

# Evidence for an interplay between cell cycle progression and the initiation of differentiation between life cycle forms of African trypanosomes



# Evidence for an Interplay between Cell Cycle Progression and the Initiation of Differentiation between Life Cycle Forms of African Trypanosomes

**Keith R. Matthews and Keith Gull**

Biochemistry Division, School of Biological Sciences, Stopford Building, University of Manchester, Manchester, United Kingdom M13 9PT

*Abstract.* Successful transmission of the African trypanosome between the mammalian host bloodstream and the tsetse fly vector involves dramatic alterations in the parasite's morphology and biochemistry. This differentiation through to the tsetse midgut procyclic form is accompanied by re-entry into a proliferative cell cycle. Using a synchronous differentiation model and a variety of markers diagnostic for progress through both differentiation and the cell cycle, we have investigated the interplay between these two processes. Our results implicate a relationship between the trypanosome cell cycle position and the perception of the differentiation signal and demonstrate that irreversible commitment to the differentiation occurs rapidly after induction. Furthermore, we show that re-entry into the cell cycle in the differentiating population is synchronous, and that once initiated, progress through the differentiation pathway can be uncoupled from progress through the cell cycle.

STUDIES of cellular differentiation events of simple eukaryotes can provide novel insights into how such organisms respond to a changing environment or control their progress through a programmed life cycle. In such microbial differentiations, this control of cell fate is commonly linked to the organism's cell cycle: the sporulation of *Bacillus subtilis* and streptomycetes, the differentiation of *Caulobacter* and *Anabaena* (Newton and Ohta, 1990 and references therein) and the mating type switch of yeast (Klar, 1992; Haber, 1992) all involve a symmetric or asymmetric division resulting in one or both cell progeny being of different cell type to the parent. In such cells, passage through at least one complete cell cycle is, therefore, a requirement for differentiation. In contrast, the differentiation of bloodstream trypomastigotes to the tsetse midgut procyclic form of the African trypanosome involves a pre-adapted nonproliferative cell type differentiating coincidentally with reentry into a proliferative cell cycle.

African trypanosomes are protozoan parasites of mammals causing sleeping sickness in man and nagana in cattle. The parasite survives in its mammalian host in the face of an immune response by its ability to undergo antigenic variation, that is, by periodically changing its variant-specific surface glycoprotein (VSG)[1] coat (reviewed by Cross, 1990). The periodic shifts in parasite antigen type and host antibody mediated destruction of former types results in ongoing waves of parasitaemia characteristic of trypanosome infection. Within these growth peaks the population of cells is not uniform morphologically, changing as each peak progresses (Vickerman, 1965; reviewed by Vickerman, 1985). Early on, trypanosomes are rapidly dividing and have a long slender morphology, whilst at the peak of a parasitaemia the cells are predominantly much shorter and fatter and do not divide. This non dividing form is known as the stumpy form and is preadapted to survival and establishment in the insect vector of African trypanosomiasis, the tsetse fly (Wijers and Willett, 1960; Turner et al., 1988; Ziegelbauer et al., 1990). When taken up in the tsetse bloodmeal the stumpy form readily differentiates through to the dividing procyclic form which colonizes the insect's midgut. This form is dramatically different from its bloodstream counterparts: it uses a full oxidative phosphorylation to generate energy rather than simple glycolysis, shows an altered cell architecture and several fundamental changes in gene expression (reviewed in Clayton, 1992). This extensive reorganization of the cell is initiated in the first 24 h after the initiation of differentiation and is, at least in stumpy cell populations, synchronous with respect to the exchange of two life cycle stage specific surface

Address all correspondence to K. R. Matthews, Biochemistry Division, School of Biological Sciences, Stopford Building, University of Manchester, Manchester, U.K. M13 9PT.

1. *Abbreviations used in this paper*: BRDU, 5'bromo 2'deoxyuridine; VSG, variant-specific surface glycoprotein.

antigens; the VSG is soon lost and a procyclic specific coat protein, procyclin/PARP (Procyclic Acidic Repetitive Protein), is rapidly gained (Roditi et al., 1989; Ziegelbauer et al., 1990, 1993). Differentiation is also coincident with progression through the first cell cycle following release from division arrest. This raises the possibility that the initiation of, or progress through, the differentiation pathway might be in some way interlinked with the trypanosome cell cycle.

The trypanosome cell cycle is well characterized at the cellular level (Sherwin and Gull, 1989*a*), with replication of the kinetoplast (the DNA of the trypanosome's single mitochondrion), nuclear DNA synthesis and elongation of the probasal body being the first discernible events (Woodward and Gull, 1990). Rigorously controlled basal body and kinetoplast segregation and subsequent mitosis then establish a highly defined cell architecture suitable for cytokinesis (Sherwin and Gull, 1989*a*; Robinson, D. R., T. Sherwin, A. Ploubidou, and K. Gull, manuscript submitted for publication). Elucidation of this cell cycle has been assisted by the derivation of several immunological probes recognizing many structural components of the trypanosome cell (Woods et al., 1989). Such tools provide a way of defining periodic events such as basal body replication and segregation, initiation of daughter flagellum growth, kinetoplast and nuclear S-phases, and mitosis. These same markers provide a means to analyze and dissect the progress of the differentiating stumpy form population through its first cell cycle and to address questions relating to the control of differentiation. Specifically, we have used a synchronous in vitro differentiation system (Ziegelbauer et al., 1990) to: (*a*) determine whether differentiating cells re-enter into their proliferative cycle uniformly; (*b*) unravel the extent to which cell cycle and life cycle progression is enmeshed; and (*c*) investigate the points of temporal commitment to differentiation within the transforming population. Our results begin to reveal the basis of control for several fundamental aspects of trypanosome bloodstream to procyclic form differentiation.

## Materials and Methods

### Trypanosomes

The pleomorphic and monomorphic lines of *Trypanosoma brucei rhodesiense* EATRO 795 (GUP 2962 and 2965, respectively), were gifts from Dr. C. M. R. Turner (Glasgow University, Glasgow, Scotland). Pleomorphic lines are those that demonstrate the ability to generate slender and stumpy cells in high numbers during the course of a peak of parasitaemia. Monomorphic trypanosome lines do not generate stumpy form cells at detectable levels. Parasites were grown in immunocompetent female BALB/c mice from an initial infection of approximately $1 \times 10^6$ trypanosomes. Monomorphic lines were harvested at peak parasitaemia (3-d after infection); pleomorphic lines were either harvested at day 3 (for slender cells) or at day 5 when the parasitaemia was just post-peak (for stumpy cells). The ratio of different cell forms was determined by examination of the morphology of 2.5% glutaraldehyde fixed cells and by NADH diaphorase assay (Vickerman, 1965): air dried blood smears were fixed for 5 min at 4°C in 2.5% glutaraldehyde/0.1 M cacodylate buffer (pH 7.2). They were then processed as described in Vickerman (1965), but were incubated in reaction buffer for 1 h. Lymphocyte staining within the blood smear was used as an internal positive control, a "without substrate" slide acted as negative control.

### Differentiation Conditions

Trypanosomes were harvested from mice by cardiac puncture using citrate as an anticoagulant (Turner et al., 1988). The cells were then normally placed immediately into SDM-79 (Brun and Schonenburger, 1979) at 27°C, containing 6 mM *cis*-aconitate at a cell concentration of $\sim 2 \times 10^6$ cells/ml. The final citrate concentration was 2 mM. The cells were not purified from blood; however, most red blood cells settled out within 2–4 h.

### Immunofluorescence

1 ml of differentiating cells were harvested from culture, concentrated at 6,500 rpm in an Eppendorf microfuge (Brinkmann Instruments, Inc., Westbury, NY), the supernatant poured off and the cells resuspended in the remaining 30–40 μl of supernate. 10 μl of cells were then spotted onto slides and smears prepared gently. The air-dried slides were fixed, generally overnight, in 100% methanol at −20°C or, for 5'bromo 2'deoxyuridine (BRDU) analyses, in 75% ethanol at −20°C. For BRDU assay, treated cells were then processed as described in Woodward and Gull (1990) and BRDU incorporation detected using anti-BRDU antibodies (Biocell, Basel, Switzerland). Other antibodies were used at appropriate dilution according to previously described methods (Sherwin et al., 1987). VSG/procyclin double labeling was achieved using fluoroscein-conjugated anti-rabbit second antibody (for VSG) and Texas red–conjugated anti-mouse second antibody (for procyclin). YL1/2 and CAP 5.5 were both detected using fluoroscein-conjugated anti-rat antibody at an appropriate dilution. Following immunofluorescence, cellular DNA was labeled by the intercalating dye DAPI at 1 μg/ml for 1–4 min. Slides were mounted in MOWIOL (Harlow Chemical Co., Harlow, U.K.) containing phenylenediamine at 1 mg/ml as an antifading agent, examined using a Zeiss Axioscope microscope and images captured on Ilford XP2 film. Counts were performed on 300–500 cells, and each experiment has been repeated on at least three separate occasions.

### Reciprocal Shift Assay

Cells were spun out of differentiation conditions at 174 *g* for 10 min. The supernatant was poured off, and the cells resuspended in 10 ml of HMI-9 (Hirumi and Hirumi, 1989) pre-warmed to 37°C. The cells were washed two more times and then cultured overnight at an initial cell density of $\sim 1 \times 10^6$ cells/ml in HMI-9 at 37°C, 5% $CO_2$. Immunofluorescence was then performed as described above.

## Results

### Cell Differentiation and Division Are Synchronous

Interpretation of the effectors for differentiation requires a detailed characterization of the process and a reproducible protocol to ensure synchrony of the process. Our initial studies concentrated upon the exchange of surface antigens associated with the change from the bloodstream trypomastigote to the procyclic form. The differentiation of bloodstream cells has been characterized previously by studies at the cell population level. Using flow cytometry, Ziegelbauer et al. (1990, 1993) have studied the loss of the surface glycoprotein coat from bloodstream trypanosomes and have detected synchrony of the process in populations derived from pleomorphic lines of trypanosomes containing high levels of stumpy form cells ("pleomorphic" and "monomorphic" trypanosome lines are defined in Materials and Methods). Our results confirm these findings at the individual cell level. Using immunofluorescence upon differentiating cells we have found that cells which have shed the VSG coat are detectable as soon as 4 h after the initiation of differentiation and that the majority of the cells have shed the coat between 6 and 8 h (Fig. 1). This trend was confirmed using densitometric analysis of Coomasie-stained protein extracts isolated through the time course; between 4–6 h there was an approximately fivefold decrease in cellular VSG (data not shown).

The procyclic form of the parasite bears a stage-specific glycoprotein coat known as either procyclin (Roditi et al., 1987) or PARP (Mowatt and Clayton, 1987). Immunofluorescence microscopy shows that procyclin is first detectable on trypanosomes fixed 2 h after culture in differentiation

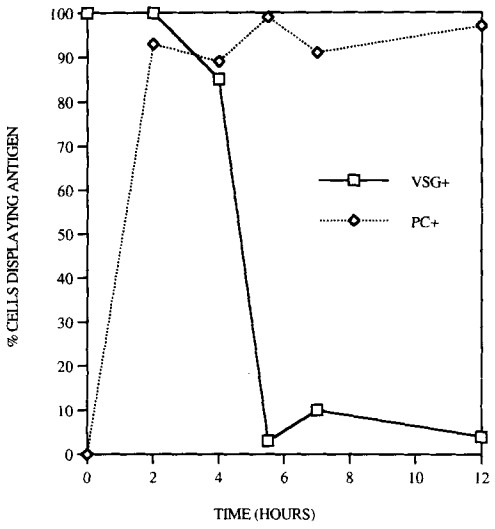


*Figure 1.* VSG loss from differentiating trypanosomes and procyclin gain. An infected mouse was sacrificed when the proportion of stumpy cells reached 80%, and trypanosomes placed under differentiation conditions at a concentration of $2 \times 10^6$ cells/ml. The ratio of coated to uncoated cells was then examined by immunofluorescence, using antibody raised against VSG GUTat 7.2. The presence of procyclin was determined using anti-procyclin antibody (a gift from Dr. Isabel Roditi). For this and subsequent figures we present the data derived from individual experiments due to temporal variation between experiments in the initiation of differentiation. Each experiment was repeated on at least three occasions, however, with the presented results being fully representative of these. VSG+ defines cells bearing the VSG coat; PC+ defines cells bearing Procyclin.

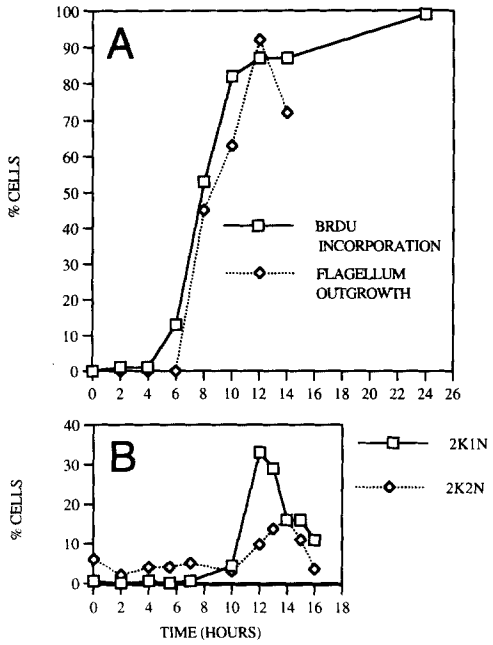


*Figure 2.* (*A*) Differentiating trypanosomes were incubated in the presence of BRDU and 2′ deoxycytidine (50 $\mu$M each). At 1-2-h intervals cells were harvested and processed for immunofluorescence. Counts represent detectable incorporation of BRDU into the cell nucleus (□) or appearance of a daughter flagellum labeled with YL1/2 and/or segregated basal bodies (◇). Kinetoplast BRDU incorporation was not scored because mouse anti-trypanosome antibody within the flagellar pocket (which is directly adjacent to k-DNA) is recognized by the fluorescent anti-mouse immunoglobulin second antibody and can confuse the analysis. (*B*) Karyotypic analysis of the differentiating population (represented in *A*); 2K1N = cells which have separated their kinetoplasts, but have not completed mitosis; 2K2N = post mitotic cells prior to cytokinesis.

medium and that by 4–6 h nearly all cells are labeled (although the intensity of fluorescence continues to increase in the first 12 h). As with the loss of VSG, the kinetics of procyclin gain can be variable, but where high levels of stumpy cells are present in the differentiating population, the gain can be extremely synchronous (95% of cells become labeled between 2 and 4 h after the initiation of the differentiation; Fig. 1). Thus, under our differentiation conditions, the exchange of stage-specific surface antigens is both synchronous and reproducible.

In pleomorphic trypanosome lines, which represent most closely natural parasite populations, the slender cells continue to divide, whilst the stumpy form apparently does not divide. This raised the possibility that differentiation to the procyclic form from division-arrested stumpy cells might be as synchronous in terms of progression through the cell cycle as is the exchange of surface antigens. We therefore scored several indicators of cell division during the differentiation of stumpy form trypanosomes. In order to follow their reentry into a proliferative cycle, we have characterized the incorporation of BRDU into the DNA of differentiating cells. This is a nucleotide analogue whose incorporation into DNA can be followed using anti-BRDU antibodies and subsequent immunofluorescence. Thus, BRDU incorporation can be used to examine the beginning and progress of kinetoplast and nuclear S-phase in the trypanosome cell cycle (Woodward and Gull, 1990).

Our results using parasite populations very high in stumpy form cells (>80% cells were scored as being positive for NADH diaphorase and having a stumpy morphology or a morphology intermediate between slender and stumpy cells) indicate that progression through the cell cycle during differentiation is indeed quite synchronous. Fig. 2 *a* shows that nuclear S-phase begins ~6–8 h after harvest from the mouse, and has occurred in most cells 2–4 h later. This S-phase is followed 4 h later by the first karyotypic signs of cell division (Fig. 2 *b*). This is in striking contrast to the BRDU incorporation of a logarithmically growing asynchronous procyclic population, which is essentially linear with time (Woodward and Gull, 1990).

We also have several monoclonal antibodies which detect antigens diagnostic for various phases of the procyclic trypanosome cell cycle. Among the most useful of these is the antibody YL1/2 which detects a posttranslational tyrosination of $\alpha$ tubulin (Kilmartin et al., 1982; Wehland et al., 1984). The trypanosome cytoskeleton is a remarkably stable structure in which newly laid down microtubules are tyrosinated, and subsequently become detyrosinated. When using YL1/2 in immunofluorescence a posterior cone of fluorescence is seen on cells, which fluctuates through the procyclic cell cycle. Additionally, trypanosomes demon-

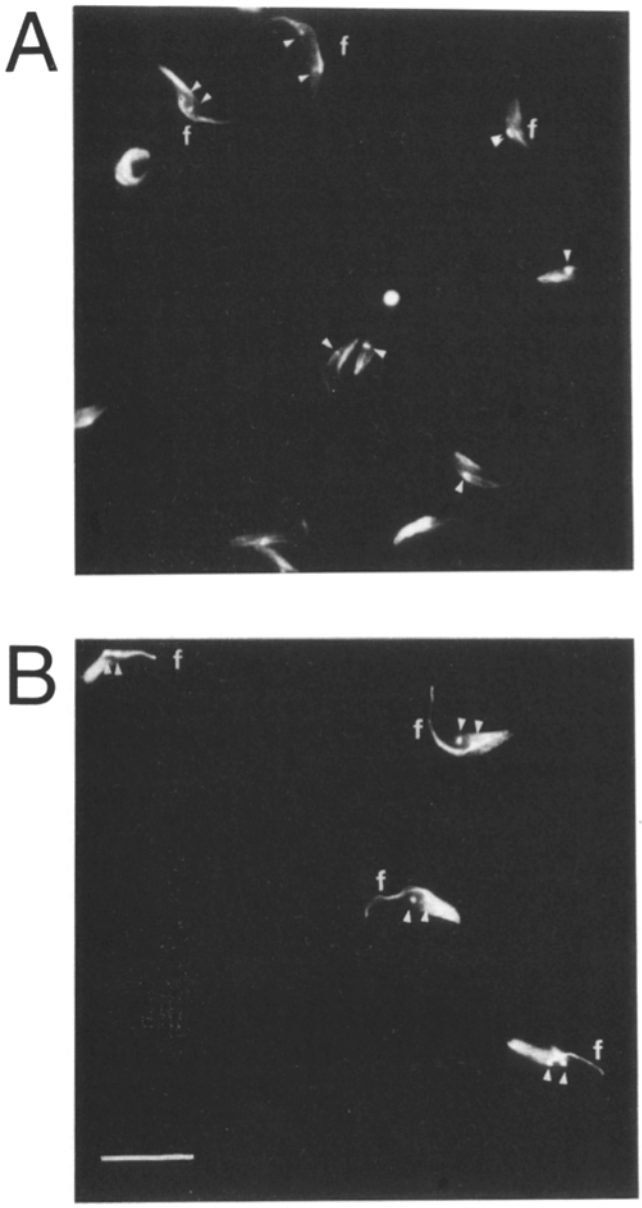


*Figure 3.* YL1/2 labeling of cells 12 h after the initiation of differentiation. (*A*) procyclic cells in exponential asynchronous growth. (*B*) Differentiating trypanosomes, 12 h following placement into differentiation medium. The cells are all at the same stage in their cell cycle, shortly after the completion of S-phase, with the basal bodies beginning their separation and daughter flagellum outgrowth being evident. Arrowheads indicate the position of the separating basal bodies; *f*, newly forming flagellum. Bar, 10 μm.

strate a bright basal body fluorescence throughout the cell cycle and show YL1/2 labeling of the newly forming flagellum in those cells emerging from S-phase. Thus, the separation of the basal bodies in preparation for kinetoplast segregation and appearance of a brightly labeled daughter flagellum when using YL1/2 are diagnostic markers for procyclic trypanosomes which have completed S-phase and are preparing for cell division (Sherwin et al., 1987; Sherwin and Gull, 1989*b*).

When we examined YL1/2 staining on differentiating bloodstream trypanosomes we found that the kinetics of daughter flagellar outgrowth in the population closely followed that of nuclear S-phase (Fig. 2 *a*, ◇) with 70–80% of cells demonstrating a newly forming flagellum (albeit of variable lengths) at 10–12 h after the initiation of differentiation. As shown in Fig. 3, the daughter flagellum arises from the more posterior of the basal bodies; the old flagellum remaining anterior and unlabeled with YL1/2 as is seen for procyclic cells (Sherwin et al., 1987). Thus, on the basis of two criteria, basal body separation and daughter flagellar outgrowth, trypanosomes emerge from their S-phase retaining a significant degree of synchrony in the population.

### *A Relation between the Trypanosome Cell Cycle and the Induction of Cellular Differentiation*

On examining the exchange of surface antigens on differentiating trypanosomes we noticed that there appeared to be a link between progression through the cell cycle and the capacity for differentiation. Specifically, until ~6 h after being placed under differentiation conditions, cells that were visibly undergoing a proliferative cell cycle, i.e., possessed two kinetoplasts and one nucleus (cells between D-phase and mitosis; Woodward and Gull, 1990) or two kinetoplasts and two nuclei (cells between mitosis and cytokinesis) were always $VSG^+$, and $procyclin^-$, that is, were not undergoing differentiation (a systematic analysis of >10,000 cells revealed 175 dividing cells, all of which were undifferentiated; see Fig. 4). In contrast, >90% of other cells had gained procyclin after 2 h and lost VSG after 4 h. We considered the failure of these early dividing cells to differentiate to be due to one of two possibilities: (*a*) There is a position in the cell cycle at which cells are receptive to the differentiation signal(s). Thus, most cells (the stumpy form) are at the receptive point and are able to differentiate, while those still in a proliferative cycle (slender cells) would generally not be at the receptive point, and would not differentiate in synchrony; (*b*) There is a difference in the capacity for differentiation between non proliferative (stumpy) and proliferative (slender) cells unrelated to position in the cell cycle. Thus, stumpy cells have the capacity for differentiation while slen-

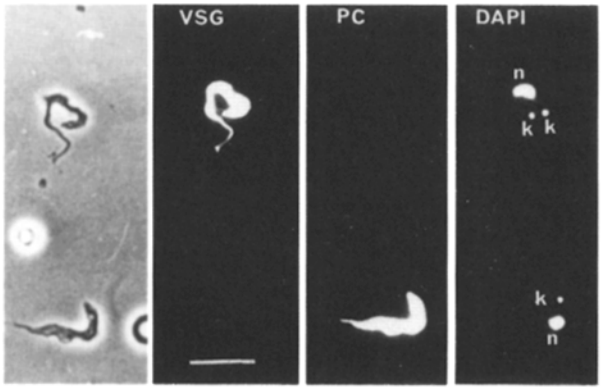


*Figure 4.* Dividing cells do not differentiate. Cells 6 h after harvest from a mouse were double labeled using antibodies directed against VSG (on the fluoroscein channel) and procyclin (*PC*, on the rhodamine channel). The DAPI staining reveals the position of each cell in their cell cycle; *n*, nucleus; *k*, labels the kinetoplast(s). Bar, 10 μm.

der cells do not, and the observed relationship with position in the cell cycle is coincidental.

In order to distinguish between these possibilities we have carried out a number of studies. First of all, we examined the comparative capacity for differentiation of slender and stumpy cells. For this we used, initially, two monomorphic lines of trypanosomes which are thought to have lost the capacity to undergo slender to stumpy transformation and in which we have never detected stumpy cells on the basis of either morphology or diaphorase assay. These were derived from pleomorphic lines by continual syringe passage through laboratory animals over a period of up to 20 years. The first was a direct derivative of the *T.b.rhodesiense* EATRO 795 pleomorphic strain used in our earlier studies (GUP 2965). The second was a line of *T.b.brucei.* (S427).

When these populations were harvested from mice and subjected to the differentiation conditions, we found that both populations were able to transform through to procyclic cells, but that the kinetics of this process were very different to that for a pleomorphic population of predominantly stumpy cells (Fig. 5; *T.b.rhodesiense* monomorphic cells are represented by *open circles*; *T.b.brucei* monomorphic cells are represented by *open triangles*); there was no synchrony, with just a few cells (~10%) becoming procyclin$^+$ in the first 24 h. Careful microscopic examination of these cells did not convince us that the differentiating cells were derived from stumpy progenitors in the predominently monomorphic population; however, such an analysis is difficult in the absence of slender/stumpy specific immunological markers.

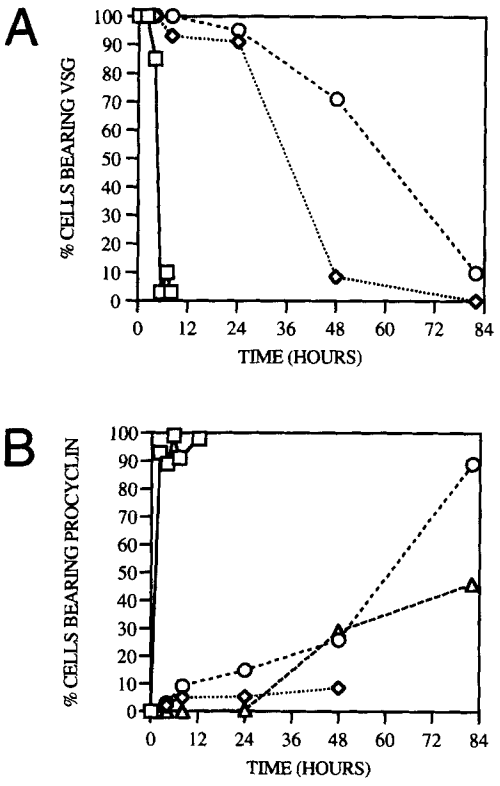


*Figure 5.* (*A*) VSG loss and (*B*) procyclin gain for different populations of trypanosomes: a high stumpy (>90%) pleomorphic population of *T.b.rhodesiense* (□), a high slender (<5% stumpy) pleomorphic population of *T.b.rhodesiense* (◇), a monomorphic line of *T.b.brucei* S427 (△) and a monomorphic line of *T.b. rhodesiense* (○). VSG loss for *T.brucei* S427 is not shown as the antigen type of our cell line is uncharacterized.

Although the monomorphic lines failed to differentiate efficiently, this may have been unrelated to their capacity to produce stumpy cells. Instead, these lines may have lost the capacity for differentiation with maintenance in the laboratory, independently of their capacity for pleomorphism. To investigate this possibility, we used our knowledge of the kinetics of stumpy production in a pleomorphic population to derive a predominantly slender population from cells known to be able to differentiate efficiently. Stumpy cells only predominate in a laboratory infection late in parasitaemia, being either not produced early on, or not selected for (Balber, 1972). We, therefore, harvested these pleomorphic trypanosomes populations from mice 48 h before our normal harvest time. In this early population the proportion of stumpy cells was judged to be less than 5% on the basis of the diaphorase assay and cellular morphology. We then asked whether these slender cells could differentiate. As shown in Fig. 5 (◇), these slender cells failed to differentiate efficiently, and instead showed kinetics of VSG loss/Procyclin gain similar to that seen for a true monomorphic line.

We next went on to investigate the basis of the failure of the monomorphic lines to differentiate efficiently under inductive conditions. Specifically, we were interested to know whether such cells were continuing through a normal cell cycle and thereby ignoring any "differentiation receptive point" within it. Therefore, we plotted the progress of the monomorphic trypanosomes through their cell cycle when under differentiation conditions by following their incorporation of BRDU. For such analysis BRDU was added either 15 min or 8 h after the cells had been placed into differentiation medium, thereby distinguishing the majority of those cells merely completing a pre-initiated cell cycle from those continuing through a proliferative cycle. Fig. 6 *a* shows the incorporation of this label occurs in ~30% of monomorphic cells during the first 2 h (Fig. 6 *a*, □), but beyond this point only ~10% of cells label (Fig. 6 *a*, ■). When we repeated the analysis using early parasitaemia pleomorphic cells, the incorporation of BRDU closely mimicked that of the monomorphic lines: 30–40% of cells labeled early on (Fig. 6 *a*, ○), representing completion of a pre-initiated cell cycle, but thereafter little further incorporation was observed (Fig. 6 *a*, ●). This was not due to cell death: both monomorphic cells and slender form pleomorphic cells remain motile in differentiation medium and are infective for mice for at least 24–48 h (data not shown).

In a final series of experiments we investigated the differentiation of monomorphic cells in a medium (DTM; Ziegelbauer et al., 1993) purported to favor bloodstream cell survival. We then measured VSG loss, procyclin gain, and BRDU incorporation for our monomorphic line of *T.b. rhodesiense.* Fig. 6 *b* shows that under these conditions we achieved a higher level of slender cell differentiation (47% of cells became procyclin$^+$ after 26-h incubation) than under our previous conditions. Furthermore, our anlyses revealed that after 2 h (during which many cells incorporated BRDU, exactly as in SDM-79) the kinetics of procyclin gain, VSG loss, and BRDU incorporation were very similar. In order to investigate the basis of this phenomenon,

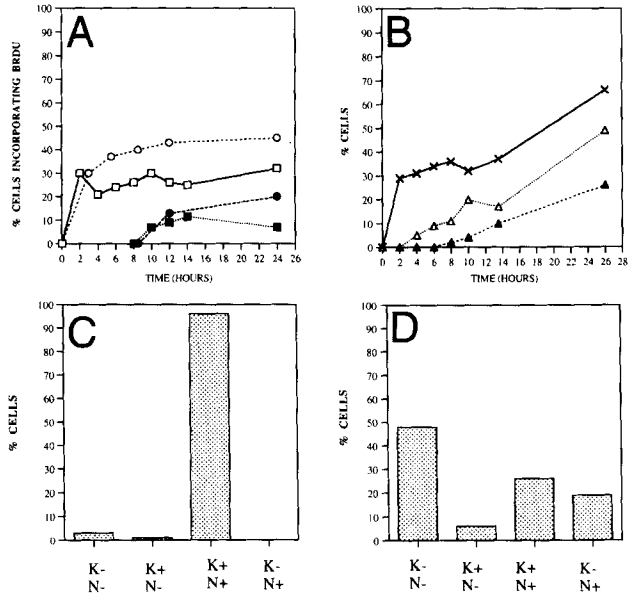


*Figure 6.* (*A*) BRDU incorporation into slender form trypanosomes under differentiation conditions. Cells were placed into differentiation conditions with BRDU being added at either 0 hours (*open symbols*) or 8 h after initiation (*filled symbols*). The latter determines cells which are continuing through a cell cycle under differentiation conditions, rather than completing a pre-initiated cycle. Boxes represent BRDU incoporation into monomorphic *T.b. rhodesiense*, circles represent BRDU incorporation into predominantly slender, low parasitaemia pleomorphic *T.b. rhodesiense*. (*B*) Monomorphic *T.b. rhodesiense* cell differentiation in the medium DTM. Cell differentiation was scored on the basis of the appearance of procyclin-bearing cells (△) or cells which had lost the VSG coat (▲). The population was assayed for cell cycle progression by following nuclear BRDU incorporation (*X*) (*C* and *D*) BRDU labeling pattern for monomorphic *T.b. rhodesiense* cells 26 h after being placed into DTM. (*C*) Cells which have lost the VSG; (*D*) Cells which are as yet undifferentiated as assessed by their retention of the VSG coat. *K*, kinetoplast; *N*, nucleus; + indicates that BRDU incorporation was detected by anti-BRDU antibody; − indicates an absence of detectable labeling. 300–500 cells were scored for each analysis.

we double-labeled cells at +26 h with antibodies against both VSG and BRDU. Interestingly, this revealed that 97% of differentiated cells (i.e., those which had lost the VSG coat) showed both nuclear and kinetoplast BRDU incorporation (Fig. 6 *c*), whilst 75% of undifferentiated cells (i.e., those still bearing the VSG coat) were either unlabeled with BRDU or showed only kintoplast or nuclear labeling (Fig. 6 *d*). Thus, under two distinct differentiation conditions, most viable trypanosomes in slender enriched populations have failed to complete one full cell cycle whether derived from monomorphic or pleomorphic lines, and this directly correlates with their ability to differentiate.

We have also discovered a further subset of cells incapable of differentiation within a pleomorphic population: "cells" without nuclei. In a normal bloodstream population there exist a small proportion (~0.5%) of apparent cells which, upon examination with DAPI fluorescence, exhibit a kinetoplast but no nucleus and which we believe to be products of aberrant cell division. We term such cytoplasts "zoids." With zoids it is possible to examine what aspects of differentiation require nuclear signaling, and to what extent the trypanosome is primed for rapid differentiation by the possession of pre-formed cytoplasmic proteins or mRNAs. Specifically, we were interested whether one of the earliest markers of differentiation—VSG loss—was an active process requiring a nuclear response. Although extremely small proportions of zoids are present in differentiating populations (we have seen 75 zoids in an examination of >10,000 cells), we have found that such cytoplasts pre-existing at 6 h into the differentiation always retain their VSG coat for up to 12 h, while >99% of nucleated cells have shed the coat at least 6 h earlier (an example is shown in Fig. 7). From this it appears the shedding of VSG during differentiation probably requires some kind of nuclear signal, although we acknowledge the caveat that many of these cells are the products of slender cell division and may, like slender populations, differentiate more slowly. NADH diaphorase positive stumpy-like zoids are also seen, however.

### *Commitment Occurs Early and Is Irreversible*

Having identified and characterized several useful differentiation markers we have used them to examine fundamental aspects of the control mechanisms underlying differentiation and its relationship to the trypanosome cell cycle. First, we

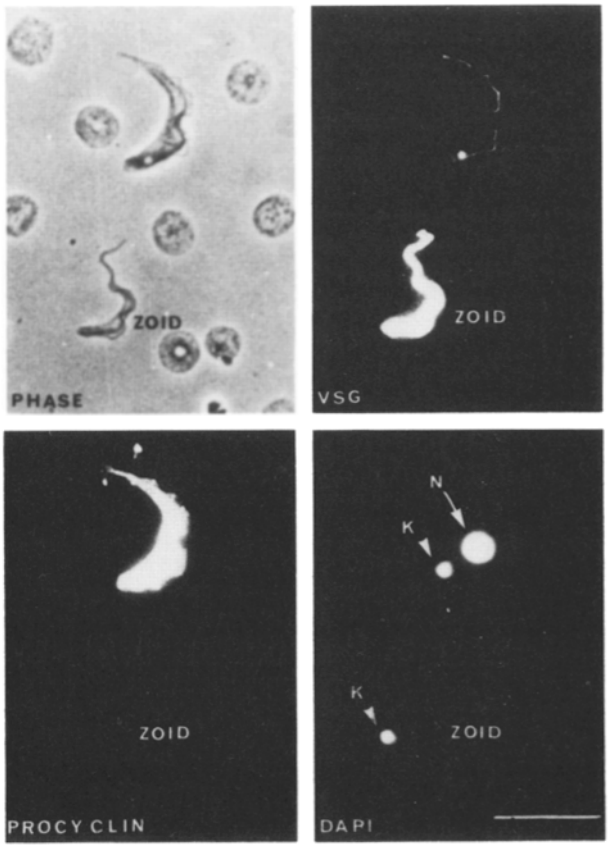


*Figure 7.* Anucleate cytoplasts do not shed VSG. Cells were harvested at 6 h into the differentiation. A differentiated (*VSG*$^-$, *procyclin*$^+$) cell is presented adjacent to an undifferentiated anucleated cytoplast (*Zoid*). *K*, kinetoplast; *N*, denotes the nucleus. Bar, 10 μm.

examined to what degree trypanosome differentiation was reversible, and at what point a commitment to any or all aspects of the differentiation occurred. We did this by transferring pleomorphic trypanosomes rich in stumpy cells back into bloodstream form culture medium (HMI-9; Hirumi and Hirumi, 1989) at various points after their incubation in differentiation medium. We followed the fate of two markers for differentiation; one that normally occurs early in the process (VSG loss) and one that occurs much later on (CAP 5.5 appearance). CAP 5.5 is a stage-regulated cytoskeleton associated protein which is induced with differentiation (Gerke-Bonet, R., and K. Gull, manuscript in preparation), appearing between 9 and 15 h after the initiation of the differentiation process. We then asked whether the VSG coat, once lost, could be re-acquired and whether and at what point trypanosomes became committed to CAP 5.5 expression.

Fig. 8 *a* shows that the differentiating trypanosomes lost the VSG coat at ~4 h after being placed in differentiation conditions (at 2 h 98% of cells were VSG$^+$, at 4 h 29% were VSG$^+$). When we examined cells shifted back into HMI-9 at 2, 4, and 6 h we found that cells did not revert to VSG coat expression when returned to bloodstream medium; cells shifted at 4 and 6 h remained uncoated, albeit with a small proportion of coated forms being retained (79–95% of cells were uncoated 20 h after transfer back into HMI-9). Cells transferred into HMI-9 at two hours still bore the VSG coat, although the debris of many dead cells were also present which may have been formerly coated or uncoated (data not shown). For CAP 5.5 appearance we found, as expected, that it was not present even after 6 h in differentiation medium (Fig. 8 *b*). However, when the reciprocally shifted cells were examined, we found that while cells shifted at 2 h remained unlabeled (although, again, many were dead), cells shifted into HMI-9 at both 4 and 6 h expressed CAP 5.5 after overnight incubation (~80% of cells transferred at 6 h were CAP 5.5$^+$). Control bloodstream cells incubated in HMI-9 directly after harvest from mice and without exposure to differentiation conditions remained VSG$^+$ and CAP 5.5$^-$. Thus, for both VSG loss and CAP 5.5 gain there was an irreversible commitment to the differentiated state between 0 and 4 h after induction.

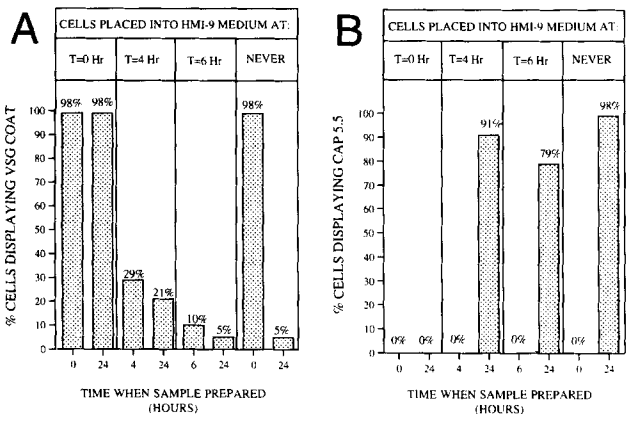


*Figure 8.* Analysis of the commitment to differentiation for high stumpy pleomorphic populations. (*A*) VSG loss (*B*) CAP 5.5 gain. Cells were harvested from mice and then placed under differentiation conditions. 2, 4, and 6 h later cells were centrifuged at 174 *g*, washed with 10 ml HMI-9 at 37°C, recentrifuged and re-washed, before being placed in 5 ml of HMI-9 (at a cell density of 1–2 × $10^6$/ml) at 37°C, 5% $CO_2$. Slides were prepared at 2, 4, and 6 h and after 24 h. The figure shows the percentage of cells displaying each antigen immediately prior to tranfer into HMI-9 and the same cell population after 24-h incubation in HMI-9. Data for cells harvested at 2 h are not shown, as many dead cells were present in this population 24 h after transfer into HMI-9.

### *Differentiation and Division Can Be Uncoupled*

We have also investigated whether differentiation and progression through the cell cycle were interdependent or merely coincident processes by examining the effect of inhibiting DNA synthesis in transforming stumpy cells. A related analysis has been performed using cell division inhibitors (Markos et al., 1989); here we wished to examine the influence of blocking an earlier cell cycle stage on our defined markers in the synchronously differentiating population. Therefore, we harvested trypanosomes from mice and placed them into differentiation medium containing the nuclear DNA polymerase inhibitor aphidicolin at 20 μg/ml. We examined these drug treated cells 24 h after the initiation of differentiation and asked whether they were still capable of inducing CAP 5.5-a marker that normally appears after the beginning of S-phase in the differentiating population. Such treated cells appear swollen (Fig. 9 *b*) and do not replicate their DNA, remaining unlabeled with BRDU (in an analysis of several hundred cells, all showed a weak kinetoplast labeling but not one cell was detected exhibiting nuclear DNA labeling). This inhibition of S-phase, however, did not disrupt subsequent cellular differentiation. First of all, the differentiation marker CAP 5.5 was readily detected on 98% of the treated cells, despite the fact that it is not normally induced until after initiation of nuclear S-phase. Secondly, analysis of the DAPI image of the transformed cells revealed that the kinetoplast was clearly lying adjacent to the cell nucleus as is seen in procyclic, but not bloodstream cells. Thus, on the basis of two markers for trypanosome differentiation, cell cycle progression and differentiation are processes that are coincident but which can be uncoupled, at least after differentiation has been initiated.

## *Discussion*

### *Entry into the Differentiation Pathway and Progress through the First Cell Cycle Are Coincident and Synchronous Processes*

In the experiments presented we have identified several novel markers for trypanosome cell differentiation and have used these to investigate the relationship between this process and transition of the trypanosome cell from a non proliferative to a proliferative form.

Our studies have revealed that populations high in stumpy cells differentiate and initiate cell division coincidentally and synchronously. By following the incorporation of BRDU we have found that nuclear S-phase is the first detectable cell cycle associated event that occurs during trypanosome differentiation. Using thymidine incorporation into the differentiating population Pays et al. (1993) have very recently obtained entirely compatible results. This S-phase is then followed by daughter kinetoplast separation, mitosis and ultimately

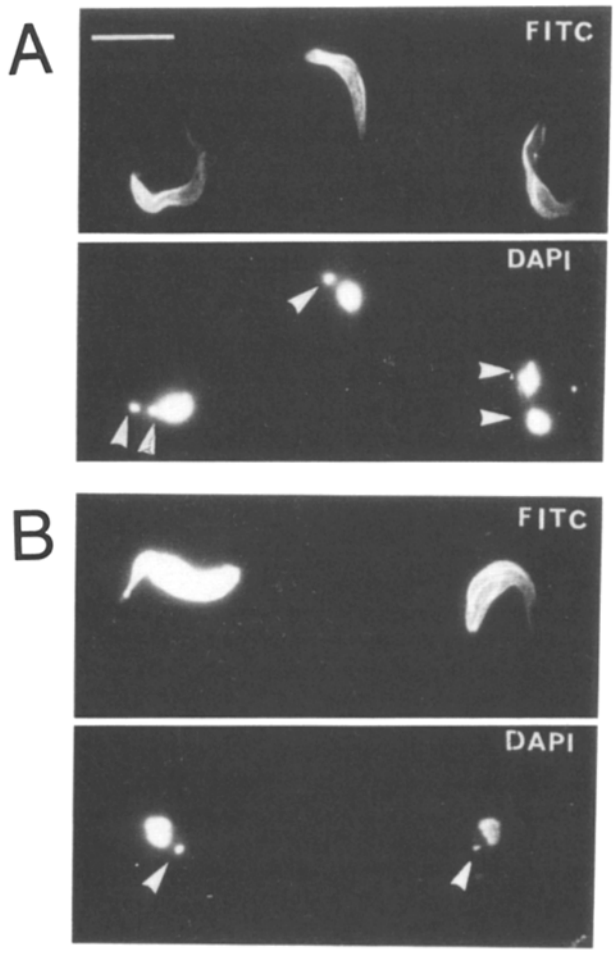


*Figure 9.* Differentiation can be uncoupled from cell cycle progression. Cells were placed into differentiation medium containing aphidicolin at 20 μg/ml. After 20-h incubation, the cells were assayed for CAP 5.5 expression (*FITC*). (*A*) Control cells, differentiated in the absence of aphidicolin. (*B*) Aphidicolin treated cells. Note that the kinetoplast (*arrowed*) in both control and the treated cells lies adjacent to the cell nucleus (*DAPI*). The right hand most cell in the control panel is postmitotic and the kinetoplasts are obscured by the nuclei. Bar, 10 μm.

cytokinesis. Although these events occur somewhat more slowly in differentiating cells than in procyclic cells, they represent progression through a normal cell cycle, initiating from stumpy cells arrested in either very early S-phase, $G_1$, or $G_0$.

Superimposed upon this first cell cycle is a gross cytological restructuring of the cell. Thus, prior to segregation of the kinetoplasts there is a migration of this organelle away from the cell terminus, to a location adjacent to the cell nucleus, its normal position in procyclic cells (Brown et al., 1973; Matthews, K. R., T. Sherwin, and K. Gull, manuscript in preparation). Later, initiating after the beginning of nuclear DNA synthesis, at least one stage specific cytoskeleton associated protein (CAP 5.5) is also deposited into the subpellicular matrix. By characterizing the relative timings of these stage regulated cytoskeletal changes with respect to the cell cycle associated events we are in a position to plot a temporal hierarchy of trypanosome differentiation and investigate the controls underlying it.

### *The Cell Cycle and Perception of the Differentiation Signal*

Our initial studies concentrated upon how the trypanosome perceives the differentiation signal. This was prompted by our observation that there existed cells in the population which failed to differentiate in synchrony with the majority, and that proliferative cells always fell into this subset. Since slender form trypomastigotes are proliferative and stumpy forms non-proliferative, we investigated the capacity for differentiation of these two cell types. Similar analyses have been performed at the population level by others and the results have been conflicting. Bass and Wang (1990, 1992) have analyzed the differentiation of monomorphic cells and pleomorphic cells either early (when predominantly slender) or late (when predominantly stumpy) and reported that they found no difference in the kinetics of differentiation between the various types. However, ambiguous morphological markers were assayed in their study. In contrast, Overath's group, using surface antigen markers, found that while predominantly slender populations do differentiate, the kinetics were slower than for populations with high levels of stumpy cells (Czichos et al., 1986; Roditi et al., 1989; Ziegelbauer, 1990). Our results, obtained at the single cell level, are compatible with those of Ziegelbauer et al. (1990). We found that populations enriched for slender cells, although able to differentiate, do so at markedly slowed rates compared to stumpy populations, whether derived from monomorphic lines or early parasitaemia pleomorphic lines. By the use of immunofluorescence we have been able to characterize the differentiation of the monomorphic lines at the individual cell level. This did not convince us that the founder population of differentiating cells represented a subpopulation of intermediate form or stumpy cells. Unfortunately the kinetics of differentiation of the monomorphic lines did not allow us to distinguish whether the final differentiated population was derived from this small founder population or was derived from a slow, asynchronous differentiation of the entire population (but see below).

Is there a point in the cell cycle at which trypanosomes receive the inductive signal for differentiation? The synchrony of the initiation of S-phase in differentiating cells suggests that stumpy cells in the bloodstream are held at the same point in the cell cycle-most likely in $G_1$ or in $G_0$. Slender populations, however, are asynchronous and would be expected to have initiated differentiation by the completion of two cell cycles under inductive conditions. This is apparently what was seen by Roditi et al. (1989) and our results in DTM medium support this. Differentiation of the population (as assessed by VSG loss and procyclin gain) begins rapidly and increases with kinetics which parallel the kinetics of BRDU incorporation, a scenario compatible with an asynchronous population passing through a receptive point and then initiating differentiation. The 30–40% of cells labeling with BRDU in the first few hours need not be passing through this window; instead they most probably represent cells originally in $G_1$ or S-phase which have become stalled or slowed in their progression through their cell cycle resulting from the change in temperature or nutrient environment (Fantes and Nurse, 1977). These cells most probably comprise the partly and fully BRDU-labeled undifferentiated cells seen at +26 h; differentiated cells were predominantly fully labeled. A similar phenomenon was seen with monomorphic

populations under SDM-79-based differentiation conditions in which there were few cells which progressed through even one complete cell cycle over 24 h and few which underwent differentiation. Thus, the conflicting rates and abilities of different trypanosome lines to differentiate seem not to reflect intrinsic inabilities in the process itself, but instead the kinetics of progress through the cell cycle of different cell lines or in different differentiation conditions. Such an explanation is simpler to envisage than heterogeneity in such a complex process as differentiation, particularly among monomorphic populations of clonal origin. Clearly sensitivity of the cell cycle to shock varies between slender and stumpy cells since the early parasitaemia, high slender, pleomorphic population was sensitive, while the stumpy derivatives of it were resistant.

In Fig. 10 we present a model to illustrate our working hypothesis based upon our observations and early ideas of Ziegelbauer et al. (1990). The model proposes the existence of a point in the trypanosome cell cycle (which may be $G_o$ or $G_1$) at which cells are receptive to the inductive signal to differentiate and that stumpy cells are already irreversibly committed to this pathway (Hamm et al., 1990). In Case I, stumpy cells are homogeneously arrested at the receptive window, and simultaneously re-enter a proliferative cell cycle and embark upon the differentiation pathway upon the reception of the inductive signal. Both processes, being perceived in a synchronized arrested population, proceed in register. In contrast, the slender bloodstream cell population is heterogeneous with respect to their position in the cell cycle; a proportion are within the receptive window (Case II), but the majority are without it (Case III). For that small proportion within the window, differentiation may proceed in an analogous manner to stumpy cells, although there may be no need for a true stumpy intermediate in the process. The remainder of the slender population (Case III), however, has two fates. In the first, they may successfully continue through their cell cycle, reach the receptive window and then initiate differentiation, as for Case II cells. Such populations, entering this window asynchronously, would differentiate asynchronously. The alternative fate is that Case III cells fail to progress through their cell cycle, do not reach the receptive window, and fail to differentiate. Such cells, although visible within the differentiating population for up to 3 d, are ultimately doomed. Case III cells may be most representative of what occurs in the tsetse fly; Turner et al. (1988) found rapid and selective death of slender cells in tsetse flies fed on blood containing pleomorphic trypanosomes.

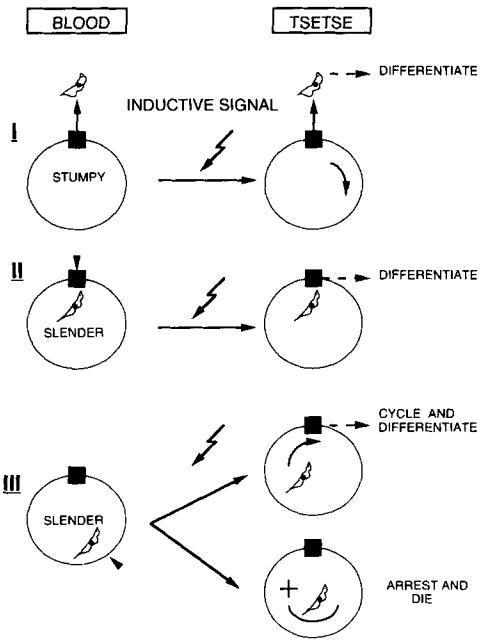


*Figure 10.* A model for the initiation of differentiation. In each case the trypanosome position in the cell cycle (*arrowheads*) is represented in relation to a "window" (*filled boxes*) in that cell cycle at which trypanosomes are receptive to the differentiation or inductive signal (*jagged arrow*). Stumpy cells are within that window, no longer cycling and irreversibly commited to differentiation (Case I cells). Case II cells are those slender cells within the receptive window and these differentiate as for stumpy cells (with or without a stumpy-like intermediate). Case III cells have two alternative fates: progression through their cycle, perception of the inductive signal and differentiation or cell cycle arrrest followed by eventual death.

Our analyses of the commitment points in differentiation begin to address its underlying control processes. We found that after 4 h VSG loss is not reversible and there is a commitment to CAP 5.5 expression. These studies support early findings of Ehlers et al. (1987) who analyzed VSG mRNA in differentiating monomorphic cell lines switched back to 37°C. In that case, VSG coat loss did not occur for at least 12 h, prior to which the depression in VSG steady state RNA levels was reversible. Our results are compatible with this, albeit within the timeframe of stumpy cell differentiation. More interesting is the commitment to the deposition of CAP 5.5 in the trypanosome cytoskeleton. In this case, the cells commit to expression of the protein some hours (at least 4–6 h) before its actual appearance. This indicates that at some point before 4 h in differentiation medium the cells embark on a temporally controlled pathway culminating on the production of the procyclic form, or cells with many procyclic features. Such a pathway may be involve a linear cascade of events, with each prior event requiring to be completed before initiation of the next step(s). Alternatively, it may a branched pathway, where some related events are coordinated, but without continual cross-checking between different elements of the differentiation. Our experiments with the DNA synthesis inhibitor aphidicolin lead us to favor the latter hypothesis, at least with respect to cell division. When cells were arrested early in S-phase, they still went on to express the marker CAP 5.5, which is normally induced during and subsequent to DNA replication. Although we do not at present know at what level control of expression of this marker operates, this would not affect the interpretation of the experiment. Whether transcriptional or post transcriptional, the temporal control of the assembly of this protein into the cytoskeleton was unaffected. Similarly, movement of the kinetoplast to a position near to the nucleus was unaffected, despite this normally being initiated during S-phase. Clearly, blocking one element of the differentiation does not block all coincident and downstream processes; cell cycle progression can be uncoupled from differentiation.

Our analyses of the monomorphic cells and anucleate cytoplasts (zoids) also address the control of the very earliest events in the differentiation. That zoids do not lose the VSG coat and monomorphic cells neither lose the VSG nor gain

procyclin with rapidity, indicates that the control of these two antigens is not simple. Clearly VSG release is an active process not reliant upon simple protein turnover because individual stumpy cells completely lose the surface coat within the space of less than two hours. This supports the implication that VSG release is carried out by a specific enzymatic process (Ziegelbauer et al., 1993). In zoids this loss does not occur, indicating that there is probably no cytoplasmic shedding mechanism which is activable without some sort of communication with the nucleus. Instead, in nucleated cells procyclin gain and VSG loss must be phenotypic responses downstream of, and secondary to, the primary perception of the differentiation signal. The basis of this initial perception of the differentiation signal is a mystery but candidates might be stumpy specific/enriched receptors and messengers. We are currently investigating such proteins and transcripts in order to understand how the trypanosome initiates its entry into the differentiation pathway and how cell cycle position impinges on this.

We thank Dr. J. D. Barry, Dr. I. Hagan, and Professor P. Overath for comments on the manuscript, and Dr. D. Robinson, Dr. T. Sherwin, and other members of the Gull lab for advice and assistance throughout the course of this work. We are also indebted to Dr. C. M. R. Turner at Glasgow University for the gift of trypanosome strains and anti-VSG antibody and to Dr. I. Roditi (University of Bern, Bern, Switzerland), Dr. J. Kilmartin (University of Cambridge), and Dr. R. Gerke-Bonet (University of Manchester, Manchester, U.K.) for the gift of antisera.

K. Matthews and K. Gull are supported by the Wellcome Trust.

Received for publication 7 December 1993, and in revised form 15 February 1994.

### *References*

Balber, A. E. 1972. *Trypanosoma brucei*: fluxes of morphological variants in intact and x-irradiated mice. *Exp. Parasitol.* 31:307–319.

Bass, K., and C. C. Wang. 1991. The *in vitro* differentiation of pleomorphic *Trypanosoma brucei* from bloodstream to procyclic form requires neither intermediary nor short-stumpy stage. *Mol. Biochem. Parasitol.* 44:261–270.

Bass, K. E., and C. C. Wang. 1992. Transient inhibition of protein synthesis accompanies differentiation of *Trypanosoma brucei* from bloodstream to procyclic forms. *Mol. Biochem. Parasitol.* 56:129–140.

Brown, R. C., D. A. Evans, and K. Vickerman. 1973. Changes in oxidative metabolism and ultrastructure accompanying differentiation of the mitochondrion in *Trypanosoma brucei*. *Int. J. Parasitol.* 3:691–704.

Brun, R., and M. Schonenburger. 1979. Cultivation and *in vitro* cloning of procyclic culture forms of *Trypanosoma brucei* in semi-defined media. *Acta Tropica.* 36:289–292.

Clayton, C. E. 1992. Developmental regulation of nuclear gene expression in *Trypanosoma brucei*. *Prog. Nucleic Acids Res. Mol. Biol.* 43:37–66.

Cross, G. A. M. 1990. Cellular and Genetic aspects of antigenic variation in African trypanosomes. *Annu. Rev. Immunol.* 8:83–110.

Czichos, J., C. Nonnengaesser, and P. Overath. 1986. *Trypanosoma brucei*: cis-aconitate and temperature reduction as triggers of synchronous transformation of bloodstream to procyclic trypomastigotes *in vitro*. *Exp. Parasitol.* 62:283–291.

Ehlers, B., J. Czichos, and P. Overath. 1987. Repression and reactivation of the variant surface glycoprotein gene in *Trypanosoma brucei*. *FEBS (Febs. Eur. Biochem. Soc.) Lett.* 225:53–58.

Fantes, P., and P. Nurse. 1977. Control of cell size at division in fission yeast by a growth-modulated size control over nuclear division. *Exp. Cell Res.* 107:377–386.

Haber, J. E. 1992. Mating-type switching in *Saccharomyces cerevisiae*. *Trends Genet.* 8:446–451.

Hamm, B., A. Schindler, D. Mecke, and M. Duszenko. 1990. Differentiation of *Trypanosoma brucei* bloodstream trypomastigotes from long slender to short stumpy-like forms in axenic culture. *Mol. Biochem. Parasitol.* 40: 13–22.

Hirumi, H., and K. Hirumi. 1989. Continuous cultivation of *Trypanosoma brucei* bloodstream forms in a medium containing a low concentration of serum protein without feeder cell layers. *J. Parasitol.* 75:985–989.

Kilmartin, J. V., B. Wright, and C. Milstein. 1982. Rat monoclonal anti-tubulin antibodies derived using a new non-secreting rat cell line. *J. Cell Biol.* 93:576–582.

Klar, A. J. S. 1992. Developmental choices in mating-type interconversion in fission yeast. *Trends Genet.* 8:208–212.

Markos, A., A. Blahuskova, M. Kalous, E. Byskova, A. Byska, and E. Nohynkova. 1989. Metabolic differentiation of bloodstream forms of *Trypanosoma brucei* brucei into procyclic forms. *Folia Parasitol.* 36:225–238.

Mowatt, M. R., and C. E. Clayton. 1987. Developmental regulation of a novel repetitive protein of *Trypanosoma brucei*. *Mol. Cell. Biol.* 7:2838–2844.

Newton, A., and N. Ohta. 1990. Regulation of the cell division cycle and differentiation in bacteria. *Annu. Rev. Microbiol.* 44:689–719.

Pays, E., J. Hanocq-Quertier, F. Hanocq, S. Van Assel, D. Nolan, and S. Rolin. 1993. Abrupt RNA changes precede the first cell division during the differentiation of *Trypanosoma brucei* bloodstream forms into procyclic forms in vitro. *Mol. Biochem. Parasitol.* 61:107–114.

Roditi, I., M. Carrington, and M. Turner. 1987. Expression of a polypeptide containing a dipeptide repeat is confined to the insect stage of *Trypanosoma brucei*. *Nature (Lond.).* 325:272–274.

Roditi, I., H. Schwartz, T. W. Pearson, R. P. Beeecroft, M. K. Liu, J. P. Richardson, H. J. Buhring, J. Pleiss, R. Bulow, R. O. Williams, and P. Overath. 1989. Procyclin gene expression and loss of the variant surface glycoprotein during differentiation of *Trypanosoma brucei*. *J. Cell Biol.* 108: 737–746.

Sherwin, T., and K. Gull. 1989*a*. The cell division of *Trypanosoma brucei brucei*: timing of event markers and cytoskeletal modulations. *Phil. Trans. R. Soc. Lond. (Biol.).* 323:573–588.

Sherwin, T., and K. Gull. 1989*b*. Visualisation of detyrosination along single microtubules reveals a novel mechanism of assembly during cytoskeletal duplication in trypanosomes. *Cell.* 57:211–221.

Sherwin, T., A. Schneider, R. Sasse, T. Seebeck, and K. Gull. 1987. Distinct localisation and cell cycle dependence of COOH terminally tyrosinated alpha tubulin on the microtubules of *Trypanosoma brucei*. *J. Cell Biol.* 104: 439–446.

Turner, C. M. R., J. D. Barry, and K. Vickerman. 1988. Loss of the variable antigen during transformation of *Trypanosoma brucei rhodesiense* from bloodstream to procyclic forms in the tsetse fly. *Parasitol Res.* 74:507–511.

Vickerman, K. 1965. Polymorphism and mitochondrial activity in sleeping sickness trypanosomes. *Nature (Lond.).* 208:762–766.

Vickerman, K. 1985. Developmental cycles and biology of pathogenic trypanosomes. *Br. Med. Bull.* 41:105–114.

Wehland, J., M. C. Schroeder, and K. Weber. 1984. Amino acid sequence requirements in the epitope recognized by the α-tubulin-specific rat monoclonal antibody YL 1/2. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:1295–1300.

Wijers, D. J. B., and K. C. Willett. 1960. Factors that may influence the infection rate of *Glossina palpalis* with *Trypanosomsa gambiense*: II. The number and morphology of the trypanosomes present in the blood of the host at the time of the infected feed. *Ann Trop. Med. Parasitol.* 54:341–350.

Woodward, R., and K. Gull. 1990. Timing of nuclear and kinetoplast DNA replication and early morphological events in the cell cycle of *Trypanosoma brucei*. *J. Cell Sci.* 95:49–57.

Woods, A., T. Sherwin, R. Sasse, T. H. MacRae, A. J. Baines, and K. Gull. 1989. Definition of individual components within the cytoskeleton of *Trypanosoma brucei* by a library of monoclonal antibodies. *J. Cell Sci.* 93:491–500.

Ziegelbauer, K., M. Quinten, H. Schwartz, T. W. Pearson, and P. Overath. 1990. Synchronous differentiation of *Trypanosoma brucei* bloodstream to procyclic forms in vitro. *Eur. J. Biochem.* 192:373–378.

Ziegelbauer, K., B. Stahl, M. Karas, Y.-D. Steirhof, and P. Overath. 1993. Proteolytic release of cell surface proteins during differentiation of *Trypanosoma brucei*. *Biochemistry.* 32:3737–3742.