This was done for 1000 cells on each of 3 days using the same samples from which the fields shown in Figure 4.3 were prepared.

Figure 4.4 shows that, at each time point assessed, the number of cells which express DHLADH is greater than the number of stumpy forms present, but is similar to the combined number of intermediate and stumpy forms. This is particularly apparent on day 4 when it can be seen that although all stumpy forms do express DHLADH, many more cells express DHLADH than there are stumpy forms. This supports the implication from Table 4.2 that DHLADH is a marker of both intermediate and stumpy forms, that it is coupled to morphological change and that it is acquired close to and perhaps somewhat earlier than the onset of morphological change. In fact, given the close correlation between the cells expressing DHLADH and the number of intermediate and stumpy forms in each of the populations assessed, I propose that DHLADH expression should be used as a defining characteristic of intermediate forms and stumpy forms. This may, however, involve reclassifying some cells which would normally be regarded (perhaps erroneously) as slender in assessments based solely upon morphology. Using DHLADH as a defining characteristic of the intermediate form would be helpful in eliminating some of the potential ambiguities in morphological evaluation, allowing intermediates to be discriminated more readily from slender forms which do not express DHLADH.

Figure 4.4

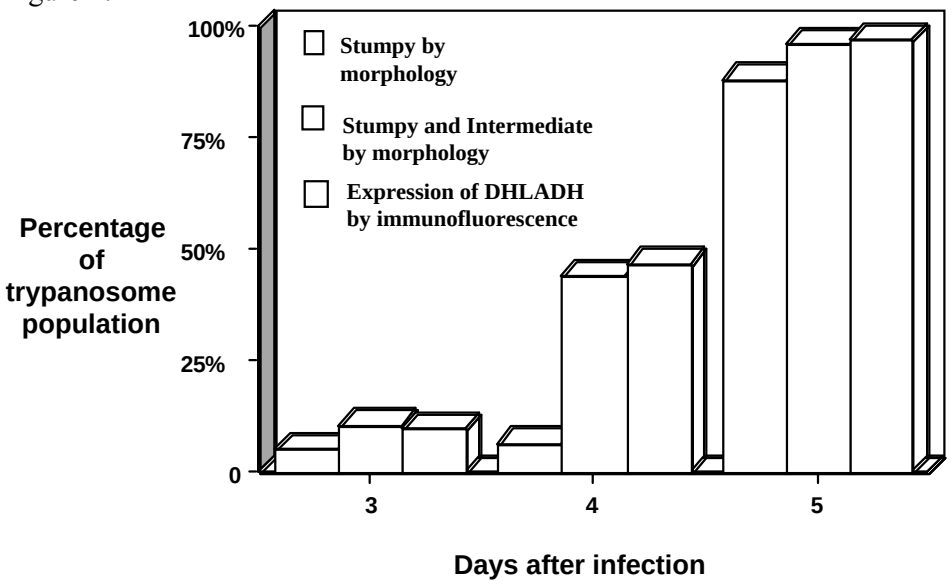


Fig 4.4 Immunofluorescence shows an increase in the numbers of trypanosomes expressing DHLADH during the primary parasitaemia This increase precedes the increase in number of stumpy forms and is very similar to the increase seen in the total number of stumpy and intermediate (non-slender) forms. 1000 trypanosomes from populations, taken 3 days, 4 days and 5 days after infection, were assessed by immunofluorescence assay for the clear mitochondrial expression of DHLADH. 1000 trypanosomes, each day, from the same populations were also assessed by morphology and categorized as slender, intermediate and stumpy.

4.2.6. Examination of whole cell cytoskeleton electron micrographs

Electron micrographs of monomorphic (from Dr. T. Sherwin) and pleomorphic (from Dr. K. Matthews) whole cell cytoskeletons had previously been prepared. Fairburn and Culwick (1946) and Hoare (1956) have previously proposed that the length of the trypanosome cell is a good marker of slender, stumpy and intermediate forms. This study bisected cell length to look at the distance between the basal body and the posterior of the cell, and the length of the whole flagellum. The study also included an established parameter used in the evaluation of the slender-to-stumpy differentiation, length of the free flagellum. For the purposes of this study, only cells with one flagellum, one basal body and one nucleus were analysed so as to minimize any cell cycle dependent variation. Since the pleomorphic slender forms, categorized by morphology, were likely to include some mistakenly identified intermediate forms; monomorphic forms were also assessed as homogeneously slender controls.

Twelve monomorphic trypanosome cytoskeletons were analysed and thirteen pleomorphic trypanosome cytoskeletons. Of the pleomorphic trypanosome cytoskeletons eight

were adjudged slender, four intermediate and one stumpy. The results shown in Figure 4.5 show mean lengths measured and include error bars calculated as plus or minus one standard error. In the case of the stumpy form however this statistic cannot be calculated so error bars are not shown. The results broadly support shortening of the flagellum (Fig 4.5A) and free flagellum (Fig 4.5B) and an increase in the distance of the kinetoplast from the posterior end of the cell (4.5C).

Figure 4.5

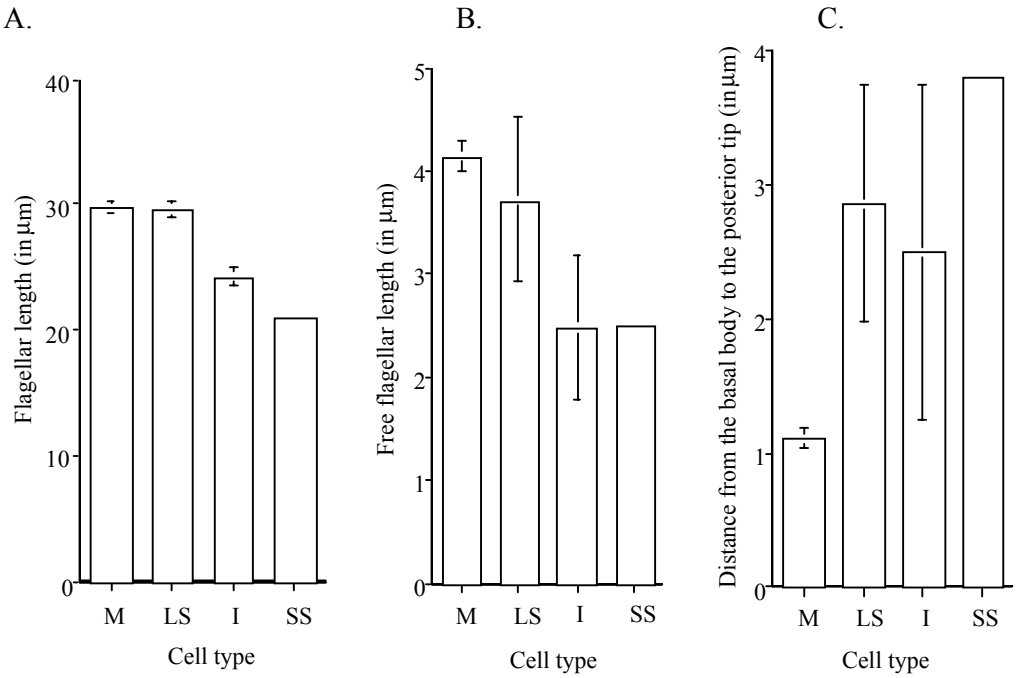


Fig. 4.5 Three parameters related to trypanosome length were assessed at the level of the electron microscope, for variation during the slender to stumpy differentiation. A. shows the length of the flagellum, B. the lengths of the free flagellum and C. the distance from the basal body to the posterior tip of the trypanosome. Cells with one basal body and one nucleus were assessed from 12 monomorphic (M) and 13 pleomorphic forms. The pleomorphic population was subcharacterized into 8 long slender forms (LS), 4 intermediate forms (IF) and one short stumpy (SS) form. The length of the flagellar axoneme appeared to vary most consistently when assessed for a small number of cells and so was investigated further.

In figure 4.5C the mean distance to the posterior tip from the basal body appears anomalous, since the mean distance of the basal body to the posterior end is longer in the slender than in the intermediate form rather than shorter, however, the standard error present in both the long slender and intermediate form is large. In contrast, the standard error in the

monomorphic long slender form is much smaller and may be a better indication of the usual positioning of the basal body in proliferative long slender forms.

In this study the sample group used was so small that any statistical inferences were of limited value. For this reason significance testing was not attempted. For any of the markers to be useful in evaluating the fate of single cells, however, a clear and consistent difference was required. Such a difference was seen only for flagellar length (Fig. 4.5A) where the flagellar lengths of all of the intermediate forms measured were smaller than the long slender form mean flagellar length. The flagellar length of the short stumpy form was the shortest flagellar length in the sample. It was flagellar length, therefore, that was selected as the primary marker for further characterization.

In addition to the small size of the sample group, it was possible that a systematic bias was being introduced, with shorter cytoskeletons being considered more intermediate than longer ones. If this was the case, it was possible that the experiment was showing a correlation between shorter cells and shorter flagella, rather than a correlation of shorter flagella with the slender-to-stumpy differentiation. It was hoped that immunofluorescence microscopy would not only facilitate the use of larger and statistically more significant sample sizes, but would allow a direct comparison with mitochondrial markers activated during the course of differentiation and, hence, eliminate the chance of a systematic bias from the use of morphological evaluation alone.

Several monoclonal antibodies are available to protein components of the paraflagellar rod (PFR). These can be used in an assay of the length of the flagellar axoneme, outside of the flagellar pocket, by immunofluorescence. PFR length was therefore tested as a marker of the differentiation on a statistically viable sample of cells, by immunofluorescence with monoclonal antibodies directed against the PFR. This testing also ensured that this region of the flagellum (rather than solely the area from the basal body to the flagellar pocket that is not associated with a PFR) varied in length during differentiation. In order to test PFR length as a marker of differentiation, a mixed population was double stained for DHLADH (selected as the best available marker for discriminating between cell types) and for staining of the PFR.

Figure 4.6 shows the results of the double staining experiment. The population used was taken five days post-infection and was predominantly stumpy so the vast majority of the forms expressing DHLADH were stumpy forms. Morphologically slender forms (Fig. 4.6A) typically did not show DHLADH expression but had longer PFRs (Fig. 4.6B). In comparison, morphologically intermediate or stumpy forms (Fig. 4.6C) did express DHLADH and had shorter PFRs (Fig. 4.6D). Figure 4.6E shows a clear difference in PFR length between cells which stain for DHLADH (intermediate and stumpy forms) and those that do not (slender forms). The results are shown as one standard error about the mean.

Figure 4.6

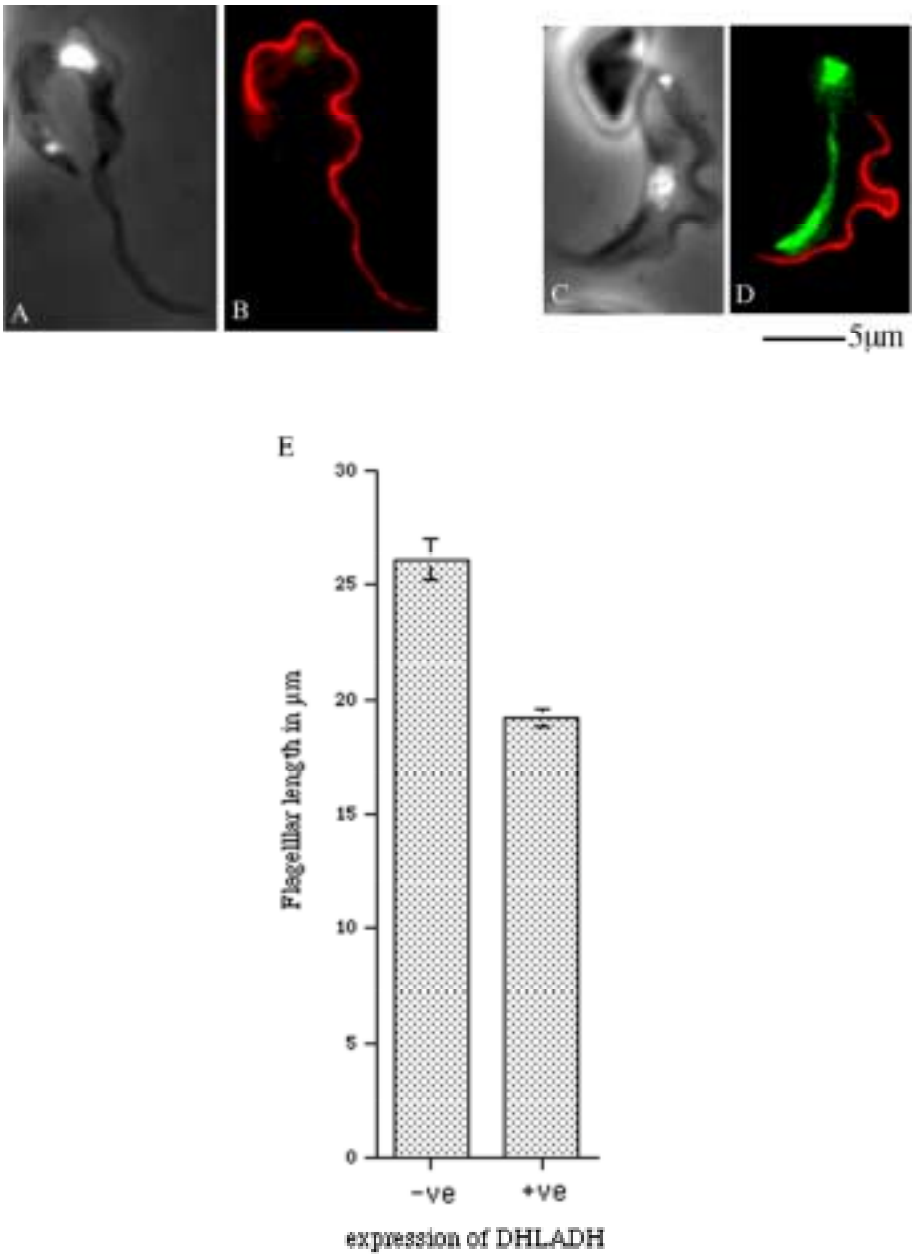


Fig. 4.6 Mean PFR length shortens during differentiation to the stumpy form. Double immunofluorescence was conducted on a mixed population of trypanosomes. PFR expression was used as a marker of flagellar length. Acquisition of DHLADH expression was used to assess cellular differentiation. A, C merged phase-contrast and DAPI fluorescence image of a slender form (A) and a stumpy form (C). B, D merged double immunofluorescence, DHLADH expression (FITC, green), PFR expression (TRITC, red). Figure shows a longer flagellum when no DHLADH is expressed (B) and a shortened flagellum when DHLADH is expressed (D). E compares the mean flagellar lengths of DHLADH expressing and non-expressing forms. Error bars represent one standard error about the mean.

Significance was tested using the t-test for two independent samples (as detailed in most basic statistics texts, e.g., Heinman, 1992). The statistics calculated were as follows:

$n_1 = 31$,	$n_2 = 34$
$mean_1 = 26.1$	$mean_2 = 19.22$
$\sum X_1 = 809$	$\sum X_2 = 654$
$\sum X^2_1 = 21{,}904$	$\sum X^2_2 = 12{,}730$
$s_1 = 5.12$	$s_2 = 2.22$
$SE_1 = 0.93$	$SE_2 = 0.39$
$t_{crit}(P<0.01) = 2.4$	$t_{obt} = 7.4$

Since the value obtained for t is considerably higher than t_{crit}, it seems that there is a highly significant, negative relationship between progression through the slender-to-stumpy differentiation, as assessed by the acquisition of DHLADH, and flagellar (and PFR) length during differentiation. Consequently, PFR length should represent a good morphological marker of the differentiation. Although the mechanism by which the perceived shortening occurs remains unknown, the molecules making up the structure are well defined. Since flagellar outgrowth is the earliest event in division and proceeds at an approximately constant rate until cytokinesis (Chapter 3), flagellar length seemed a well suited parameter with which to examine biogenesis of stumpy forms (Chapter 5).

4.2.7 Messenger RNAs upregulated in the stumpy form

Increased gene expression in the stumpy form has not yet been linked to increased levels of mRNA in the stumpy form for any given protein. There has, however, been one previous report of increased levels of a protein encoding transcript in the stumpy form (Murphy and Pelle, 1994). In searching for differentially expressed mRNAs, which might encode stumpy specific proteins useful as markers, a differential display based approach was adopted.

Differential display employs the polymerase chain reaction to amplify cDNA fragments, generally using short primers of arbitrary sequence. Template cDNAs are prepared from the poly $(A)^+$ selected mRNA of specific cell types by reverse transcription. All cell type specific cDNA templates are then amplified with the same mixture of primers under the same conditions. Different cDNA fragments are amplified depending on the cell type from which the cDNA is made and reflect the relative abundance of the corresponding mRNA in that cell type. By comparing patterns of amplified cDNA fragments between cell types, fragments which may correspond to differentially expressed genes can be identified and subsequently cloned and characterized. The screen was performed in collaboration with Dr. R. Docherty who was searching for trypanosome cell cycle regulated proteins.

First strand cDNA (prepared by Dr. K. Matthews) was used as a template and was amplified using two defined but arbitrary primers and the spliced leader sequence. Mitochondrial mRNA is not trans-spliced to the (nuclear encoded) spliced leader sequence. Consequently, the inclusion of a spliced leader sequence biased the results towards nuclear encoded sequences, however, the other two primers ensured that kinetoplast encoded RNA might also be amplified. The sequence of the other two primers had been selected to lack stop codons in all potential reading frames, to show little secondary structure and to have a 60-70% GC content (Sokolov and Prockop, 1994). Following amplification, reaction mixtures were separated by agarose gel electrophoresis. Fragments that were specific to the stumpy form were excised from the gel, purified, and cloned in a T-vector (for details see Chapter 2).

Figure 4.7

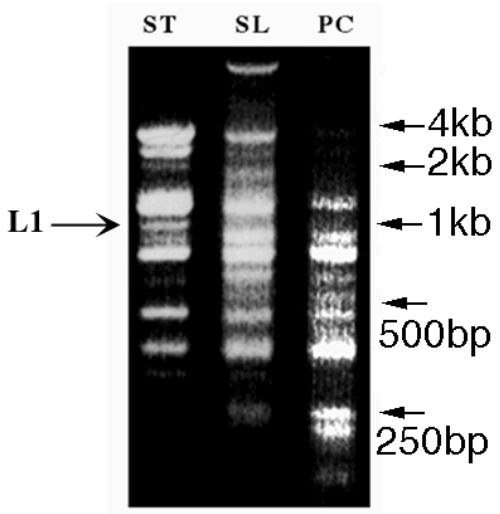


Fig 4.7 Differential display of cDNA from stumpy (ST), slender (SL) and procyclic (PC) trypanosome populations. The cDNA fragment L1 (arrowed) was cloned from this gel. ST22 was cloned from a similar gel by Dr R.Docherty. Bands were excised from the agarose gel and ligated into pBSK. The fragments were partially sequenced and used to search available databases for homologues (Fig. 4.8). Riboprobes were generated by in vitro transcription of the cloned fragments and used for northern blots (Fig. 4.9) and in situ hybridization (Fig. 4.10).

Ten cDNA fragments from putatively regulated transcripts were initially cloned and sequenced. These were found to be made up of three groups: three copies were of an approximately 240bp fragment named ST22; two copies were of an approximately 1kb fragment named L1; and five copies showed exact identity to the plasmid cloning vector, pGEM. Figure 4.7 shows the differential display pattern of the agarose gel from which the L1 fragment was cloned; ST22 was cloned by Dr. R. Docherty from a similar gel.

L1 and ST22 sequences were compared to the nucleotide database, dbest, using a BLASTn search on the NIH ENTREZ server. The highest homology sequences obtained for L1 and ST22 showed extremely high identity with expressed sequence tags from *T. brucei* transcripts in the database. In the case of L1, this sequence tag was listed in the database as showing substantial homology to the ribosomal protein P13A. Moreover, the sequence tag corresponding to ST22 was also listed in the database as showing substantial homology to the ribosomal protein L9 (Fig. 4.8).

Figure 4.8

a, for ST22 cDNA fragment

```
gb|T26832|T26832      T512 Trypanosoma brucei rhodesiense ...   404  1.3e-43   2
gb|AA003479|AA003479 T3151 MVAT4 bloodstream form of sero...   341  2.2e-26   2
gb|T26833|T26833      T073 Trypanosoma brucei rhodesiense ...   290  1.3e-16   1

gb|T26832|T26832 T512 Trypanosoma brucei rhodesiense cDNA 5' similar to
           Ribosomal protein L9.
           Length = 353

  Minus Strand HSPs:

 Score = 404 (111.6 bits), Expect = 1.3e-43, Sum P(2) = 1.3e-43
 Identities = 84/88 (95%), Positives = 84/88 (95%), Strand = Minus / Plus

Query:   143 CGCGTGAACAAGAAGCTCCGTACCTTCACTGCGGTTCGTTGGTTTGGTAACAAGATCAAT 84
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   118 CGCGTGAACAAGAAGCTCCGTACCTTCACTGCGGTTCGTTGGTTTGGTAACAAGATCAAT 177

Query:    83 AACTCAACCATCAACACGGCTCTCGCAC 56
             ||||||||||||||| || ||| | |
Sbjct:   178 AACTCAACCATCAACACCGCGCTCTCGC 205

 Score = 236 (65.2 bits), Expect = 1.3e-43, Sum P(2) = 1.3e-43
 Identities = 48/49 (97%), Positives = 48/49 (97%), Strand = Minus / Plus

Query:    52 CGAAACATGATCATGGGTGTTACAAAAGGGTTCCGCTTCAAGGTGCGTT 4
             ||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct:   210 CGAAACATGATCACGGGTGTTACAAAAGGGTTCCGCTTCAAGGTGCGTT 258
```

b, for L1 cDNA fragment

```
                                                                  Smallest
                                                                    Sum
                                                           High  Probability
Sequences producing High-scoring Segment Pairs:            Score  P(N)       N

gb|T26824|T26824      T266 Trypanosoma brucei rhodesiense ...   190  3.3e-08    1
gb|T26823|T26823      T045 Trypanosoma brucei rhodesiense ...   186  6.9e-08    1
gb|AA255332|AA255332 T3514 Bloodstream form of serodeme I...   159  1.3e-05    1

gb|T26824|T26824 T266 Trypanosoma brucei rhodesiense cDNA 5' similar to
           Ribosomal protein L13A.
           Length = 386

  Plus Strand HSPs:

 Score = 190 (52.5 bits), Expect = 3.3e-08, P = 3.3e-08
 Identities = 42/48 (87%), Positives = 42/48 (87%), Strand = Plus / Plus

Query:     1 AAGAGAGTAAGCTCCCGGAATGGTTTACCAAGGCCGCAGTCGCTGAAT 48
             ||||| |||||||||||| |||||||  | | |||||||||||||||
Sbjct:     2 AAGAGNGTAAGCTCCCGGNATGGTTTTACCAAGCCGCAGTCGCTGAAT 49
```

Fig 4.8 Sequence from cDNA fragments, cloned from the differential display, were screened against the dbest data base (at NCBI via the internet) using a BLASTn search optimized for near identical sequences. Sequences from two fragments, ST22 and L1, recognized expressed sequence tags with very high identity. The sequence tags themselves showed homology to ribosomal proteins L9 and L13A when back translated to protein sequence in their open reading frames.

To determine whether these cDNA fragments corresponded with stumpy specific RNAs, the fragments were transcribed as riboprobes and used to probe northern blots of total RNA (Fig. 4.9). The pattern of expression was almost identical for both L1 and ST22 showing moderately increased expression in the stumpy population compared with slender and procyclic forms.

In the case of ST22 however two bands of an approximately equal intensity were observed in each lane. These two bands may represent allelic variants (since they are present at similar levels), they may result from alternative post-transcriptional modifications of mRNAs transcribed from the same gene, or they may represent two different genes which both have regions of high homology to the probe.

In the absence of an obvious constitutive RNA (most structural proteins such as tubulin have reduced levels in the stumpy form), the loading control used for the northern blots was the three bands of ribosomal RNA. Loading was assessed (by eye) for equal fluorescence between bands after staining with ethidium bromide and on exposure to ultraviolet light. There is no direct evidence, however, that ribosomal RNA is equally expressed in all trypanosome cells regardless of life cycle stage. It was therefore possible that the RNA loadings used were biased toward one stage or another. In order to establish that these transcripts actually were more abundant in stumpy forms than in slender forms, RNA abundance was compared at the level of the single cell using an in situ hybridization assay. In situ hybridization was conducted (by the method of Ersfeld et al., 1996) using the sense and antisense riboprobes for L1 and ST22 in addition to riboprobes for tubulin and VSG controls (Fig. 4.10).

Figure 4.9

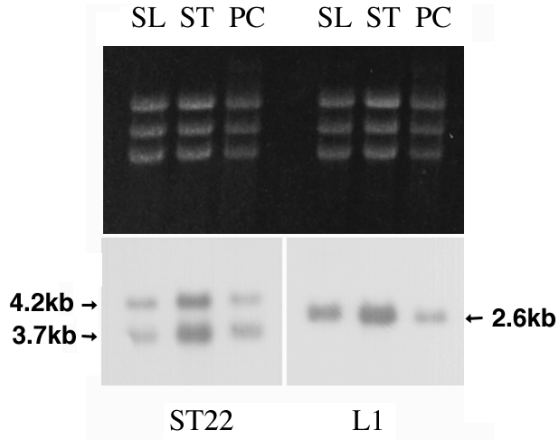


Fig. 4.9 Northern blots probed with cDNA fragments derived from differential display. Loading was controlled for by the fluorescent intensity of the ribosomal bands on the formaldehyde/ agarose gel, stained with ethidium bromide. Blots were probed with sense and antisense riboprobes of each fragment. The antisense only is shown since the sense probes gave no signal.

The riboprobe for L1 failed to generate a detectable signal from either the slender or stumpy forms on in situ hybridization, perhaps because the target RNA was not abundant enough. The ST22 probe however gave a clear and differential signal between the slender and stumpy forms (Fig. 4.10A-F), with staining in the region of the nucleus being clearly visible in the stumpy but not the slender form. Phase images of fields with slender and stumpy forms are shown (Fig. 4.10 A, C, E) for assessment of morphology, alongside corresponding fluorescence images of the in situ hybridization visualized in the FITC channel (Fig. 4.10 B, D, F). The in situ images shown in Figure 4.10 are not of the same quality as those obtained in regular immunofluorescence preparations due to the harsh conditions used in preparing the samples, which includes acid treatment and prolonged hybridization at relatively high temperature. Nevertheless, morphology is preserved to a great enough degree to discriminate between slender and stumpy forms.

Control staining for α-tubulin (Fig 4.10J, K) and VSG -GUTat 7.2- mRNA (Fig. 4.10L, M) shows clear cytoplasmic staining of almost all cells. The tubulin probe in particular showing a region of reduced intensity in the nucleus which contrasted with the strong stumpy specific nuclear signal of the ST22 probe. A few cells did not stain for GUTat 7.2 mRNA and

these were assumed to be VSG heterotypes in the population (arrowed in Fig. 4.10L). The sense riboprobes were used as negative controls, although only in the case of tubulin (where there are 16 copies) were they sensitive enough to show the location of the gene locus visualized as two (or in dividing forms four) distinct points (Fig. 4.10 G, H, I). Figure 4.10I shows the fluorescence image (Fig. 4.10G) merged with the phase/DAPI image (Fig. 4.10H) in order that the points can bee seen to localize to the nucleus.

The results from the in situ hybridization serve to confirm the evidence from northern blotting that L1 and ST22 hybridize to RNAs that are more abundant in stumpy forms. It is intriguing that what are apparently transcripts of ribosomal proteins show increased staining in proximity to the cell nucleus, in contrast to other expressed transcripts (e.g.: VSG, tubulin and histones) which show reduced staining in the nucleus (See also Ersfeld et al., 1996).

Figure 4.10

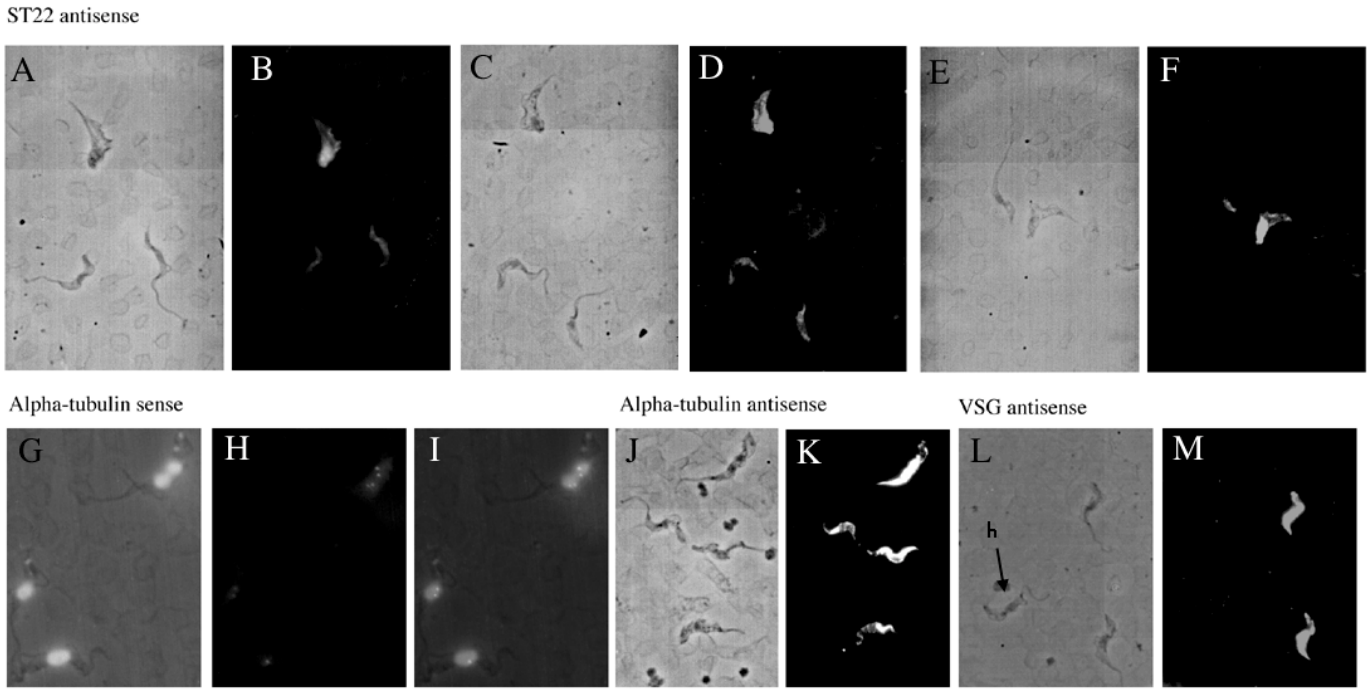


Fig 4.10 In situ hybridization with ST22 antisense probe shows increased levels of a specific mRNA in the region of the stumpy form nucleus relative to nearby slender forms (A-F). The lower panel shows α-tubulin (G-K) and VSG 7.2 (L, M) riboprobe controls. Tubulin sense probe can be visualized binding to nuclear DNA at the multiple tandem repeats of the tubulin locus (G, H, I). Tubulin antisense shows typical cytoplasmic distribution of a highly expressed mRNA (J, K). VSG 7.2 was also used; h marks what was believed to be a non-7.2 heterotype expressing trypanosome.

4.3 Discussion

Phenotypic markers are acquired during the differentiation to the division arrested stumpy form, which is morphologically and biochemically distinct from the slender form. Characterization of these markers is an essential first step in understanding how the process of differentiation is co-ordinated and how this co-ordination is regulated. Nevertheless, markers of this critical differentiation have remained scarce and for the most part ill-defined. This part of the project was initiated with a search for stage regulated molecules and consistent changes in phenotype (particularly during morphogenesis) which could lend definition to the slender-to-stumpy differentiation.

4.3.1 Screen for known antigens

As expected the development of the mitochondrion proved to be the most fruitful source of markers for the slender-to-stumpy differentiation. Several mitochondrial activities have previously been shown to increase during differentiation to the stumpy form and the project hoped to equate some of these with perceived increases in enzyme expression, by immunofluorescence and western blot. Unfortunately, the majority of these enzymes, which are well characterized in organisms close to the metazoan lineage, did not show enough conservation of epitopes with their trypanosomal homologues to allow detection. Where antibodies raised to kinetoplastid proteins and the highly conserved mitochondrial heat shock proteins were available a greater degree of success was achieved, but it was only with trypanosome antigens that antibodies showing good specificity for the target proteins were identified (Table 4.1). Unfortunately, few of the enzymes that mediate activities known to increase during the slender-to-stumpy differentiation have thus far been cloned and, consequently, there were few antibodies available.

An exception to this is DHLADH, a component of the pyruvate dehydrogenase and α-ketoglutarate dehydrogenase enzyme complexes and which is thought to have a central role in the maintenance of trypanosome redox. DHLADH has been perceived as a drug candidate particularly for *T. cruzi* and *Leishmania* sp. (Krauth-Siegel and Schoneck, 1995; Schoneck et al., 1997), consequently the *T. cruzi* protein is well characterized and high quality antibody was available for this study. The gene for DHLADH has been previously cloned in trypanosomes and the sequence is available (Else et al., 1993). Whilst increased levels of this protein in the stumpy form have not been previously recorded, the suggestion that it was an increase in the activity of this enzyme which mediates the diaphorase assay has been previously made (Giffen and McCann, 1993). This conclusion, however, was apparently contradicted by the localization of the enzyme's activity to the slender form plasma membrane in cell fractionation studies (Danson et al., 1987; Jackman et al., 1990).

The immunofluorescence localization obtained here (Fig. 4.1), correlates strongly with the results from the diaphorase assay. There was no detectable immunofluorescence from DHLADH at the plasma membrane in slender forms, although there was a low level of staining of trypanosome nuclei which may be non-DHLADH specific antibodies present in the polyclonal antibody. It seems likely, therefore, that lipoamide dehydrogenase, when expressed, is normally resident in the trypanosome mitochondrion and that any enzyme activity at the plasma membrane is either at a very low level or attributable to a second enzyme. It should be remembered that the low level of DHLADH detectable by western blotting (Fig. 4.2) in slender populations is attributable, at least in part, to intermediate and stumpy forms, since the populations are not pure.

Mitochondrial heat shock proteins also provided markers of differentiation. In this case the antibodies came from *T. cruzi* homologues which show a high degree of identity with *T. brucei,* even at the DNA level (Klein et al., 1995b). The

mitochondrial heat shock proteins hsp60 and mhsp70 both demonstrated elevated expression in stumpy forms, although in the case of hsp60 this demarcation was less clear by western blotting. I do not know the basis for this difference between immunofluorescence and blotting results: it may be that hsp60 epitopes are sterically masked in the slender mitochondrion. A published characterization of hsp60 in *T. brucei* (Bringaud et al., 1995) also showed a marginal increase in expression between bloodstream and procyclic forms consistent with the western blotting results presented here.

Members of the hsp70 family of proteins are associated with protein assemblies, often referred to as chaperone machines, which are conserved throughout evolution. The mitochondrial homologue, mhsp70, is a central component of the mitochondrial import chaperone machine and has a key role in mitochondrial biogenesis (reviewed in Martin, 1997; Cyr, 1997). Since mhsp70 must be present in the mitochondrion first, in order to facilitate the import of other mitochondrial proteins, it is a good candidate for an early marker of differentiation.

Previous reports of mhsp70 in trypanosomes (using the same antibody as used in this study) have localized the protein solely to the kinetoplast in both slender and procyclic forms (Klein et al., 1995ab). While the immunofluorescence results presented here in Figure 4.1 confirm this localization in some slender forms, evidence of staining throughout the mitochondrion of some slender and all intermediate, stumpy and procyclic forms was also observed. Furthermore, the pattern of mitochondrial staining observed in this study was reproduced with antibody raised to the leishmanial homologue, lmhsp70, which gave a higher background level of staining. This is supported by another published study in which lmhsp70 has been shown to localize throughout the mitochondrion of *Leishmania major* by immunogold labelling (Searle et al., 1993).

I believe the differences observed between experiments in the immunolocalization mhsp70 is one of sensitivity and may be explained by the

differences in the method of sample preparation: wet fixation of attached cells shown here, as opposed to a dry smear used by others, and omission of up to 4% protein block used in other methods. Using air dried smears and a 4% bovine serum albumin blocking step (published method of Klein et al., 1995ab) I obtained similar results to those previously published (Klein et al., 1995ab), with mitochondrial staining very much reduced but kinetoplast staining still clearly visible. It is clear, however, that more than one member of the hsp70 family is resident in the mitochondrion of most organisms (Schilke et al., 1996), so the presence of one mhsp70 at the kinetoplast and a different one at the mitochondrial membrane, both of which are recognized by the same antibody, could resolve apparent differences in localization. Although western blots (Fig. 4.2) with the mhsp70 antibody lend no support to this idea, it may be that the two proteins comigrate and therefore cannot be resolved in one dimension.

Although the trypanosome alternative oxidase was a poor marker of the slender-to-stumpy differentiation, showing a substantial degree of expression in both forms, it proved to be a useful marker of both the slender and stumpy mitochondrion. In particular, TAO provided a control for the presence of a mitochondrion in cell types which were negative for mitochondrial staining with the differentially regulated mitochondrial markers (slender forms, dividing forms, slender zoids) and a control marker for the level of regulation of other mitochondrial proteins.

Characterization of expression of TAO also highlighted other points of interest. The level of nuclear encoded TAO is apparently constant, but the level of nuclear encoded DHLADH is increased during the slender-to-stumpy differentiation. This indicates that the developmental control of mitochondrial biogenesis is protein specific and not a general post-translational mechanism directed at nuclear encoded mitochondrial proteins (e.g. a non-specific control of mitochondrial import). Western blots for TAO show a pattern with two distinct

bands separated by a distance consistent with a cleaved mitochondrial leader sequence (Fig. 4.2). Such sequences have been demonstrated in other trypanosome proteins including DHLADH (Hauser et al., 1996). DHLADH did not, however, show detectable levels of the nascent protein. This may indicate that the precleaved TAO protein is more stable than precleaved DHLADH. It is interesting to speculate whether protein specific regulation in mitochondrial biogenesis may be linked to the stability of the precleaved protein.

A non-mitochondrial antigen, the glycosylated epitope CB1, was localized by immunofluorescence to the lysosomal/endosomal compartment as previously published (Brickman and Balber, 1993, 1994). As a result of substantial expression in some slender forms and no expression in some stumpy forms, however, the pattern of expression was too complex to be useful as a marker of the slender-to-stumpy differentiation. It is not clear whether increased expression of the CB1 epitope is directly associated with the developmental program producing a stumpy form. Investigation in immunosuppressed hosts, or using the in vitro system of Vassella and Boshart (1996), might determine whether increased endocytosis at the flagellar pocket, influenced by the presence of antibody, is responsible for the increased expression of CB1, or whether CB1 expression is under direct developmental control.

In summary the screen of antibodies was successful in demonstrating that the antibody recognizing DHLADH provided the best available marker for the slender-to-stumpy differentiation and could be used in a range of fixation conditions and sample preparations. By an immunofluorescence criterion, most slender forms showed no mitochondrial expression of DHLADH but the protein was clearly expressed throughout the mitochondrion of intermediate and stumpy forms. Expression of mhsp70 is consistently, but marginally, increased in the stumpy form relative to the slender form. Furthermore, there is a clear difference in distribution of mhsp70 in the mitochondrion during differentiation to the stumpy

form ensuring that mhsp70 should also be a useful marker by an immunofluorescence criterion. Another protein associated with mitochondrial import, hsp60, is a good marker of differentiation, however, there is no evidence of regulated expression of hsp60 which is apparently present in slender forms undetected by immunofluorescence.

4.3.2 Screen of morphological parameters

For nearly a century morphology has served as the primary basis by which the slender-to-stumpy differentiation has been assessed. Previous cellular studies which have assessed cell type using morphology have used a range of parameters which included the length of the cells, the basal body to nucleus distance, nuclear position, length of free flagellum and more recently the cell volume. In assessing cells by light microscopy, several of these criteria were used to establish a cellular profile upon the basis of which cells were defined as slender, intermediate or stumpy. The reason for multiple criteria rather than any single feature lies in the high degree of heterogeneity in cellular morphology which has made it difficult to rely on a single morphological parameter to define cell type. In this study morphological parameters were sought which were altered with consistency through differentiation, so that a single morphological parameter could be compared to the acquisition of mitochondrial markers and to exit from the cell cycle. Cell length has been used as such a parameter in the past and this study dissected its readily measured, linear components (flagellar length, free flagellar length, distance from basal body to the posterior tip).

This part of the project was initiated at the level of the electron microscope with analysis of small numbers of cells to determine whether a general rule could be established. Three linear parameters were assessed - flagellar length, free flagellar length and distance from the posterior end to the basal body. Free flagellar length and the distance from the posterior end to the basal body both showed high

degrees of variation in the slender and intermediate forms, the slender form measurements overlapping with those for intermediate and stumpy forms. A general trend based on mean values was, however, confirmed for each parameter evaluated. Interestingly, the monomorphic line used for comparison had more consistent values that did not overlap with intermediate and stumpy form values, which may indicate that some of the forms adjudged slender by morphology were actually early intermediate forms (Fig. 4.5).

One parameter did appear to change consistently with morphological type. This was the length of the flagellar axoneme and this parameter was selected for further characterization (Fig. 4.5A). As the trypanosome flagellum emerges from the flagellar pocket it acquires a paraflagellar rod (PFR - reviewed by Bastin et al., 1996) which is antigenically distinct from other structures in the cell. To look at flagellar length, on statistically viable numbers of trypanosomes, immunofluorescence for the PFR was performed on pleomorphic populations of trypanosomes. Bloodstream form trypanosomes were taken from a mixed (day 4) population and the length of the PFR was compared directly with acquisition of the best mitochondrial marker, DHLADH. The results show significantly shorter PFRs in DHLADH expressing forms (Fig. 4.6). PFR length was therefore chosen as the best available marker of morphogenesis because of the demonstrable consistency with which it varies during differentiation and its accessibility to assay at the level of the electron and fluorescence microscope. PFR length also has the advantage of being easily followed into division since the outgrowth of the new PFR in slender (day 3) populations was already documented (Chapter 3) and questions about stumpy form biogenesis are therefore accessible using this marker. For example, it is possible to look for restriction of the outgrowth of the new PFR in dividing forms as evidence of precommitment to the production of a stumpy form (Chapter 5).

Trypanosomes are a well defined system in which to study morphology. The trypanosome is almost certain to mediate morphological change via the

microtubules of its subpellicular array, since microfilaments and intermediate filaments seem to be absent. During the differentiation to the procyclic form, acquisition of a microtubule associated protein - CAP5.5 (Matthews and Gull, 1994a) - has been localized to structures cross-linking the subpellicular microtubule array (R. Gerke-Bonet, A. Bagerzadeh and K. Matthews; unpublished observations). Similar proteins may also be involved in morphological control of the slender-to-stumpy differentiation. Tubulin itself shows reduced expression in the stumpy form relative to the slender and procyclic forms (Fig. 4.2) and this has been previously reported to be reflected in reduced mRNA levels of the tubulins investigated (Gull et al., 1986). In fact, a number of isotypes of tubulin are present in trypanosome microtubules (Sherwin et al., 1989b, Schneider et al., 1997). Reduced tubulin expression and changes in the ratio of these tubulin isotypes is likely to reflect the transition from a proliferative cell type to a quiescent stumpy form. This is because a stumpy form does not require microtubule synthesis for construction of mitotic spindles or a new microtubule corset for a daughter cell. It is possible, however, that these fluctuations may also have a direct role in mediating the change in morphology as has been suggested for other kinetoplastids (Gallo et al., 1987). In the future it will be important to investigate changes in morphology, exerted at the level of the subpellicular array, as potential markers of differentiation. This is important since it is likely that the mechanisms controlling the change in cell shape differ considerably from the mechanisms limiting flagellar outgrowth or mediating flagellar shortening.

4.3.3 Screen for increased mRNAs in the stumpy form

Differential display analysis has had widespread use in the investigation of RNA levels between two populations and has previously been applied to differentiation in trypanosomes (Murphy and Pelle, 1994). In this project an agarose gel based technique was adapted from a method previously described for mammalian tissue (Sokolov and Prockop, 1994). The primary advantage of this technique was ease of use, specifically because it allowed the identification of stage specific transcripts without involving the incorporation of a radiolabel. The technique is biased toward abundant transcripts which are nuclear encoded. Of the bands isolated, cloned, and sequenced only two trypanosome sequences were obtained. In both cases BLASTn searches, of the dbest database on the National Institute of Health ENTREZ server, identified *T. brucei* expressed sequence tags (ESTs) with nearly complete identity. Back translated (to their predicted amino acid sequence) these ESTs showed good homology to the riboproteins L13A and L9. On Northern blot analysis, probes generated from the original cloned bands showed a pattern of increased mRNA in the stumpy form relative to the slender and procyclic forms. This was confirmed in the case of one of the two transcripts by in situ hybridization which showed increased levels of staining in the stumpy form relative to slender forms.

It was surprising that the quiescent stumpy form should have increased levels of ribosomal protein mRNA since it is unlikely that these cells would show increased levels of translation. In preparation for rapid morphological change, new surface protein expression and re-entry into proliferative growth, however, a pool of sequestered, untranslated ribosomal mRNA in the stumpy form might be advantageous and has a precedent in embryogenesis (Palis and Kingsley, 1995). The possible nuclear localization observed by in situ hybridization of the putative L9 homologue (Fig 4.10A-F) lends support to such a hypothesis, since a nuclear localization would serve to segregate the mRNA from ribosomes and limit

translation of the protein. There is however no direct evidence to support this idea and the mechanisms by which such a sequestration may be achieved are neither clear nor precedented. A nuclear localization of the ribosomal protein transcript would, however, be in contrast to the localization of other transcripts such as VSG and tubulin, which are known to be expressed in the stumpy form: these transcripts localize to the cell's cytoplasm and normally show a region of nuclear exclusion.

4.3.4 Summary

DHLADH, hsp60 and mhsp70 were defined as markers of mitochondrial biogenesis in *T.b.rhodesiense.* The project was able to establish immunofluorescence detection as an effective assay for the appropriate expression of these markers during the slender-to-stumpy differentiation. Immunofluorescence is an assay, permissive of the addition of other markers for co-expression studies, which allows ready assessment of cell cycle position by DAPI staining of nuclei and kinetoplasts (Chapter 3), thereby facilitating temporal studies on the acquisition of the stumpy phenotype (Chapter 5).

Change in PFR length was also established as a marker of morphological change during the slender-to-stumpy differentiation. This flux in the length of the new PFR during division is characterized (Chapter 3), making this a good marker with which to examine the biogenesis of stumpy forms (Chapter 5). The length of the PFR can be accurately assayed by immunofluorescence microscopy as a marker of flagellar length using image analysis software. This is, therefore, a morphological marker likely to be less prone to subjective bias and readily correlated to other fluorescence markers of cell cycle and mitochondrial biogenesis.

Finally, two cDNA fragments, of putative ribosomal proteins, were obtained that recognized mRNAs with increased levels in the stumpy form. This result is suggestive of preadaptive mRNA accumulation for rapid differentiation and proliferative growth.

Chapter 5

The differentiation-division of *Trypanosoma brucei*

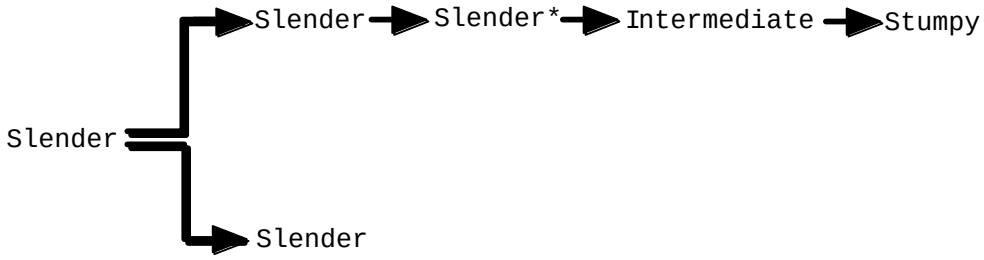
5.1 Introduction

At the outset of this project, general models by which differentiation to a cell cycle arrested phenotype could occur had been described. These models were directed specifically towards the slender-to-stumpy differentiation of *T.brucei.* It is important to distinguish between models such as these in order to gain insight into the mechanisms and coordination of the differentiation process itself. A correct model should also lend insight into the manner by which trypanosome populations are limited and replenished during the course of a parasitaemia. In this Chapter, the mitochondrial and structural, stage-regulated markers established in Chapter 4 are employed. These markers are used to exploit the in vivo system established and cell types observed, in Chapter 3, for the investigation of models based on those originally proposed by Matthews and Gull (1994b) and as reinterpreted in Models 1, 2 and 3.

Stumpy forms arise from proliferating slender forms which are undifferentiated and uncommitted to differentiation. It is generally held that one slender form can give rise to two new slender forms, each of which can give rise to two new slender forms and so on. In this way a single slender form can establish a clonal infection in a host. This sort of division can be referred to as proliferative division. Slender forms can also give rise to stumpy forms which are arrested in G_1 of the cell cycle. This arrest presumably takes place before a restriction point, referred to as START, when commitment to a new round of cell division occurs. At the outset of the project it was not known how slender forms give rise to stumpy forms, but it was envisaged as happening in one of two ways.

1) A proportion of slender forms in G_1 of the cell cycle may simply exit the cell cycle and undergo differentiation to the stumpy form. If this is the only way in which differentiation occurs then all division, including the division which immediately precedes the formation of a stumpy form, will consist of homogeneous, proliferative divisions as described in Model 1. Model 1 describes the normal program of slender form cell division giving rise to two slender forms in the same way as previous divisions. Subsequent to this division, the two undifferentiated and uncommitted slender form progeny would be competent either to undergo subsequent rounds of division or to receive a signal to differentiate. If the signal to differentiate was received by one of the slender daughter cells before commitment to another round of division, then the trypanosome would exit the cell cycle and go on to become intermediate and then stumpy. However, slender forms receiving a differentiation signal later in the cell cycle, after commitment to another round of division, would be required to complete a normal proliferative division before being able to initiate differentiation.

Model 1



Model 1: Proliferative division.
In this model a slender form commits to become stumpy (* denotes commitment to differentiation) and does so without dividing. This implies that the division from which a stumpy form arises is no different from previous divisions.

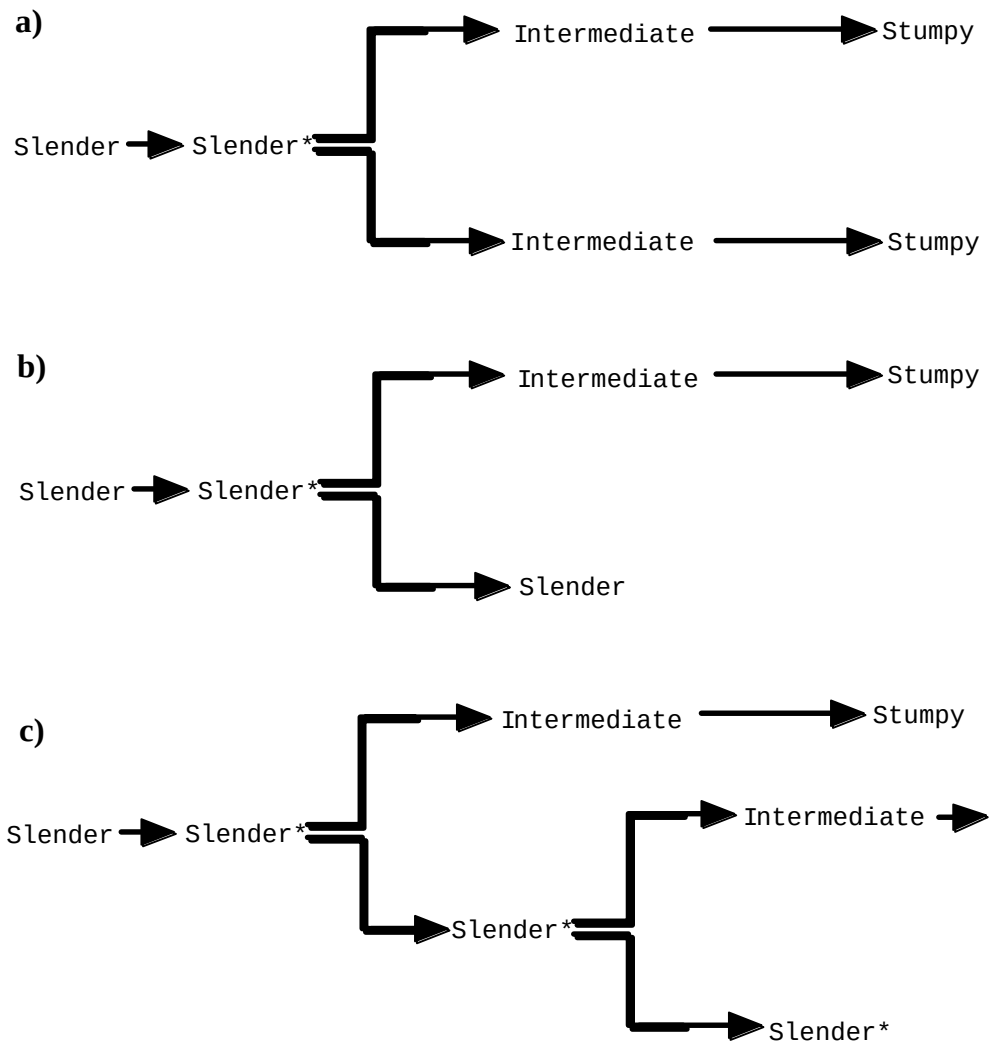
2) Alternatively the slender-to-stumpy differentiation may be initiated before or during cell division. In this case a round of cell division would form part of the slender-to-stumpy differentiation. This cell division would be heterogeneous to previous rounds of proliferative cell division, and could be referred to descriptively as a "differentiation-division" (Model 2). If this was the case, daughter cells would exit the cell cycle after completion of the differentiation-division and then go on to complete the differentiation to the stumpy form. A differentiation-division might be expected to show differences to earlier divisions, such as a heterogeneous rate of production of stumpy forms relative to slender form production. A differentiation-division might also show acquisition of some of the markers of the arrested cell (stumpy) phenotype, such as those characterized in Chapter 4.

A differentiation-division can be modelled either as symmetrical division, both progeny going on to produce stumpy forms without further division (Model 2a), or as asymmetrical division. Asymmetrical division models show one sibling going on to be stumpy without further division. The second sibling, however, may be capable of undergoing proliferative or differentiation-divisions depending on the continued presence of a stimulus to differentiate (Model 2b). Alternatively, the second sibling may already be committed to another similar differentiation-division (Model 2c). Unlike Models 1 and 2a which are straightforward, Models 2b and 2c are simplified and can accommodate further degrees of complexity.

If differentiation must occur solely after division (Model 1), then all dividing-forms will be phenotypically slender. Models of a differentiation-division (Models 2a, 2b, 2c), however, do not fix the temporal onset of an intermediate form phenotype relative to the differentiation-division. This is because an intermediate form is in the process of differentiating and is defined by the acquisition of some,

but not all, of the morphological markers of the stumpy form. Such markers may begin to be acquired at any point after commitment by the slender form to the production of a stumpy form. Consequently, a dividing form might have a predominantly slender, intermediate or stumpy phenotype.

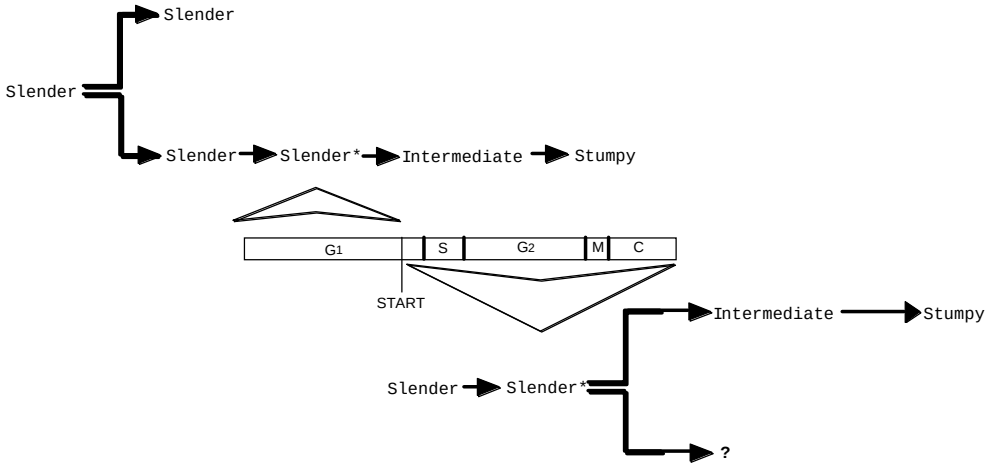
Model 2



Model 2: Differentiation-divisions.
In this model a slender form commits to produce a stumpy form (* denotes commitment to differentiation) and does so by way of division. This implies that the division from which a stumpy form arises is different from previous divisions. The differentiation-division can be symmetric as in Model 2a or asymmetric as in Models 2b and 2c. If a slender form is the product of an asymmetric division it may be uncommitted (Model 2b) or committed to another similar round of division (Model 2c)

It is important that the proliferative division and differentiation-division models are not seen as mutually exclusive. They can be reconciled in a unified model based on the cell cycle (Model 3). It may be that trypanosomes which commit to differentiation prior to commitment to a new round of division (before START) do not undergo a differentiation-division; whereas, trypanosomes that commit after this point must undergo a differentiation-division.

Model 3



Model 3. Cell cycle dependent or unified model.
Stumpy cells may arise by both proliferative and differentiation-divisions if the method of differentiation is dependent on the phase of the cell cycle occupied when the commitment to differentiate (denoted by *) is made. START is the position in the cell cycle after which a cell becomes committed to completing a new round of cell division. If the decision is made prior to START then the slender form (having arisen from a proliferative division) differentiates to the stumpy form without dividing. If the decision is made after START, then the trypanosome undergoes a differentiation-division according to one of the three options shown in Model 2 and summarised here by using ? for the fate of one of the daughter cells produced.

5.2 Results

5.2.1 All bloodstream division occurs prior to substantial morphological or mitochondrial differentiation

Stage regulated antigenic markers were used to address the relative order of events during the slender-to-stumpy differentiation and so distinguish between the models detailed above. To determine whether cells ever initiated differentiation prior to, or during, cell division, dividing-forms were sought during day 4 and day 5 of the primary parasitaemia. At these time-points most trypanosomes showed clear expression of DHLADH by immunofluorescence and so presumably had already exited the cell cycle. It was reasoned that in these populations, the daughter cells of the few dividing-forms remaining would produce trypanosomes that would become stumpy forms without dividing again. Dividing-forms were recognized by the possession of two kinetoplasts and one or two nuclei, as viewed by DAPI fluorescence (See Section 3.2.3). If a substantial number of dividing-forms in these populations had acquired stumpy specific markers or characteristics, then that acquisition would indicate that the commitment to the production of a stumpy form could take place prior to, or during, a differentiation-division .

Using immunofluorescence, dividing-forms were assessed for mitochondrial expression of DHLADH, hsp60 and mhsp70. In Table 5.1 the results from two time-points taken late (day four and day five post-infection) in the primary parasitaemia are shown. Since most cells in these populations had exited the cell cycle, it was necessary to search through over 10,000 cells at some time-points in order to locate just one hundred dividing-forms. It was realized that a subpopulation of the dividing-forms, occurring very late in the primary parasitaemia, might actually represent VSG heterotypes beginning to establish the secondary parasitaemia, before the immune response had cleared the primary parasitaemia, and that the daughter cells of these dividing-forms might continue to proliferate rather than become

stumpy. It was for this reason that dividing-forms were also assessed at the earlier time-point on day 4 of the primary parasitaemia.

For the studies with DHLADH and hsp60 it was possible to categorize most cells as being either "positive" (showing clear mitochondrial staining) or "negative" (showing no clear mitochondrial staining). For the study with mhsp70, however, many cells were assigned to a +/- category of intermediate fluorescence. The results of these studies were then tabulated. With a few exceptions, dividing-forms showed no expression of either DHLADH or hsp60. Most of the exceptional cells expressing DHLADH or hsp60 and having two kinetoplasts and one or two nuclei had an unusual morphology in terms of size, shape and nuclear/kinetoplast positioning. This may indicate that these were cells that had failed to segregate in an aberrant division.

Table 5.1

Antigen assayed	Days post-infection	No. of cells counted	Percent of population +ve for antigen	Dividing cells seen	Dividing cells +ve for antigen	Dividing cells +/- for antigen	Dividing cells -ve for antigen
DHLADH	4	2700	60%	100	0	0	100
	5	13200	92%	100	1	0	99
hsp60	4	2000	67%	103	3	3	97
	5	10000	95%	102	2	4	96
mhsp70	4	2000	95%	100	5	58	37
	5	10000	99%	106	21	23	62

+ trypanosomes showing strong mitochondrial expression by immunofluorescence
- trypanosomes not showing mitochondrial expression by immunofluorescence
+/- trypanosomes showing a weak/intermediate mitochondrial expression by immunofluorescence

Table 5.1 Trypanosomes in the process of division assayed by immunofluorescence do not generally show staining for DHLADH or hsp60. Immunofluorescence for DHLADH, hsp60 and mitochondrial hsp70 was performed on populations of trypanosomes taken from mouse blood 4 days and 5 days post-infection. A search was made for dividing-forms possessing 2 kinetoplasts and one or two nuclei visualized by DAPI stain. For each sample, approximately 100 dividing-forms, were assessed for staining of the mitochondrion. When assessed for DHLADH and hsp60 very few of these cells showed detectable mitochondrial staining, however, mhsp70 staining was detected in a substantial number of cells.

DHLADH and hsp60 expression is detectable by immunofluorescence in the vast majority of intermediate forms and some slender forms (Section 4.2.3, Table 4.2), but not in the dividing trypanosomes of differentiating populations (Table 5.1). Taken together these observations imply that the majority of mitochondrial and morphological changes, which occur during the course of the slender-to-stumpy differentiation, take place after completion of the cell division directly preceding the formation of the stumpy form.

Significantly, staining of dividing-forms for mhsp70 showed distinct, albeit weak, staining of the mitochondrion in many such cells (Table 5.1 and Fig. 5.1F). In fact, in both the populations assayed, the level of mhsp70 appeared to have increased in a majority of dividing-forms. This may be indicative of expression beginning to increase in some dividing-forms. Since mhsp70 expression is increased in some but not all dividing-forms there must be at least some degree of heterogeneity in cell division. Moreover, since mhsp70 is not expressed in most slender forms but is expressed in most intermediate and stumpy forms, the expression of mhsp70 during division strongly suggests that commitment to differentiation occurs prior to or during a differentiation-division. It does appear that fewer dividing-forms show increased levels of mhsp70 in the day 5 post-infection population relative to the day 4 post-infection population. This may be because the day 5 population contains a larger proliferative subpopulation of VSG heterotypes than the day 4 population and that dividing-forms of this population tend not to be in a differentiation-division.

In Figure 5.1 the intermediate level of staining for mhsp70 in some dividing-forms is compared with the corresponding lack of staining seen for DHLADH (Fig. 5.1B) and hsp60 (Fig. 5.1D) and with the localization of the dividing mitochondrion visualized using immunofluorescence for TAO (Fig. 5.1H). This figure is presented to reinforce the idea that bloodstream divisions are heterogeneous, that differentiation can proceed by a differentiation-division , but that most of the phenotypic changes probably occur only after cytokinesis of the differentiation-division.

Figure 5.1

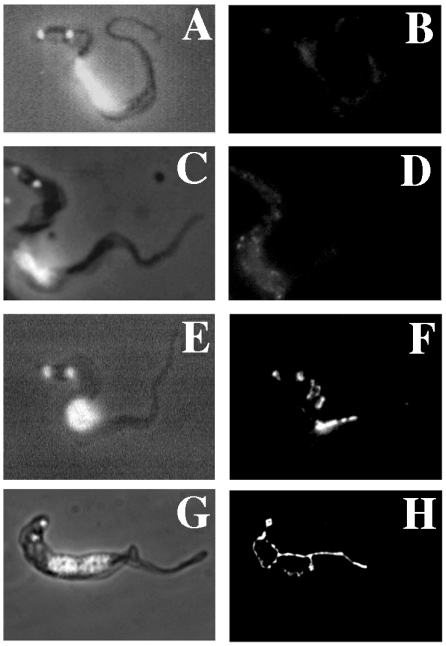


Fig. 5.1 Expression of markers in dividing-forms, when assayed by immunofluorescence. The figure shows phase-contrast images merged with DAPI fluorescence to allow the kinetoplasts and nuclei to be used as markers of the cell cycle and define dividing-forms (**left column**). The corresponding images (**right column**) are the results from immunofluorescence assay. When immunofluorescence assay was performed for DHLADH (**A, B**) the vast majority of cells showed no significant staining of the mitochondrion. This was also the case for hsp60 immunofluorescence assay (**C, D**). Immunofluorescence assay for mhsp70 (**E, F**) showed an increase in expression in some but not all dividing- forms. Immunofluorescence assay for TAO (**G, H**) stained the mitochondrion in all such cells revealing its branched appearance during cellular division.

5.2.2 Commitment to the production of a stumpy form

The significant mhsp70 expression in many dividing-forms indicated that the commitment to make a stumpy form occurred prior to or at the time of a differentiation-division. To confirm this result we sought additional evidence that some dividing-forms had already made a commitment to production of a stumpy form. Such evidence appeared to be available from examination of anucleate cytoplasts (termed "zoids") which occur naturally in trypanosome populations (Matthews and Gull, 1994b; Section 3.2.2) and arise by aberrant cytokinesis (Robinson et al., 1995). Lacking a nucleus, zoids cannot transcribe mRNA for nuclear encoded genes *de novo* and must receive mRNAs for any such proteins from the "mother cell" prior to cytokinesis. The vast majority of mitochondrial proteins are nuclear encoded, but despite this zoids clearly all possess a mitochondrion as revealed by immunofluorescence with the antibody to TAO (Fig. 5.2F, H), which is also nuclear encoded (Chaudhuri et al., 1996). It was therefore decided to look at bloodstream trypanosome populations by immunofluorescence microscopy to determine whether zoids express DHLADH (Fig. 5.2B, D).

In populations predominantly composed of slender forms, most zoids had a slender morphology and did not express levels of DHLADH detectable by immunofluorescence (Fig. 5.2B). By contrast in populations predominantly composed of stumpy forms, most zoids shared some of the morphological characteristics of the stumpy form (Fig. 5.2C, G) and so have been termed "stumpy zoids" (Matthews and Gull, 1994b). These "stumpy zoids" were present at levels up to 0.5% of the population and were observed to show clear mitochondrial staining for DHLADH expression by immunofluorescence microscopy (Fig. 5.2D). This result was confirmed using diaphorase staining and immunofluorescence for hsp60 and mhsp70 and, in each case zoids, were observed which clearly showed mitochondrial staining with these nuclear encoded markers (Fig. 5.2E, F, G).

Figure 5.2

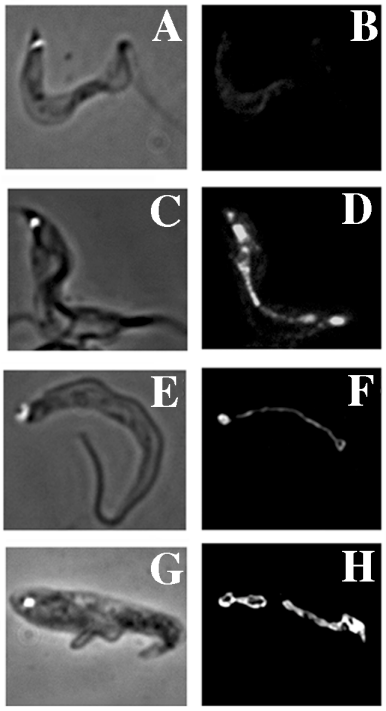


Fig. 5.2. Anucleate forms, "zoids", can show clear staining for DHLADH by immunofluorescence assay. Populations of trypanosomes from the bloodstream of mice 3 days and 5 days after infection were assayed by immunofluorescence (**right column**) with DHLADH (**B, D**) and TAO (**F, H**): the corresponding images (**left column**) are images obtained by phase-contrast merged with DAPI staining, these images show the morphology of these forms and that each form has a kinetoplast but no nucleus. Zoids observed in the day 3 population appeared slender and showed mitochondrial staining with TAO (**F**) but not with DHLADH (**B**). Zoids observed in a day 5 population showed some morphological characteristics of a stumpy trypanosome and showed mitochondrial staining with both TAO (**H**) and DHLADH (**D**).

Since DHLADH mRNA cannot be transcribed *de novo* by an anucleate zoid, these results indicate that at least some of the nuclear encoded stumpy specific mRNA, including DHLADH mRNA, is made prior to (or during) the differentiation-division. The existence of stumpy zoids implies that the mRNA of stumpy specific proteins can accumulate prior to cytokinesis of the cell cycle preceding the formation of a stumpy form. This prefabrication of mRNAs in turn

suggests that some dividing-forms have already decided to produce stumpy forms and that differentiation can therefore proceed by a differentiation-division. In the case of DHLADH, translation of these mRNAs would follow cytokinesis and would then contribute to the establishment of first intermediate and then stumpy phenotypes.

5.2.3 Expression of DHLADH by zoids is not proof of a differentiation-division

Stumpy zoids are evidence that nuclear encoded trypanosome transcripts, for stumpy specific proteins such as DHLADH, are present at the division from which the stumpy form arises. They are not, however, necessarily evidence that a transitional or commitment point, in differentiation to the stumpy form, is reached at or prior to division. Instead it is possible that the mRNA encoding stumpy specific proteins are present in all slender and dividing-forms. If this is the case, the observation of a stumpy zoid could mean that no mRNA production, or nuclear interaction, is required for the acquisition of stumpy mitochondrial and morphological markers. For instance a membrane bound receptor could be triggered to release a secondary messenger cascade which would be capable of activating the translation of stumpy specific proteins from mRNA already present in the cell. If levels of DHLADH mRNA were regulated during differentiation, however, then such possibilities could effectively be eliminated.

To determine whether DHLADH mRNA levels were increased during the slender-to-stumpy differentiation, mRNA levels in slender and stumpy populations were compared on a northern blot of total mRNA. Partial cDNA of the DHLADH gene was generated as a probe. The cDNA fragment was cloned by amplification of first strand cDNA using primers selected by analysis of the known gene sequence (Else et al., 1993). The fragment was cut at two internal restriction sites and ligated into the multiple cloning site of pBSK+. The fidelity of the cloned fragment was then confirmed by sequencing.

Sense and antisense riboprobes were then generated and used to probe a preliminary northern blot (Fig. 5.3). The antisense probe detected a single transcript of approximately 3Kb in both the slender and stumpy form (Fig. 5.3B). The loading control (Fig. 5.3A) shows that the stumpy lane may be somewhat underloaded, and this may explain why the stumpy signal appears somewhat weaker than the signal from the slender sample. Although this blot is of relatively poor quality and must be repeated in the future, it seems to show a substantial amount of DHLADH mRNA in the slender form and clearly provides no evidence of increased mRNA levels in stumpy populations. The sense probe (Fig. 5.3D), included as a negative control, did not hybridize to the blot. With both the sense and the antisense probe a negative image of the ribosomal bands could be discerned. It is not clear why this is the case but it may be because the ribosomal RNA blocked non-specific binding of either the probe or anti-DIG antibody to the membrane. Since this result indicated that there was no obvious upregulation of the mRNA level during the slender-to-stumpy differentiation; it would seem to follow that the regulation of DHLADH expression is controlled either translationally or by post-translational mechanisms such as rapid degradation of the newly formed protein.

This northern blot implies (Fig. 5.3) that DHLADH mRNA is present in all slender forms and that DHLADH expression is not related to an increase in the mRNA levels. Consequently, the expression of DHLADH by stumpy zoids cannot be taken as direct evidence of a differentiation-division. It remains possible that the existence of stumpy zoids may provide evidence of a differentiation-division. The lack of a marker regulated at both the mRNA and protein levels means, however, that another explanation remains a possibility. In effect, the possibility that slender zoids can receive a differentiation stimulus and differentiate to stumpy zoids has not been eliminated.

Figure 5.3

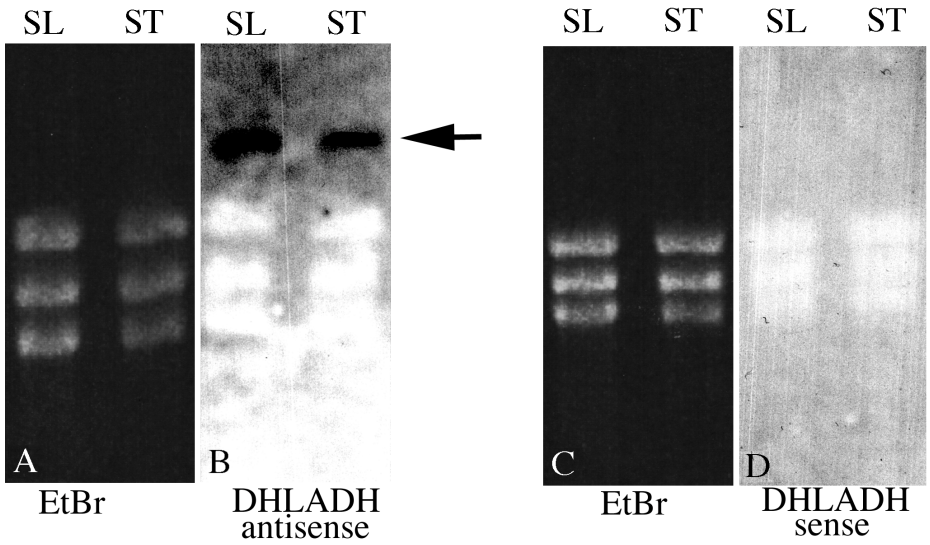


Fig. 5.3 Preliminary northern blot for DHLADH. Total RNA from slender (SL) populations (day 3 post-infection) and stumpy (ST) populations (day 5 post-infection) was blotted and then probed with antisense (B) and sense (D) riboprobes. These were generated by in vitro transcription of a cloned fragment of DHLADH. Although the stumpy lane is underloaded relative to the slender lane, it is clear that there is no obvious upregulation of the approximately 3Kb DHLADH mRNA transcript which is indicated with an arrow. Indeed from the appearance of the blot the level of mRNA seems to be constant between the slender and stumpy forms. The sense probe shows no evidence of hybridization under identical hybridization conditions. Ribosomal RNA bands were used to assess loading in the absence of an obvious loading control.

5.2.4 The new PFR does not reach the same length during a differentiation-division as during proliferative division

Flagellum length is a component of the bloodstream form trypanosome's total length, which is consistently shorter in differentiating (intermediate) and differentiated (stumpy) forms (see Section 4.2.6). Outgrowth of the new flagellum is itself a marker of division in slender form trypanosomes, and flagellar outgrowth is one of the first discernible cytological events in division and continues until cytokinesis. In Section 3.2.3 outgrowth of the PFR, which is a structure associated with the flagellar axoneme once it exits the flagellar pocket, was followed during the later stages of the cell cycle and was shown to occur at an apparently linear rate.

In slender populations, by the time of cytokinesis the new PFR has reached a mean length of approximately 26μm. Although this is still (on average 15%) shorter than the old PFR, it is considerably longer than the mean PFR length of DHLADH expressing forms which is approximately 19μm (compare Fig. 5.4b with Fig. 4.6). It would seem that this difference in lengths could be explained in two ways. 1) It could be that after cytokinesis was complete one or both daughter cells would begin a program of flagellar shortening to produce shorter cells. 2) It could also be that the new PFR of dividing-forms in a differentiation-division did not reach the full length which was reached by the new PFR of preceding proliferative divisions.

Indeed, if dividing-forms had already committed to the production of a stumpy form then the prediction that the new PFR would not grow to the full length reached in earlier rounds of proliferative division could make sense biologically. This is because, if the PFR reached full length it would have to undergo subsequent shortening which would be inefficient. Production of a new PFR shorter than that produced in previous rounds of division would also be consistent with similar observations made of asymmetric division in both the promastigote to amastigote differentiation in *Leishmania sp*. (LeBowitz, Bastin and Gull; unpublished

observation) and during proventricular to epimastigote transformation in *T.brucei* (Van Den Abeele and Le Ray, personal communication).

106 dividing-forms, assessed to be committed to division by the possession of two paraflagellar rods, were assayed from a population which was taken late on day 4 post-infection and of which 64% of cells expressed DHLADH by immunofluorescence. The new and old PFRs were highlighted, by immunofluorescence assay for PFR proteins, captured photographically for each cell and then measured using IPlab software. Dividing-forms were then categorized into each of the five phases of division described in Figure 3.6. Outgrowth of the new PFR in the dividing-forms from this differentiating population was compared with the data for the 100 dividing-forms (described in Figure 3.5) derived from a slender population. The data from each population was broken down into the mean PFR lengths at each of these five phases of division which were then compared.

Figure 5.4 shows this comparison; the error bars represent one standard error about the mean. Only in the near-cytokinesis forms, stage 5, was the mean new PFR length of dividing-forms drawn from a differentiating population found to be considerably (16%) shorter than the mean new PFR length of dividing-forms (at the same stage) in slender populations. However, Figure 5.4 does indicate that the onset of morphogenesis, with regard to reduction in new PFR outgrowth, could begin even earlier. It is also interesting that the proportion of cells in each of the five phases is considerably different between populations, in particular over 50% of the dividing-forms in the early population occupied phase 1, whereas less than 40% of dividing-forms in the differentiating population were in phase 1. Also, phase 5 made up only 6% of the dividing-forms in the slender population but over 15% of dividing-forms in the differentiating population. Although these differences may not be statistically significant given the small number of cells assessed, they may indicate a heterogeneous rate of progress through the cell cycle by dividing-forms in different populations. In particular they may indicate that progress through the cell-cycle is

slowed close to cytokinesis in a differentiation-division. This would be consistent with the heterogeneous rates of division observed by Turner and colleagues (Turner et al., 1995).

Statistical analysis of the differences in the mean PFR length during phase 5 was performed using a t-test for independent samples. In spite of the quite large difference between the means, the small sample numbers involved (Table 5.2) meant that the difference was not significant and might still have been a result of sample error. The statistics used for the t-test are shown below Table 5.2. Ideally the measurement of the PFR lengths from at least 30 phase 5 cells from a slender population and, at least 30 phase 5 cells from a differentiating population should be compared in the future. Even so, the constitution of all populations are mixed with regard to the slender, intermediate and stumpy cell types. Consequently, if phase 5 cells of a differentiation-division are long lived compared with phase 5 cells of a proliferative division, it may be that phase 5 cells of differentiation-divisions are more frequent than might be expected in slender populations. This may affect the ability of significance testing to distinguish two types of division.

In spite of the lack of a statistically significant result, this data, when coupled with other evidence of a heterogeneous differentiation-division, implied that phase 5 cells in a differentiation-division might be discriminated from phase 5 cells undergoing proliferative division using the length of the new PFR. Near-cytokinesis (phase 5) forms were assessed by the length of their new PFR and putatively categorized as being in either a differentiation-division or a proliferative division. The two putative categories were then compared directly in photographs and the other morphological differences which were observed were then evaluated.

Figure 5.4

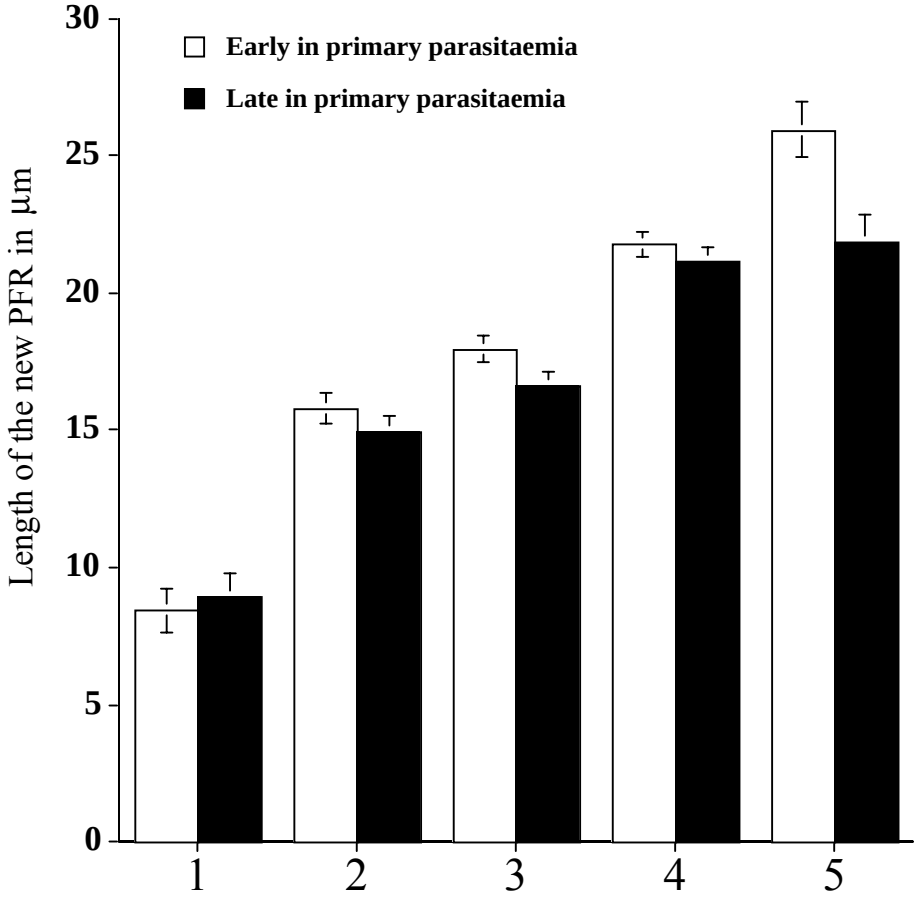


Fig. 5.4 Outgrowth of the new PFR appears to be reduced during division in differentiating populations. From each population, immunofluorescence for the PFR was performed and the new and old PFR lengths of 100 dividing-forms measured. The phases of division were as previously defined in Chapter 3, Figure 3.6. The data which shows how outgrowth of the new PFR is reduced in the near-cytokinesis forms (phase 5) is presented as the mean values with standard error bars (5.4b). Trypanosomes from the later population are represented by the dark bars, those from the earlier population by open bars.

Table 5.2

	1	2	3	4	5	Total
n_1	52	11	15	16	6	100
n_2	39	12	23	17	16	106

Table 5.2 shows the number of cells measured for new PFR length in the slender, day 3, population (n_1) and late day 4 population (n_2). A t-test for two independent samples was conducted on the two populations for phase 5. The statistics used are shown below. In spite of the substantial difference between the means no significance can be shown, due to the low values of n.

$n_1 = 6$ $n_2 = 16$

$mean_1 = 25.94$ $mean_2 = 21.87$

$\sum X_1 = 155.6$ $\sum X_2 = 350$

$\sum X^2_1 = 4044$ $\sum X^2_2 = 7926$

$s_1 = 1.16$ $s_2 = 4.25$

$SE_1 = 0.52$ $SE_2 = 1.1$

$t_{crit}(P<0.05) = 2.080$ $t_{obt} = 1.284$

Figure. 5.5 shows cells which appear to be undergoing the two different types of division and which differ morphologically, particularly with respect to the nuclear shape. In the case of near-cytokinesis forms apparently undergoing proliferative division, the nuclei appear oval as do slender nuclei. In contrast, the near-cytokinesis cells apparently undergoing a differentiation-division show the characteristic roundness of stumpy and intermediate form nuclei. Trypanosomes in their differentiation-division also appear to be predominantly broader, and shorter, than the proliferative division forms, although these parameters have not yet been quantitatively analyzed.

Figure 5.5

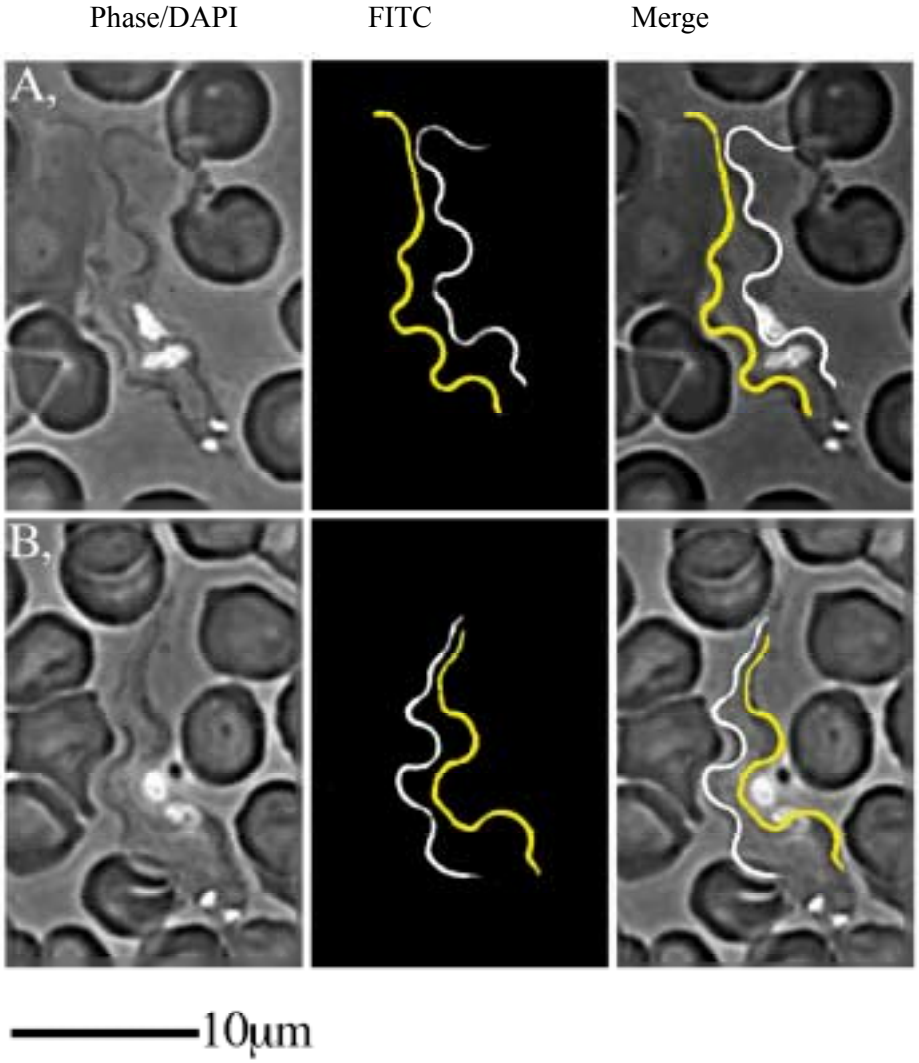


Fig 5.5 New PFR length can be used to identify cells undergoing the differentiation-division.
Following staining of the PFR by immunofluorescence, dividing-forms were photographed and the length of the new PFR measured. The new PFR is shown in yellow and the old PFR in white for convenient identification. Near-cytokinesis forms, which had new PFR lengths more than 25μm, were proposed to be in a proliferative division (A). Those that had a new PFR length of less than 21μm were proposed to be in a differentiation-division (B). The Figure shows a direct comparison of representative cells of each type in order to highlight the differences in morphology. In particular the appearance of the nuclei is considerably different. The nuclei are stained with DAPI and merged with the phase contrast image in the left-hand panels.

5.2.5 Shortening of the old PFR

The evidence of mhsp70 expression in dividing-forms, of DHLADH expression by stumpy zoids and of a shortened new PFR in the near-cytokinesis (phase 5) forms of differentiating populations, indicated that the slender-to-stumpy differentiation encompassed a differentiation-division. It seemed possible that the length of the old PFR might be useful in determining which sort of differentiation-division was occurring.

In Model 2a both of the siblings of the differentiation-division go on to become stumpy without further division. If this is the case then the PFR of the daughter cell which inherited the old flagellum would have to shorten considerably during differentiation, in order to approach the mean PFR length of a stumpy form. Such a flagellar shortening program might be well under way during the differentiation-division. Consequently, the lengths of the old flagella in near-cytokinesis forms were measured in a differentiating population and compared with data from a slender population for evidence of shortening. There is a very large degree of variation in old PFR length and the population used may not have been large enough to show that shortening of the new PFR was occurring during the differentiation-division. The mean length of old PFR from early populations, in near-cytokinesis forms, was 27.3μm (n = 6, standard error of 2.5μm). The mean length of the old PFR from late populations, in near-cytokinesis forms, was 26.7μm (n = 16, standard error of 1.6μm). The marginal reduction in mean old PFR length observed could still be consistent with old PFR beginning to shorten during division. A much larger sample group would be required to look for evidence of shortening in near-cytokinesis forms.

If only the trypanosome which receives the truncated new PFR during a differentiation-division went on to become a stumpy form (which would be consistent with Models 2b and 2c), then intermediate forms would never be longer than the length of the PFR at cytokinesis of the differentiation-division (mean length 22μm). Consequently, "long" intermediate forms, defined by a slender morphology and the acquisition of DHLADH, were sought. In samples taken from a parasitaemia four days after infection these long intermediate forms were found to make up over 4% of the population. Long intermediate forms were defined for the purposes of these counts as expressing DHLADH and having a PFR length in excess of 24μm. The example shown in Fig. 5.6 has a PFR length of 28μm.

Fig 5.6

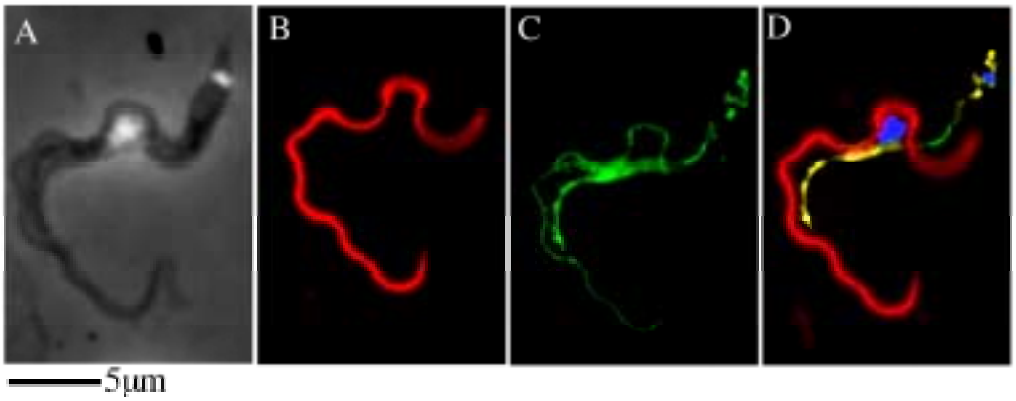


Figure 5.6 shows double immunofluorescence assay performed on a "long intermediate" form trypanosome. A is the phase contrast DAPI image of the trypanosome which typically has a slender morphology, B (TRITC) is stained with α-PFR antibody which marks the flagellum, PFR lengths were measured using IPlab software. The PFR of this example is over 28μm long. C (FITC) is stained for DHLADH expression in the mitochondrion. The PFR also shows some "leaky" fluorescence from the TRITC channel. D shows the merged fluorescence images, allowing the mitochondrion (green) to be clearly discriminated from the flagellum (red). Also included is the localization of the nucleus and kinetoplast by DAPI fluorescence (blue).

This result focuses the debate on two possible mechanisms. Long intermediate forms may arise from the differentiation-division as the cell possessing the old flagellum (Model 2a). These would then subsequently shorten the flagellar length as the cell completes morphogenesis. Alternatively, the differentiation-division may not be obligatory and some of these long intermediate forms may be derived from the direct differentiation of a slender to a stumpy form without intervening division, as in the unified model (Model 3).

5.3 Discussion

5.3.1 Cell division and commitment during stumpy form generation studied with mitochondrial markers

Two basic models describe the way in which slender forms give rise to stumpy forms. Model 1 entails a slender trypanosome committing to become stumpy after the completion of a proliferative division, such that differentiation occurs without further cell division. In this scenario, the expectation would be that none of the stumpy enriched markers would show substantially increased expression in any dividing bloodstream trypanosomes. By contrast, in Model 2 slender forms may commit to differentiation prior to a differentiation-division, a division that is different to previous proliferative divisions and in which stumpy specific markers may begin to be expressed. It is possible that stumpy forms arise in both ways and so these models can be combined in a cell cycle dependent manner (Model 3).

To distinguish between these models, the expression of DHLADH, hsp60, and mhsp70 in populations late in the primary parasitaemia was investigated. Such populations consist predominantly of intermediate and stumpy forms and it was reasoned that many of the dividing-forms in these populations would be in flux between the slender and stumpy form. When DHLADH and hsp60 expression was examined, less than 5% (1% for DHLADH) of trypanosomes that were in the process of division showed detectable mitochondrial fluorescence. In contrast to this, the vast majority, if not all of the cells which were morphologically intermediate or stumpy, demonstrated clear expression of these markers. This indicates firstly that DHLADH and hsp60 expression are events that occur after division. Secondly, the observation that all intermediate forms express these markers, whereas dividing-forms never express them, indicates that the intermediate forms do not divide and are a product downstream of division. Together, this suggests that the major phenotypic changes associated with production of the stumpy form occur after the terminal bloodstream

division and, that cells in this division are predominantly slender by morphological criteria.

Although the hsp60 and DHLADH analyses are compatible with both Models 1 and 2 describing the generation of a stumpy cell, analysis of mhsp70 expression distinguishes between them. Expressly, when a differentiating population was examined for dividing-forms, it was found that approximately one half of these demonstrated increased mhsp70 expression with respect to the weakly staining slender population. This suggests that early differentiation events may precede, or coincide with, the differentiation-division and positions the commitment to differentiation in the cell cycle prior to this terminal division (i.e. Model 2). A model in which commitment occurs prior to the differentiation-division is consistent with data from growth rates and mathematical modelling (Turner et al., 1995). These mathematical models accommodate a final division which is heterogeneous with previous divisions, taking longer to complete. This may suggest that the decision to differentiate is made prior to, or at, the time of this division. These mathematical models have also suggested a substantial time delay in the emergence of stumpy forms. This time delay could be explained by the time taken after the differentiation-division, for the transition between the morphological extreme of the slender and the stumpy form, via heterogeneous intermediate morphologies. If as proposed, commitment precedes, but most morphogenesis follows division, it would mean that a differentiation-division would not have to accommodate much concomitant structural change associated with the slender-to-stumpy differentiation.

Commitment to differentiation may be marked by the prefabrication of mRNAs specific to the next life cycle stage. If commitment to the production of a stumpy trypanosome is made prior to the differentiation-division, it was reasoned that this might be reflected in a peculiarity of trypanosome biology, the zoid. Zoids are motile, anucleate cytoplasts with a flagellum, kinetoplast and basal body and which apparently arise by an aberrant mitosis (Robinson et al., 1995). Lacking a nucleus,

zoids, are unable to transcribe the nuclear gene DHLADH *de novo*. It was therefore reasoned that if zoids did exist which expressed this protein, the mRNA must have been transcribed during or prior to the differentiation-division. Zoids which showed clearly detectable DHLADH expression by immunofluorescence were therefore sought, successfully. Since, in dividing-forms DHLADH expression was not detectable (and in zoids DHLADH expression is detectable) the mRNA for increased DHLADH expression must be transcribed prior to cytokinesis of the differentiation-division and then translated and processed subsequently by the zoid. Although the release from a nuclear encoded negative regulator of differentiation could generate this result, this seems unlikely since 'slender zoids' also exist earlier in the parasitaemia and these never demonstrate the expression of stumpy specific markers. Instead, the expression of stumpy specific markers enriched in zoids may imply that the commitment to differentiation occurs prior to the terminal division and that this is then followed by morphological transformation.

The primary marker used, DHLADH, demonstrated no difference in mRNA levels between the slender and stumpy form. This result meant that the zoid data was open to alternative interpretation. It is possible that solely slender zoids are produced from aberrant cytokinesis during proliferative division. These slender zoids would not be committed to becoming stumpy forms but rather would differentiate, in response to a differentiation stimulus, to stumpy forms since all the mRNA necessary would already be present in the cytoplasm of a slender zoid. The implication would be that the slender-to-stumpy differentiation was achieved solely by a plasma membrane/cytoplasm mediated mechanism, presumably involving activation of stumpy specific protein translation. To test this hypothesis it would be necessary either to develop markers which are regulated at a mRNA as well as a protein level, or to determine whether exposure of slender zoids, from slender populations, to a differentiation stimulus would induce differentiation to stumpy zoids. It should be possible to determine whether slender forms can differentiate to stumpy forms

without the need for additional transcription by using the cAMP analogue, pCPT, as recently used by Vassella and colleagues for cultured bloodstream forms (Vassella et al., 1997), in conjunction with a transcriptional inhibitor.

5.3.2 Length of the new PFR defines the differentiation-division

Previous studies of trypanosome flagellum and PFR length have been conducted almost exclusively on the procyclic form (Sherwin et al., 1987; Robinson et al., 1995). These have shown that the majority of PFR outgrowth occurs during division and that this outgrowth can continue outwith of division for some time but then stops. Consequently, there is minimal difference in the PFR lengths of most procyclic forms with a single flagellum.

This is not true in the case of slender form trypanosomes which show a large degree of heterogeneity in their PFR lengths. PFR lengths may vary by over 40%, and potentially continue to grow for several cell cycles after formation. This is reflected in heterogeneity of the old PFR of dividing-forms (Figure 3.5). It is this heterogeneity which makes the old PFR a poor marker with which to study the differentiation-division.

In contrast, the consistency in the rate of new PFR outgrowth (Figure 3.5) makes the new PFR a suitable marker of division. PFR length can also be used as a marker of the slender-to-stumpy differentiation since it is shorter in stumpy forms than in slender forms. My results indicated that the mean PFR length of forms expressing DHLADH was less than the mean new PFR length of near-cytokinesis forms in slender populations. I reasoned that if a cell was already committed to producing a stumpy form, it would be inefficient to construct a long PFR and then shorten it during differentiation. In a differentiation-division, the new PFR was likely to be shorter than in a proliferative division. Near-cytokinesis forms can be defined by the proximity of their kinetoplasts which, like the nuclei and other organelles, align themselves as the cleavage plane is established (Fig. 3.6). In differentiating

populations such cells did indeed appear to have shorter new flagella than in slender populations. Unfortunately sample sizes were too small to determine whether this difference was significant. Nevertheless, supported by evidence of a differentiation-division from the studies with mhsp70 and DHLADH, an assumption was made that the difference was significant. Given this assumption it was possible to categorize those near-cytokinesis forms, as being in a differentiation-division or a proliferative division, by measurement of the new PFR length.

When near-cytokinesis forms were categorized by the length of their new PFR it was possible to directly compare cell types putatively categorized as undergoing a differentiation-division or a proliferative division. This kind of direct comparison (Fig. 5.5) highlighted other morphological differences. The oval nuclei of cells undergoing proliferative division are characteristic of slender forms, whereas, the nuclei of differentiation-division forms are round like stumpy form nuclei, although apparently smaller and more condensed. Proliferative division forms were also longer and narrower than those in differentiation-division.

Evidence of a shortened new PFR close to cytokinesis, and morphological differences in division supported by increased expression of mhsp70 in some dividing-forms and by the incidence of DHLADH expressing zoids taken together strongly suggests that cell division can occur as part of the program of differentiation to the stumpy form. As detailed in the introduction of this Chapter, however, several different types of differentiation-division are possible (Models 2a, 2b, 2c).

It seems reasonable that if the new PFR is shorter during a differentiation-division, that this implies that the daughter cell which inherits the new PFR is destined to become stumpy. Since the mean new PFR length of near-cytokinesis forms from differentiating populations (22μm) is within the normal range of stumpy form PFR lengths (16-23μm), it may be that there is little flux in PFR length thereafter. If the other daughter cell of the differentiation-division, which has inherited the old PFR, effects further division (as in Models 2b or 2c), it could

follow that intermediate or stumpy forms would never have a PFR length longer than 22μm. To determine if this was the case trypanosomes in a differentiating population were assayed by double immunofluorescence for DHLADH expression and for PFR length.

Trypanosomes with an apparently slender morphology, which expressed DHLADH and possessed PFRs which were substantially longer than 22μm, were readily found. This result may further refine the models since, if a differentiation-division is an obligatory part of differentiation, it implies that models 2b and 2c are incorrect. This means that an obligatory differentiation-division would produce two daughter trypanosomes that would then go on to produce stumpy forms without further division (Model 2a). The daughter trypanosome which inherited the old PFR would be longer than the other daughter which inherited the new PFR and would need to activate a program of flagellar shortening during subsequent differentiation.

Support for Model 2a can be drawn from recent in vitro work in which a population of slender forms were accelerated through differentiation by the presence of the cAMP analogue pCPT. The study showed that 100% of the population occupied G_1 within one cell cycle (Vassella et al., 1997). This implies that the products of a differentiation-division must both arrest (and go on to become stumpy) without further division. Neither set of experiments, however, rules out the possibility of a unified model.

In my opinion, it is more likely that a tightly co-ordinated process like differentiation will proceed by one mechanism only since a second mechanism would seem to add an unnecessary tier of complexity. I have, therefore, proposed a working hypothesis based on Model 2a and summarized in Figure 5.7 In summary, the work to date on investigation of the mechanism of differentiation has refined the original models to a choice between Model 2a alone and Model 3 (which may incorporate Model 2a).

Figure 5.7

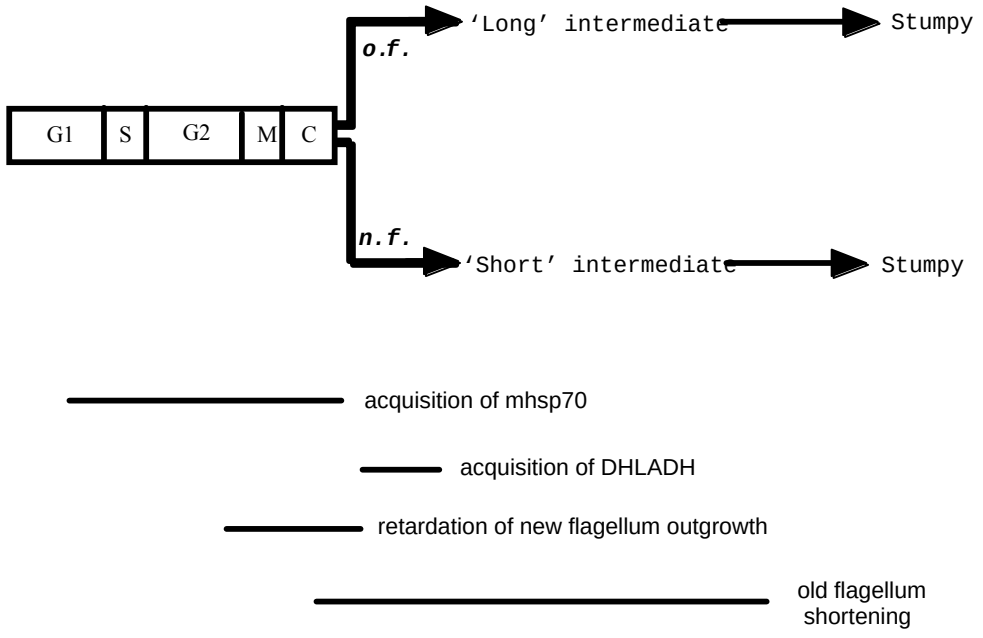


Fig. 5.8 Working hypothesis for the temporal events of the slender-to-stumpy differentiation. It is proposed that the slender-to-stumpy differentiation incorporates an obligatory differentiation-division and that both daughter cells go on to become stumpy without further division, as in Model 2a. This division is asymmetric in so far as the daughter cell inheriting the old flagellum ***(o.f.)*** can be considerably longer than the one inheriting the new flagellum ***(n.f.)***. This necessitates a program of flagellar shortening which may not be required by the daughter which inherits the new flagellum. A relative map of the differentiation events examined is shown. Acquisition of mhsp70 and retardation of the new flagellum is detectable during cell division. Acquisition of DHLADH and old flagellar shortening occurs predominantly thereafter.

Chapter 6

Addendum:

Differentiation and division of the bloodstream forms of *T. brucei* in mammalian hosts - A theoretical and speculative discussion of related topics

6.1 Mechanism of the slender-to-stumpy differentiation

In pleomorphic trypanosomes a lethal peak parasitaemia is normally avoided by the differentiation of the proliferative long slender form to the nonproliferative short stumpy form. Exit from the proliferative cell cycle during the slender-to-stumpy differentiation limits population expansion. This is thought to allow time for the host immune system to react and to eliminate the vast majority of trypanosomes. In comparison monomorphic lines, which have lost the ability to differentiate to the stumpy form and do not exit the cell cycle, reach a lethal parasitaemia before they can be controlled by the immune system.

Modulation of the slender-to-stumpy differentiation is therefore implicated in the virulence and pathogenicity of the African trypanosomosis and it would be highly desirable to define any signal which initiates this differentiation. The nature of the differentiation signal however remains controversial. The slender-to-stumpy differentiation does not occur following a fixed number of divisions (Michelotti and Hajduk, 1987), and is not mediated by the immune system (Balber, 1972; Luckins, 1972). The signal to differentiate must be labile, in order that the slender heterotypes can establish a new homotype once the immune system has eliminated the old one and the signal must also be non-immunogenic, or it would be neutralised by the immune system during a prolonged infection. It also seems increasingly likely that the signal to differentiate is transduced primarily by cAMP as a second messenger (Mancini and Patton, 1981; Vassella et al., 1997).

It is thought that the slender-to-stumpy differentiation is density dependent (Seed and Sechelski, 1989) although this is controversial (see Turner et al., 1995). If the differentiation is density dependent, the differentiation signal is likely to fall into one of three categories. A host derived factor which accumulates during the ascending parasitaemia, a host derived factor which is depleted during the ascending parasitaemia or a parasite derived factor which is accumulated during the ascending parasitaemia.

Monomorphic trypanosome lines represent a potential tool for the investigation of the slender-to-stumpy differentiation. Monomorphic lines are unable to undergo complete differentiation to the stumpy form in a specified (typically rodent) host. This monomorphic phenotype may result from lesions in one of three pathways producing either an inability to sense (or transduce) a stimulus to differentiate, an inability to manufacture a stimulus to differentiate or an inability to exit the cell cycle. In theory, co-infection of monomorphic and pleomorphic lines can be used both to identify the lesion of a monomorphic line and the nature of the signal to differentiate.

Depletion of a host derived factor is suggested as the mechanism of the slender-to-stumpy differentiation by two observations (Black et al., 1983).
1) When a monomorphic line was co-infected with a pleomorphic line in a murine host, the monomorphic forms remained monomorphic and the pleomorphic forms differentiated, just as they would in the absence of the co-infecting monomorphic line. This result apparently implies that the monomorphic line either does not itself cause the depletion or accumulation of a host derived factor to which trypanosomes of the pleomorphic lines are sensitive, or alternatively that it does not itself produce a factor to which the trypanosomes of the pleomorphic lines are sensitive.
2) When the same monomorphic line used in the co-infection experiment was inoculated into a bovine host, it was able to establish a pleomorphic infection (Black et al., 1983). This result was taken to imply that it was sensitivity to the depletion of a host factor that was involved rather than accumulation of a parasite derived factor, although accumulation of a host derived factor was not ruled out. The reasoning for this was that if the monomorphic lines were able to produce a signal in a bovine host it was unlikely that they would not be able to do so in a murine host.

More recent reports from in vitro studies apparently contradict these interpretations and indicate that differentiation may be induced by accumulation of a parasite derived stumpy induction factor, or SIF (Hesse et al., 1995; Vassella and

Boshart, 1996; Vassella et al., 1997). It is, however, possible to reconcile these apparently conflicting results in several ways. It may be for instance, that the SIF is somewhat altered in monomorphic forms compared with pleomorphic forms, so that monomorphic form SIF is more rapidly cleared from murine hosts but is stable in other hosts. An alternative hypothesis is that a host specific factor can mediate the competence of a slender form trypanosome to undergo differentiation to the stumpy form. An appealing candidate for such a factor is transferrin.

The transferrin receptor is encoded by ESAG6 and ESAG7 (Steverding et al., 1994; Salmon et al., 1994) and is co-transcribed with VSG at telomeric expression sites. The receptor is polymorphic between expression sites, apparently showing differences in affinities for any given transferrin molecule between expression sites. The structure of transferrin also varies between species of mammalian host. It has therefore been proposed in a recent report, that upon transfer to a new mammalian host the transferrin receptor may mediate selection for trypanosomes which express a transferrin receptor that allows most efficient uptake of the host transferrin (Borst et al., 1997) and that, if the mammalian host is changed, this would result in selection of trypanosomes which had undergone an appropriate in situ switch of expression site. Since such a switch would be concomitant with the host specific pleomorphism, most recently observed by Black and colleagues (Black et al., 1983 but also see Herbert and Parratt, 1979 for a review of earlier such observations), it may be that there is a gene, whose expression is associated with the slender-to-stumpy differentiation, which is situated at the expression site. If this gene contained a lesion which made a strain monomorphic, then when the host species was changed and following the resulting in situ switch, the strain would express an undamaged gene from the new expression site and so the strain would regain pleomorphism.

The hypothesis proposed above, that the gene encoding the receptor for the stumpy induction factor is located at the expression site and is therefore one of the

ESAG genes, may prove incorrect. However, the ESAG genes are relatively few in number, are increasingly well characterized and are accessible to genetic manipulation. A systematic disruption of each plasma membrane associated ESAG gene of unknown function using reverse genetic approaches would seem to be a reasonable approach to adopt in searching for the SIF receptor. Interestingly, a silent phenotype was recently reported when ESAG1, a bloodstream-specific surface-membrane protein that shows considerable polymorphism between expression sites, was recently disrupted in a monomorphic line (Carruthers et al., 1996). A negative result such as this might be expected for a gene associated with the slender-to-stumpy differentiation and this emphasizes the importance of doing disruptions of ESAG genes in pleomorphic strains.

6.2 The effect of the slender-to-stumpy differentiation on parasitaemia

Antigenic variation explains the mechanism by which the African trypanosome can evade the immune system. On its own, however, antigenic variation cannot explain the maintenance of a chronic undulating parasitaemia during the infection. Monomorphic lines can undergo antigenic variation and do not necessarily have a faster growth rate than pleomorphic lines but invariably go on to kill their host (Herbert and Parratt, 1979; Turner et al., 1995). This implies that the transition from the slender-to-stumpy form is critical to the establishment of an effective immune response by the host. The reasons for this may be that the slender-to-stumpy differentiation limits population growth, to a sublethal level, until the immune response is sufficiently potent to clear the vast majority of trypanosomes (Balber, 1972). A second reason is that stumpy forms may be more able to provoke an immune response than the slender forms (Sendanshonga and Black, 1982).

The undulating parasitaemia is therefore generated as a result of three components; antigenic variation, the immune response and the slender-to-stumpy differentiation. In the bloodstream of the mammalian host, pleomorphic populations are never completely homogeneous with respect to stumpy forms. Populations of predominantly stumpy forms always contain within them some slender forms. Superficially, it appears that some of the population are refractory to the signal which causes the majority of the population to differentiate. In predominantly stumpy populations these remaining slender forms are normally VSG heterotypes. This has led to speculation that recently switched trypanosomes may be able to ignore a signal to undergo the slender-to-stumpy differentiation (Pays, 1988).

I favour an alternative explanation, however, where the presence of slender VSG heterotypes in a stumpy population is explained by heterogeneity in the concentration of the signal to differentiate, in the different compartments of the host and by the preferential destruction of slender forms by the immune response. It has been observed that even when the vast majority of bloodstream form trypanosomes

are stumpy, trypanosomes in lymphoid compartments are slender (Ssenyonga and Adam, 1975; Tanner et al., 1980). This implies that the compartments of a mammalian host are not identical with regard to the stimulus for differentiation. It may be that the slender forms are themselves at lower concentration in some compartments of the host and so do not differentiate, or that the signal to differentiate may be differentially labile in different compartments (e.g., reaching higher concentration in the blood than in the lymphoid tissues). Movement of trypanosomes between compartments is to be expected, particularly, since the trypanosome is a highly motile, flagellated organism. Re-invasion of the bloodstream by trypanosomes, in locations such as the lymphoid tissue, would ensure that bloodstream populations always contained some slender forms. Indeed, efficiency of extravascular tissue invasion may be a reason for the morphology and high degree of motility of slender forms.

Slender forms are more susceptible to immune killing than stumpy forms, so slender forms from the VSG homotype are eliminated prior to stumpy forms (McLintock et al., 1993). Slender forms in the lymphoid tissue are also eliminated by the VSG specific immune response (Turner et al., 1986). Elimination of slender form homotypes by the immune response should leave a population of stumpy forms (predominantly of the homotype but containing at least some heterotype stumpy forms) and some slender form VSG heterotypes in the bloodstream. In the lymphoid tissue only slender form heterotypes should survive and these should continue to multiply and maintain a flux with the bloodstream.

Late in the course of the parasitaemia, the concentration of trypanosomes in the blood becomes more constant, an obvious homotype can be lost and the ratio of slender to intermediate to stumpy forms becomes more constant (reviewed in Murray et al., 1982). Co-infection experiments at time-points late in the infection show reduced growth of the second strain compared with time-points early in the

infection. It has been proposed that these observations imply a growth inhibitor associated with the chronic disease (Turner et al., 1996).

There is now growing evidence for a stumpy induction factor (Hesse et al., 1995; Vassella and Boshart, 1996; Vassella et al., 1997) and the latter course of the parasitaemia can be explained by a stumpy induction factor alone, without the need for a second growth regulator. During the chronic phase, the parasitaemia can cease undulating (Murray et al., 1982; Turner et al., 1996). The reason for this is not clear; it may be in part due to the immune system being less able to clear each wave of infection, or alternatively, it may be that the trypanosome population loses homogeneity with respect to VAT (Murray et al., 1982; Black et al., 1985). Whatever the reason, the constant parasitaemia and ratio of cell types indicates that an equilibrium may have been established.

In this equilibrium, it can be hypothesised that stumpy induction factor reaches a concentration which allows only enough replication to replace trypanosomes as they die or are killed. The equilibrium is self-correcting since, if too many trypanosomes are killed, the level of stumpy induction factor is reduced and replication increases. If too many trypanosomes are produced, the level of stumpy induction factor increases restricting replication.

A constant level of stumpy induction factor at equilibrium would ensure both a constant parasite concentration and that the ratio between cell types is maintained, since these levels are effectively a function of the concentration of stumpy induction factor. If stumpy induction factor is differentially labile between hosts, then the equilibrium reached will be host specific. In this chronic phase (when equilibrium has been established), outgrowth of a co-infecting second trypanosome line would also be restricted, by differentiation of a proportion of the new line to stumpy forms, and this might appear as the growth inhibition reported by Turner and colleagues (1996).

In summary, the slender-to-stumpy differentiation may bias the parasitaemia towards a chronic equilibrium. It is the balance between the immune system and antigenic variation which perturbs this equilibrium and contributes to the characteristic fluctuations in trypanosome levels and cell type. Later in the parasitaemia the balance between the immune system and antigenic variation may be disturbed, consequently, the slender-to-stumpy differentiation would become the predominant factor in controlling the course of the chronic parasitaemia and an equilibrium may be established.

6.3 A simulated primary parasitaemia limited by the slender-to-stumpy differentiation

Figure 6.1

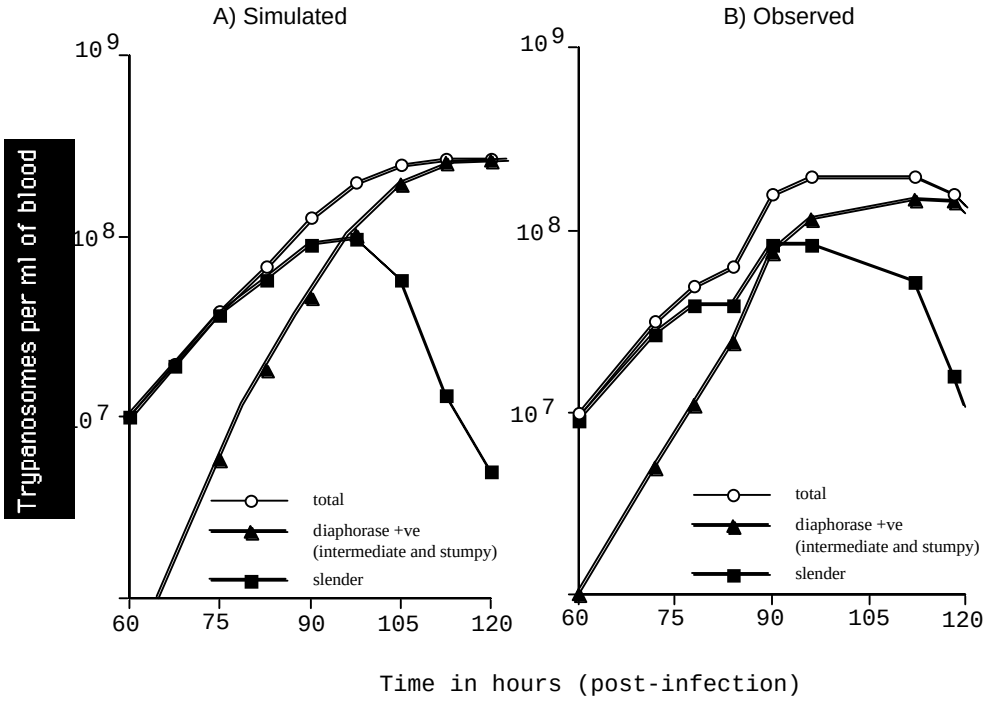


Fig 6.1. A comparison of simulated and observed primary parasitaemias. Fig. 6.1A. The simulated parasitaemia assumes that the rate of differentiation is proportional to trypanosome density up until an arbitrary maximum when 90% of divisions are differentiation divisions. $5x10^{-9}$ was assumed as the differentiation rate constant and 7.5 hours as the doubling time of the proliferative forms. Acquisition of diaphorase staining was assumed to occur 3.25 hours after exit from the cell cycle. Fig 6.1B shows the same data set as fig 3.2 redrawn on appropriate axes.

It has been implied that the slender-to-stumpy differentiation occurs as an intrinsic function of pleomorphic slender form growth (Turner et al., 1995). This idea is, however, apparently contradicted by earlier experiments which found differentiation to be a function of parasite density (Seed and Sechelski, 1989), by more recent reports of a stumpy induction factor (Hesse et al., 1995; Vassella and Boshart, 1996; Vassella et al., 1997) and by the complete lack of stumpy forms in pleomorphic cell cultures at low density (Section 3.3.4). It was felt by Turner et al. (1995) that the growth curves obtained for the pleomorphic lines that they had monitored did not fit with a density dependent model. I sought to examine density

dependent models further to determine if this was indeed the case. In doing so a simulation of the primary parasitaemia which assumes the rate of differentiation to be a function of parasite density was developed.

Bloodstream trypanosomes can be considered to be present in the bloodstream in two forms; a proliferative form, which has a long slender morphology, and a non-proliferative form which may have a long slender, short stumpy or intermediate morphology.

The total number of trypanosomes (**T**) is the sum of the proliferative (**P**) and non-proliferative (**N**) forms

$$\mathbf{T=P+N}$$

In mathematical terms proliferative forms can be considered to divide by way of binary fission. This division can be of two types; either a proliferative division producing two proliferative forms or a differentiation-division producing two division arrested forms. If the proportion of cells undergoing a differentiation division (**D**) is itself proportional to the total number of cells, then where **k** is the constant of proportionality:

$$\mathbf{D = kT}$$

It is clear, however, that it is never the case that all dividing-forms undergo a differentiation-division. A maximum point is reached and this can be described as $\mathbf{D_{max}}$**.** It would also be possible to assign a minimum value as it is likely that below a certain density differentiation is insignificant. However, given the likely range of the constant of proportionality, the rate of differentiation at low parasitaemia will be so low that this omission seems a reasonable approximation in order to minimize complexity.

From a theoretical perspective, cells are best considered in terms of residence in a proliferative cell cycle. Markers of the non-proliferating forms are not, however, acquired until some time after exit from the cell cycle. One marker for instance,

diaphorase, is thought to be acquired some 3-4 hours after cell cycle arrest (Vassella et al., 1997).

The total number of trypanosomes observed is the sum of the slender diaphorase negative forms (**L**) and the intermediate and stumpy/diaphorase positive forms (**S**).

$$\mathbf{T = L+S}$$

Further, the number of non dividing-forms will be equal to the sum of the diaphorase positive forms plus those that have exited the cell cycle but have not yet acquired diaphorase (**E**).

$$\mathbf{N = E + S}$$

If time points are defined by the period of time taken for the proliferative component of the parasitaemia to double, then since the population arises by binary fission, at any time point (**t**)the total number of trypanosomes is the sum of the number of non-proliferating forms and twice the number of non-proliferating forms at the previous time point.

$$\mathbf{T_t = 2(P_{t-1}) + N_{t-1}}$$

The number of newly formed non-proliferating forms (**n**) will be equal t0 the product of the rate of differentiation and twice the previous number of proliferating forms

$$\mathbf{n_t = (2P_{t-1})D_{t-1}}$$

The total number of non proliferating forms is equal to the sum of all the proliferating forms

$$\mathbf{N_t = n_t + N_{t-1}}$$

If the time taken for acquisition of diaphorase as a marker is half of one (proliferative form) doubling time, then the total number of diaphorase positive forms will be

$$\mathbf{S = (N_t+N_{t-1})/2}$$

In formulation of an algorithm for this theoretical model, the key constants are: the constant of proportionality (**k**), the doubling time of the proliferative form (**t**), the time taken for acquisition of diaphorase (**a**) after cell cycle arrest and the maximum proportion of dividing-forms undergoing differentiation ($\mathbf{D_{max}}$). In the simulated parasitaemia (fig 6.1A) these were estimated as:

$$\mathbf{k = 5x10^{-9}, t = 7.5h, a = 3.25h, D_{max} = 90\%}$$

In the simulation logarithmic growth continues for approximately 90 hours before the curve shallows and reaches a plateau. It is also at about 90 hours that the numbers of slender forms begin to be reduced in number and that the rate of increase in stumpy form numbers also begins to slow. All of this closely resembles the example given. It is noteworthy however that the number of stumpy forms in the example is initially higher than in the simulation. This may reflect the composition of the inoculum used, which contained 10^6 trypanosomes, some of which may already have been committed to the production of stumpy forms. This varies from the founder population, which can be theoretically obtained from the simulated curve, which would be much smaller and made up of only uncommitted slender forms.

Although the simulated primary parasitaemia apparently shows a close fit to the observed data, this is artificial. It does indicate, however, that a density dependent model is able to emulate many aspects of experimental data sets. Indeed, it highlights the difficulty that a model based on differentiation as an intrinsic function of growth would have in explaining the observation that the number of proliferative forms first increases and then decreases, even in immunosupressed models. This is because if the rate of differentiation is less than 50% per generation the number of proliferating forms increases throughout, while if it is more than 50% it falls throughout.

The arithmetic simulation shown is limited; it incorporates no role for removal of stumpy forms by cell death. It also makes no accommodation for the possibility that stumpy forms lose the ability to influence the density dependent regulation, for instance by becoming non-secretors of a stumpy induction factor. There is also no attempt to incorporate the effect of the immune system. In the simulated primary parasitaemia it is likely that the differentiation constant (**k**) and the maximum rate of differentiation ($\mathbf{D_{max}}$) are both artificially high, which has the effect of emulating immune killing.

Introduction of new parameters and the variation of the parameters established here is cumbersome using arithmetic simulation. In collaboration with Dr. P. Higgs (Bioinformatics, University of Manchester) continuous time equations were derived from these arithmetic principles for use in computer modelling of trypanosome parasitaemias. These were that:

$$\mathbf{1)\ \delta T/\delta t = rP}$$

$$\mathbf{2)\ \delta P/\delta t = rP(1-2D)}$$

$$\mathbf{3)\ \delta N/\delta t = rP(2D)}$$

$$\mathbf{4)\ \delta E/dt = (rP.2D) - aE}$$

$$\mathbf{5)\ \delta S/\delta t = aE}$$

T - total parasite numbers. **P** - number of proliferative forms. **N** - number of non-proliferative forms. **S** - number of diaphorase positive forms. **E** - number of forms which have stopped dividing but have not yet acquired diaphorase. **r** - rate of proliferative form division. **a** - rate of diaphorase acquisition. $\mathbf{D_{max}}$ - maximum rate of differentiation. $\mathbf{T_{sat}}$ - concentration at which 1/2 $\mathbf{D_{max}}$ is reached .

These equations and parameters have been used in generating a computer program to emulate the primary parasitaemia and solve the key variables **t, a, k,** and $\mathbf{D_{max}}$ for best fits with available data sets. Having established a computer emulation it should now be possible to add in additional parameters for stumpy cell death and (hypothesized) loss of SIF secretion. As these parameters become defined experimentally the computer simulation will become more refined and more adept at calculating missing variables.

As can be seen in figure 6.1B, until about 90 hours post-infection both the theoretical and observed growth curves show approximately logarithmic increase in slender and diaphorase positive forms. This is because of the progressive nature of differentiation in this model, taking place over days rather than as a rapid all or nothing response, following a threshold concentration for differentiation being reached. If differentiation was uniformly triggered in all accessible trypanosomes, when a critical concentration of differentiation signal was reached, differentiation would be extremely rapid. Presumably, differentiation would be complete in little more than one cell cycle (as seen in vitro, Vassella et al., 1997). It is clear, however, from growth curves (such as 6.1B) that differentiation takes place over approximately 42 hours.

It is also clear that slender forms are always present which indicates that the rate of differentiation never reaches 100%. In the model presented a maximum of 90% differentiation per mean doubling time of proliferating forms is assumed. However this is an arbitrary figure, the variation of which affects the “plateau” of the growth curve. In the continuous time, computer generated simulation the maximum is reached more gradually by adding a constant, which is negligible at low parasite density but restrictive at high parasite density.

The mean doubling time of proliferating forms shown in the model is 7.5 hours; this was not based on direct experimental evidence but rather was selected to produce a time-course similar to the observed time-course in mice. This value is,

however, in line with previous estimates of population doubling times (Herbert and Parratt, 1978; Turner et al., 1995) which show considerable strain dependent variation. Finally, the time taken between exit from the proliferative cell cycle and acquisition of the diaphorase marker was approximated, to the nearest half cell cycle, as 3.25 hours from values obtained in vitro by Vassella et al. (1997).

The development of a predominantly stumpy form population during the primary parasitaemia of a rodent occurs with progressive, complex and comparatively slow kinetics. In Chapter 5, however, it was proposed that at the level of the single cell, differentiation occurs by a mechanism which is straightforward and rapid. This apparent contradiction can be reconciled since complexity can be generated by the addition of variables and these variables are generated at the level of the interaction between the slender form and the signal to differentiate. In this way, the mechanism by which differentiation occurs may be straightforward, but the trypanosome's commitment to undergo differentiation may result from a complex and stochastic choice.

There are several explanations why the decision to differentiate might be stochastic, but with the chance of commitment to differentiation for individual trypanosomes increasing proportionally with the increase in parasite density. For instance, if differentiation is mediated by a single factor and the factor has a low affinity receptor, or is present at low concentration, then increase in the number of parasites and hence the amount of factor increases the chance of a differentiation signal being generated from a receptor/ligand interaction. Complexity may also be generated by differences in individual trypanosome susceptibility to the differentiation signal, for instance, if trypanosomes were only susceptible during some parts of the cell cycle then the asynchrony of differentiation could reflect the asynchrony of the population with regard to cell cycle position. Even marginal differences in the level of signal required to make individual trypanosomes differentiate could greatly affect the timespan of differentiation at a population level.

Complexity may also be introduced at the level of the host. The mammalian bloodstream and lymphatics do not represent a homogeneous culture system, but rather a series of linked compartments which are separate microenvironments. Parasites may be resident in some compartments at higher densities than in others and the signal to differentiate may be differentially labile between compartments. Even if differentiation follows directly from a critical concentration of a factor being reached, this concentration will be reached at different times for different groups of parasites. It is also possible that initial progress towards commitment to differentiation is a stochastic process, progress being dependent in part on the continued reception of a differentiation signal. If so, differentiation would be highly susceptible to concentration gradients in the signal. This would make the exact rate of differentiation specific to individual trypanosomes, regardless of the model by which stumpy forms arise.

Recently, Seed and Black (1997) proposed an arithmetic model which is similar to, though less sophisticated than, the model proposed here and which likewise assumed that differentiation occurred in a parasite density dependent fashion. There are three important differences between the Seed and Black model and the one presented here. First, in the model of Seed and Black, the rate of differentiation was related to the number of proliferating forms. In an immunosupressed murine model (Balber, 1972), however, the parasitaemia reaches a plateau and this is maintained for 72 hours which is well beyond the time that the population has become predominantly stumpy. This seems to indicate that, at least initially, stumpy forms are able to inhibit trypanosome replication by inducing the slender-to-stumpy differentiation. For this reason the model proposed here assumes proportionality to total parasite number. Second, Seed and Black allow no time between exit of the cell cycle and acquisition of the intermediate/stumpy phenotype, which is likely to dramatically affect the ratio of cell types observed.

Finally, the model presented here is presented in the form of algorithms which are subject to straightforward manipulation of the key variables. This should facilitate the emulation of host specific primary parasitaemias. Moreover, additional tiers of complexity can be added, to what is essentially a model in the absence of an immune response. Consequently, by compiling sets of data on the onset of immune response to the trypanosome homotype and its rates of differential killing, and then by adding in data on switching rates, it is even possible that a model of the entire parasitaemia in immunocompetent animals could be constructed.

6.4 Cellular differentiation, altruism, immortality and cell death

The study of cellular differentiation remains loosely defined. Differentiation relates to a change from one cell type to another. At a microscopic level this change may be obvious, because of morphological differences, but intrinsically differentiation relates to differences in gene expression and gene product modification. It has been argued in the past, that differentiation should refer exclusively to metazoan lineages (See Fulton, 1977). Differentiation has been used to describe the changes in cell types which occur during progression from the undifferentiated cells of the fertilized embryo, to terminally differentiated effector cells and to gametes (for instance see Smith and Woods, 1997). The cellular characteristics which change and the mechanisms by which change is brought about during metazoan differentiation are, however, analogous to dramatic cellular changes which occur in protozoons and even in prokaryotes. Consequently, the definition of differentiation can be extended to encompass all cellular transformations, from one stable cell type to another, occurring during the normal development of any organism (Fulton, 1977).

I consider metazoan differentiation to differ from protozoan differentiation, not primarily mechanistically, since similar levels of mechanistic diversity are apparently seen within and between both groups, but rather functionally. Metazoan differentiation is essentially altruistic; billions of specialized cells are programmed to die and so are effectively sacrificed for the survival of a few gametes, which carry only half of the organism's DNA through to the next generation. When this altruism breaks down, as in the case of neoplasia, the resulting cellular proliferation is often lethal. In metazoons then, the well being of the organism is more important than the well being of individual cells.

In protozoons the cell and the organism are the same. It would seem to follow that anything analogous to the sacrifice of a programmed cell death, such as terminal differentiation or apoptosis, would be selectively disadvantageous and

would not occur. Altruism at the level of the organism, however, occurs readily in colonies of genetically homogeneous individuals. For instance in colonies of fertile halictids (sweat bees), all bees in a colony possess the same genetic make-up but the vast majority of individuals forego reproduction to maximise the efficiency of reproduction for a small minority of “dominant” individuals (Wilson, 1975; Craig, 1980). It seems, therefore, that protozoan colonies should likewise be able to evolve altruism where such a trait is advantageous, particularly if extinction was the likely result of a breakdown in altruism.

In order for parasites to be effective, an equilibrium must exist between host and parasite. The death of the host prior to successful transmission would lead to parasite extinction. Likewise, extinction of all suitable hosts would lead to the extinction of the parasite. Parasites have therefore evolved to maintain chronic infections in order to avoid killing their hosts and maximise their transmission potential. Protozoan parasites have evolved mechanisms not only to avoid host defences, but also to avoid proliferating to such an extent that they kill their host. Trypanosomes replicate freely in the energy rich bloodstream of the host mammal but restrict their proliferation to a sublethal level to avoid killing the host and hence being killed. Whilst growing in the bloodstream of a mammalian host, the aim of each trypanosome must be to maximise its chance of transmission before the death of its host. Each trypanosome therefore has two potential worthwhile fates, either continued proliferation within the host or transmission to a tsetse fly.

Slender form trypanosomes appear to have evolved a density sensing mechanism, which informs the parasite that the infection is close to reaching a level which would kill the host and hence result in its own death. It is in the interest of each trypanosome that receives such information to exit the cell cycle. This is akin to starvation responses in other protozoons, such as yeast cells, and can be viewed as a selfish act, since although the chances of being taken up by a tsetse fly are slim they are better than the chances of survival if the parasite continues to replicate.

An ancestral slender form would exit the cell cycle to avoid killing the host. Since trypanosome population density was maintained, within the limiting environment of the host bloodstream, there would be no chance for an arrested slender form to re-enter the cell cycle at a later point. This may explain the unidirectional unipotency of stumpy form differentiation. At high population density, in the mammalian host, an arrested slender form has only one possible productive fate - transmission to a tsetse-fly. From an evolutionary perspective it was, therefore, in the best interests of arrested forms to optimise their potential for transmission to and survival in the tsetse fly. This optimisation is reflected in the evolution of preadaptions, such as the partial activation of the mitochondrion seen in the stumpy form.

The vast majority of bloodstream form trypanosomes are not transmitted but are removed by the host's immune response. A few slender forms, however, continue to divide. The heterotypes within these slender populations propagate a new wave of infection once the previous homotype has been eliminated, hence generating chronicity. At a population level, this system of maintaining a chronic parasitaemia is beneficial to all of the individual trypanosomes within it, yet there is no real evidence that it has evolved through the sacrifice of individual trypanosomes and so cannot truly be regarded as an altruistic phenomenon (as was previously proposed by Vickerman, 1989).

The longevity of a stumpy form has been estimated as about two days (and no more than three) from the time of cell cycle arrest (Turner et al., 1995). This is superficially quite a short period but it cannot be known whether stumpy longevity is of an artificially short duration. It may be that stumpy forms are programmed to die early and that this is directed towards removing stumpy trypanosomes in hosts that fail to mount an effective immune response in order to maintain a chronic parasitaemia. If this is the case then abbreviation of the longevity of the division arrested stumpy form would be an example of altruism, since it sacrifices individual

transmission potential in the interests of maintaining a parasitaemia. Work in immunocompromised mice, however, suggests that stumpy longevity is not so short as to prevent the death of the host in the absence of an immune response (Balber, 1972).

In the tsetse midgut the procyclic population is also limited to a level which is sublethal to the tsetse fly and relatively constant, even after a blood meal when nutrients are not limiting (Welburn and Maudlin, 1997). It has been suggested that this is achieved not just by cell cycle arrest, but actually by activation of a programmed cell death pathway which is akin to apoptosis in metazoons. Evidence has been presented which focuses on characteristic blebbing of the nucleus and on the production of the characteristic apoptotic "ladder" of DNA fragments obtained when DNA of these dying forms is purified and run on agarose gels (Welburn et al., 1997). These reports have understandably been met with a degree of caution. It is not necessarily clear why endonuclease ladders of this type should occur since trypanosome chromatin, although containing nucleosomes, is far more susceptible to the effects of endonucleases than that of metazoons. Furthermore, from an evolutionary perspective, the necessity for apoptosis is largely lacking. Even in the confined environment of the tsetse midgut, the establishment of an equilibrium between proliferative forms, arrested forms and natural elimination of cells would suffice, without resort to more complex mechanisms such as apoptosis.

It is not yet clear whether all procyclic forms that arrest in the tsetse midgut become potentially invasive proventricular forms. Once the tsetse salivary gland has been colonised the existence of the remaining procyclic trypanosomes in the midgut is apparently futile. From the evolutionary perspective of the trypanosome population, once the salivary gland is colonized the midgut infection would ideally be eliminated, promoting fly longevity. This is a level of altruism which clearly has not evolved. Procyclic forms which have arrested at high density and cannot invade the salivary gland must eventually die (not suicide since they would presumably

initiate a salivary gland infection if they had the chance). It is likely that as this occurs, the nucleus fragments and it is possible that this is seen as blebbing, moreover, the fragmented DNA may, when purified, show a “ladder” effect on an agarose gel. Although these phenomena are taken as characteristic of apoptosis in metazoons, in the evolutionarily disparate trypanosome they may just be characteristic of cell death.

The activation of the BAX/BCL-2 pathway is considered an integral part of apoptosis. Recently, evidence of BCL-2 homologues and of conservation of this pathway in yeast has been presented (Ink et al., 1997): over-expression of heterologous BCL-2 leading to yeast cell death in a manner akin, but not identical, to apoptosis of mammalian cells (Jurgensmeier et al., 1997). The implications of this work support a universally conserved programmed cell death mechanism. It can be speculated that the reason for activation of such a mechanism would not be starvation (yeast cells arrest growth when this occurs), rather this may represent defence against spread of a pathogen (typically a virus) which might otherwise wipe out a yeast colony. Such a mechanism may even extend to prokaryotes (Shub, 1994). If this turns out to be the case, such a mechanism is truly altruistic. It will be interesting to determine whether the BCL-2 pathway extends to trypanosomes.

In summary then, one of the major differences between protozoan differentiation and metazoan differentiation is that protozoan cells normally retain potency during differentiation so such differentiations are normally not altruistic. It is true, however, that protozoons have the potential to evolve altruistic traits particularly when growing as genetically homogeneous populations. Trypanosome infections are usually (very nearly) genetically homogeneous and so it is possible that altruism does occur during the trypanosome life cycle. To date, however, there is no unequivocal evidence of altruism in *T.brucei* and the heralding of altruistic phenomena such as atrophy, terminal differentiation and apoptosis in trypanosomes may be premature.

Chapter 7

Overview

7.1 General discussion

African trypanosomes are important pathogens of both domestic livestock and human beings. They proliferate freely in the bloodstream and lymphatics of a mammalian host and in doing so they can cause pathology and would cause the death of the host if the proliferation went unchecked. Arrest of division occurs in pleomorphic infections at high parasite density. The slender-to-stumpy differentiation acts in part as an intrinsic mechanism for ensuring maintenance a chronic parasitaemia. Study of the slender-to-stumpy differentiation represents an opportunity to examine the co-regulation of mitochondrial expression with morphological change and cell cycle arrest, in a single cell system which is increasingly tractable to investigation. In the long term, however, the study of the slender-to-stumpy differentiation is concerned with the need to emulate this natural arrest chemotherapeutically. As an initial step towards this objective, the work presented here has set out to provide better understanding of a medically important differentiation process. The following approach was adopted.

1) To establish a reliable and reproducible system, in which to observe and manipulate the slender-to-stumpy differentiation and from which homogeneous populations of each cell type could be gathered.
2) To establish stage specific markers for each form, with which to assess the differentiation.
3) To apply these markers in the system established, to dissect the cytological events that accompany the slender-to-stumpy differentiation. Specifically, by looking for evidence of heterogeneity between the dividing-forms of low parasitaemia and high parasitaemia populations.

7.2 Establishment of trypanosome culture and differentiation systems

Parasitaemia is affected both by the host used and by the infecting strain. Consequently, I standardized parameters associated with both the host and the inoculum, which led to highly reproducible rodent parasitaemias. Trypanosomes were harvested when over 95% were slender forms (three days after infection) and when over 90% were stumpy forms (five days after infection).

An in vitro culture system was developed for the maintenance of pleomorphic, bloodstream form trypanosomes in liquid culture. However, this failed to show efficient transformation to the stumpy form. This system should prove complementary to a recently reported agarose plate based culture system, which does support efficient slender-to-stumpy differentiation of at least one pleomorphic *T.b.brucei* strain (Vassella and Boshart, 1996). In particular, the in vitro system developed here should allow growth and selection in both liquid and solid culture (as colonies) and will allow transgenic, pleomorphic trypanosomes to be selected. The effect of such experiments on differentiation and on pathogenicity, can then be monitored by raising the transgenic strains in rodent hosts.

Using the in vitro culture system it was observed that slender forms obtained from culture at low density were almost completely unable to differentiate to procyclic forms. This was in contrast to slender forms cultured in vivo, which showed a significant degree of differentiation under the same differentiation conditions (27^{o}C and in the presence of cis-aconitate). This finding may imply that these cultured bloodstream forms cannot enter a G_0 state requisite for differentiation to the procyclic form. Alternatively, it may indicate that cell cycle position alone is not responsible for the competence of bloodstream forms to differentiate to the procyclic form (see Matthews and Gull, 1994ab).

7.3 Division of bloodstream forms

In order to determine whether bloodstream division is heterogeneous, it was necessary to characterize slender form division rather than relying on analogy with previous studies which describe procyclic form division (Sherwin and Gull, 1989a; Woodward and Gull, 1990; Robinson et al., 1995). Study at the level of the fluorescence and electron microscope (E.M. micrographs kindly provided by T. Sherwin and K. Matthews) established a relative order of events, similar but not identical to division in the procyclic form. This order of events represents a primary characterization of some of the events occurring during slender form division. In the future it will be useful to fit these and other events to a temporal map of the cell cycle using BrdU incorporation to define the kinetoplast and nuclear S-phase.

Despite the preliminary nature of this cell cycle characterization, two sets of observations were of particular interest. The first study highlighted the dynamic nature of mitochondrial segregation, showing that it proceeds by a mechanism of branching and fusion. This immunofluorescence study complements earlier work performed by electron microscopy (Vickerman, 1966). In the second study, immunofluorescence was again used to follow outgrowth of the new flagellum in dividing-forms. New flagellum outgrowth was found to be one of the first cytological events marking commitment to cell division and new flagellum outgrowth was shown to continue at an approximately constant rate until cytokinesis. As with procyclic forms kinetoplast segregation was found to precede nuclear segregation. Unlike procyclic forms both kinetoplasts were positioned posterior to the nuclei throughout the cell cycle. The mature flagellar length was found to be highly variable. However, at the time-point immediately preceding cleavage, the mean length of the new flagellum was 12% shorter than the mean length of the mature flagellum, indicating that the division was into two unequal cells.

7.4 Markers of differentiation

Stage specific morphological markers and markers of mitochondrial biogenesis were sought, to combine with markers of the cell cycle, in order to triangulate the slender-to-stumpy differentiation with markers of each major event. Three approaches were taken to obtain such markers.

- A comparison of established morphological parameters known to vary during differentiation to the stumpy form.
- A screen of pre-existing, predominantly mitochondrial antibodies for stumpy specific antigens.
- A screen for stumpy enriched mRNA transcripts by a differential display technique.

Each of these techniques proved successful in producing markers that varied during the slender-to-stumpy differentiation. Structurally, the most consistent and significant variation assessed was found to be flagellar length, the mean length of the stumpy form being approximately 30% shorter than the mean length of the slender form. The mean stumpy form flagellar length is also shorter than the length of the new flagellum immediately prior to cytokinesis of a proliferative slender division. Flagellar length was adjudged to be a valuable choice of marker with which to examine models relating to division and differentiation, because of its additional role as a marker of cell cycle progression.

From the antibody screens, which were assessed by immunofluorescence and western blot, DHLADH was found to be the antigen most clearly regulated between the slender and stumpy forms. Two other mitochondrial markers, mhsp70 and hsp60, were also shown to be useful immunofluorescence markers of the slender-to-stumpy differentiation.

Differential display and subsequent screening for stage specificity by northern blot and in situ hybridization, yielded two cDNA fragments apparently encoding homologues of ribosomal proteins. The localization of these transcripts by in situ hybridization was peculiar, being more nuclear than cytoplasmic. Antibodies to these proteins would be

desirable to determine whether ribosomal protein levels, as well as mRNA levels, are increased in the stumpy form.

7.5 Evidence for a differentiation-division

Stumpy, intermediate and some slender forms show mitochondrial expression of DHLADH. By combining immunofluorescence with DAPI fluorescence it was found that dividing-forms do not normally show mitochondrial DHLADH expression. Together this data indicates that all dividing-forms have a predominantly slender phenotype at division and that the daughter cells of all bloodstream form divisions initially look like slender forms. The round of cell division that precedes the formation of a stumpy cell appears superficially like all preceding divisions. This is not surprising since the more different this round of cell division is, the more the basic program for slender cell division would have to be amended.

The demonstration of the mitochondrial expression of mhsp70, in the dividing-forms of differentiating populations provided evidence of a differentiation-division different to preceding proliferative divisions. This was apparently supported by the observation of anucleate forms (zoids), which showed increased mitochondrial expression of (the nuclear encoded) DHLADH by immunofluorescence. The incidence of these stumpy zoids indicated that, at the time of division, the mRNA for DHLADH was already present. In a preliminary assay of DHLADH mRNA, however, the levels of mRNA were constant between slender and stumpy forms. If this is correct then it would seem that DHLADH expression is not regulated transcriptionally or at the level of mRNA stability. It follows, that all of the nuclear encoded mRNA necessary for the mitochondrial and morphological changes during the slender-to-stumpy differentiation may be present in slender forms; that the slender-to-stumpy differentiation does not require de novo mRNA synthesis. The implication of this is that all the events producing a stumpy phenotype are controlled translationally or post-translationally. This would be consistent with the translational control of the only other nuclear encoded protein which has been shown to increase during

the slender-to-stumpy differentiation, the *nrk* gene product (Gale et al., 1994). However, this hypothesis would be in contrast to the stumpy-to-procyclic differentiation which is generally held to employ mRNA stability as the primary level of control for most proteins (Clayton, 1992; Priest and Hajduk, 1994a; Vanhamme and Pays, 1995).

When flagellar length was compared between dividing cells of slender and differentiating populations, evidence of a reduced new flagellum length was found in cells approaching cytokinesis. This was taken to imply that the dividing trypanosome, having already decided to produce a stumpy form, produced a shorter new flagellum than in preceding rounds of cell division. The morphology of cells which were defined as being in their differentiation-division by the length of their new flagellum, was compared directly to trypanosomes in previous proliferative divisions. The comparison showed that cells in their differentiation-division appeared shorter and wider and contained nuclei which were nearly spherical. In contrast, cells in a proliferative division were longer, narrower and contained ovoid nuclei.

It was possible to speculate on the fates of the two daughter cells from the differentiation-division. The daughter cell which inherits the new flagellum has a flagellar length encompassed within the range of stumpy flagellar lengths and need only maintain this length during differentiation. The daughter cell inheriting the mature flagellum may need to undergo considerable shortening of the flagellum and so must activate a flagellar shortening program in order to become a stumpy form. This explains the observation of long intermediate forms with flagellar lengths intermediate between the slender and stumpy forms and which showed mitochondrial expression of DHLADH. Although this work strongly supports the slender-to-stumpy differentiation occurring solely by means of a differentiation-division, it does not preclude direct differentiation of some early G_1 cells to the stumpy form without an intervening differentiation-division.

A working hypothesis for the slender-to-stumpy differentiation was formulated. The hypothesis proposes an obligatory differentiation-division producing two cells which undergo subsequent morphological and biochemical change, but no further division, to

become stumpy forms. This hypothesis was then used as one of the assumptions in a mathematical simulation of trypanosome primary parasitaemia.

In summary I would like to propose the following pathway for normal transmission through a mammalian host (Fig. 7.1).

1. After being transmitted in the bite of a tsetse-fly, the metacyclic trypanosome differentiates to the slender form and enters a proliferative cell cycle. Following multiple rounds of proliferative cell division, a level of parasitaemia is reached at which a proportion of slender forms commit to differentiation to the stumpy form.
2. The committed slender forms then undergo a round of cell division that is heterogeneous to previous proliferative divisions. This differentiation-division may take longer than proliferative divisions. Dividing-forms of a differentiation-division show evidence of the acquisition of early mitochondrial markers such as mhsp70. These forms are also different to dividing-forms undergoing proliferative division in that they have a shorter new flagellum close to cytokinesis and show cytological changes such as the more rounded appearance of the nuclei following mitosis.
3. After cytokinesis of the differentiation-division the two daughter cells do not divide again in the bloodstream. Each then undergoes most of the morphological and metabolic changes associated with the slender-to-stumpy differentiation. This includes acquisition of markers such as DHLADH and hsp60. In addition at least one of the two daughter cells of the differentiation-division will undergo substantial flagellar shortening.
4. Once formed the stumpy cells persist in the bloodstream for 48-72h (Turner et al., 1995).
5. If stumpy forms are taken up in the bite of a tsetse-fly they will differentiate in the fly midgut. During this differentiation cells will gain a procyclin surface coat and shed VSG, continue mitochondrial biogenesis and will initiate morphological change.
6. Finally, cells will re-enter a proliferative cell cycle. In doing so it may be that the initial round of cell division will be different to ensuing rounds of procyclic form cell division.

It is therefore possible that there will also be a differentiation-division during the stumpy-to-procyclic differentiation.

Figure 7.1

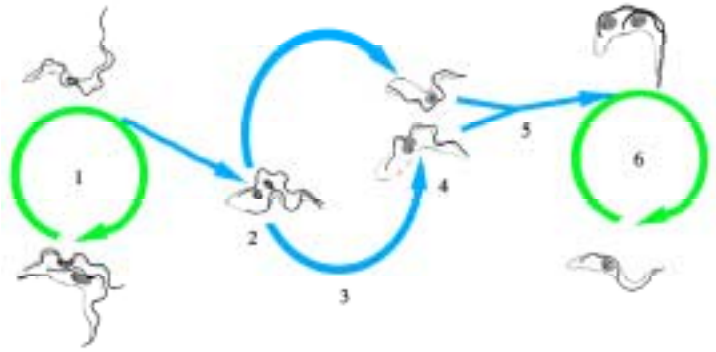


Fig. 7.1 Overview of the proposed pathway of differentiation from a slender via stumpy to the procyclic form.
1) The slender form can continue to undergo rounds of proliferative cell division.
2) The slender form can become committed to differentiation to the stumpy form. When this occurs the slender form undergoes a heterogeneous cell division, the differentiation-division. At this stage early mitochondrial markers such as mhsp70 are recruited to the mitochondrion.
The length reached by the new flagellum prior to cytokinesis is shorter than in previous rounds of proliferative division, and the nuclei formed after mitosis appear more rounded.
3) The daughter cells formed from a differentiation-division then differentiate via a range of morphological intermediates. Most mitochondrial markers, including DHLADH, are recruited during this period and flagellar shortening occurs.
4) Both of the daughter cells of the differentiation-division become stumpy forms.
5) Under appropriate conditions, or when taken up by a tsetse-fly, these stumpy forms will acquire procyclin, shed VSG, complete mitochondrial biogenesis, undergo morphogenesis and re-enter the proliferative cell cycle.

7.6 Future work

This work has provided the foundation for ongoing projects at the University of Manchester. It complements recently published cell culture protocols from Dr. Boshart's laboratory. The advent and application of cell culture and differentiation systems, and of molecular markers, to the study of the slender-to-stumpy differentiation has significantly broadened this field of research. It seems that it should now be possible:

- To define a temporal map of events for the bloodstream form cell cycle.
- To determine differences in mitochondrial structure during the differentiation-division, using fluorescence and electron microscopy.

- To isolate and study mitochondrial associated, kinesin-like molecules which may prove particularly interesting with regard both to mitochondrial division and biogenesis.
- To define a temporal map for the acquisition of markers during the slender-to-stumpy differentiation.
- To determine the point at which trypanosomes become competent to undergo synchronous differentiation to the procyclic form at 27^{o}C in the presence of cis-aconitate.
- To define transitional and commitment points using inhibitors of transcription and translation in an in vitro system.
- To study the regulation of markers individually and, by defining control molecules, look for unifying mechanisms of control.
- To compare regulation of nuclear encoded mitochondrial molecules, such as TAO and DHLADH, and use parts of each molecule, such as leader sequences along with reporters or epitope tags to define control mechanisms.
- To make antibodies to ribosomal proteins and thereby investigate the role of the upregulation in ribosomal protein mRNAs during the slender-to-stumpy differentiation.
- To devise further screens to define more markers of the slender-to-stumpy differentiation, particularly directed towards cytoskeleton associated proteins.
- To further refine and test the theoretical model of the primary parasitaemia.
- To further investigate autofluorescence of the mitochondrion and of cytoplasmic vesicles found during the course of the project (data not shown).

Chapter 8

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