

# Commitment to differentiation and cell cycle re-entry are coincident but separable events in the transformation of African trypanosomes from their bloodstream to their insect form

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## SUMMARY

**African trypanosomes undergo extensive changes in cellular morphology, biochemistry and surface antigen expression as they differentiate from their bloodstream form to those forms that colonise the midgut of their tsetse fly vector. If initiated with stumpy-form cells, a non-dividing sub-type of the bloodstream parasite, differentiation and cell cycle re-entry occur synchronously in the population and provide a means to dissect the respective controls of proliferation and transformation. We have exploited this synchrony to determine the respective importance and hierarchy of the known triggers for differen-**

**tiation (cis aconitate, temperature drop) for individual components of both differentiation and the cell cycle. This has revealed the pre-eminence of cis aconitate as a primary trigger for parasite differentiation, and has allowed us to determine that the cellular commitment to both differentiation and cell-cycle re-entry are precisely co-incident processes**

Key words: Differentiation, commitment, Cell-cycle, Trypanosome, *Trypanosoma brucei*

## INTRODUCTION

Cellular differentiation events in both prokaryotic and eukaryotic organisms are often intimately associated with the regulation of cell proliferation. Most commonly, cells exit from the cell cycle and then undergo differentiation leading to either a terminal, irreversible cell specialisation (e.g. in multicellular organisms) or a particular developmental step in the life cycle (e.g. unicellular organisms). The controls of the cell-cycle can therefore share common features and themes with the processes that control cell differentiation (overviewed by Derynck and Wagner, 1995). One such theme is that there is a requirement for particular triggers for successful entry or exit from the cell cycle and for differentiation. For example, mammalian cells require particular mitogen triggers for successful completion of the cell cycle (Zetterberg et al., 1995), and yeast cells are stimulated to exit the cell cycle and initiate differentiation in response to starvation and the  $\alpha$  factor pheromones (Kurjan, 1993). A second common theme is that progress through the cell cycle and differentiation pathways involves passage through a point of commitment, at which further advance becomes independent of the original triggers for the process (Maclean and Hall, 1987). In many cases the molecular constituents that regulate these respective pathways can also share common components.

The African trypanosome, *Trypanosoma brucei*, is a protozoan parasite in which cell division and life cycle differentiation are closely linked. The parasite is spread between

mammalian hosts by the blood feeding tsetse fly, with there being multiple developmental stages within both the mammal host and the fly vector (Vickerman, 1985). Progression through these life cycle stages involves an alternation in the proliferation status of the parasite, with dividing forms adapted to the colonisation of particular host or fly environments alternating with non dividing parasite forms adapted to transmission between these environments (Mottram, 1994; Matthews and Gull, 1994a). In the mammalian bloodstream, the parasites proliferate extensively as morphologically 'slender' forms. However, as each wave of parasitaemia progresses the parasite population shifts from being predominantly slender to being predominantly 'stumpy' in morphology. The stumpy form of the parasite is non-dividing and is pre-adapted for differentiation through to the parasite's procyclic form, which colonises the midgut of the tsetse fly vector (Wijers and Willett, 1960; Turner et al., 1988). This differentiation is initiated when bloodstream parasites are ingested by the tsetse fly.

The differentiation of bloodstream-form trypanosomes to procyclic-forms can be induced in vitro, by the addition of cis aconitate/citrate and by the reduction in temperature from 37°C to 27°C (Brun and Schonemberger, 1981; Overath et al., 1983, 1986; Simpson et al., 1985; Czichos et al., 1986). Under these conditions, both slender-form and stumpy-form parasites can undergo differentiation (Overath et al., 1983; Roditi et al., 1989; Bass and Wang, 1991), although stumpy cells do so most efficiently both in vivo and in vitro (Turner et al., 1988; Zielgelbauer et al., 1990; Matthews and Gull, 1994b). There are

now a number of markers which allow the progress of this differentiation to be followed and their temporal order of expression has been mapped at both the cellular and population level (Roditi et al., 1987; Mowatt and Clayton, 1987; Pays et al., 1993; Matthews and Gull, 1994a,b; Matthews et al., 1995). Detailed analysis of slender and stumpy-form differentiation using these markers has demonstrated that a homogeneous population of stumpy form parasites are able to undergo this differentiation process with great synchrony (Ziegelbauer et al., 1990). This synchrony has permitted the temporal order of both differentiation and cell cycle events for bloodstream derived trypanosome cell populations to be described (Matthews and Gull 1994a; Pays et al., 1997). This revealed that re-entry into the proliferative cell cycle occurs concomitantly with progress of the cells through the differentiation pathway and raised the possibility that these events might be co-ordinated, at least in their early stages (Ziegelbauer et al., 1990; reviewed by Matthews and Gull, 1994a).

In this paper we have investigated the degree of interplay between the cell division and differentiation pathways for stumpy cells as they develop toward tsetse-midgut procyclic form cells *in vitro*. In particular, we have investigated the respective response of these two pathways to the identified triggers for *in vitro* differentiation and have determined the point at which the pathways become independent of these stimuli. The results demonstrate that commitment to differentiation and cell cycle re-entry are coincident processes which, although separable under defined conditions, follow a highly predictable temporal programme. The results describe a situation in which there is an initial reversible perception of the differentiation signal, rapid passage through a point of commitment and then progression into a precisely regulated developmental pathway culminating in the proliferative procyclic form.

## MATERIALS AND METHODS

### Trypanosomes

A pleomorphic line of *Trypanosoma brucei rhodesiense* EATRO 795 was used throughout (GUP 2962; kind gift of Dr C. M. R. Turner, University of Glasgow, Scotland). These cells retain the ability to generate both slender and stumpy bloodstream form parasites when grown in rodents and express the GUTat 7.2 variable surface glycoprotein (VSG) coat to near homogeneity. Trypanosome populations were harvested from infected BALB/c mice when the proportion of stumpy cells was >80% in the population, as assessed by cellular morphology and the NAD diaphorase assay (Vickerman, 1965). This was typically 5 days post-infection. Procyclic cells were either derived from *in vitro* differentiation of the EATRO 795 bloodstream forms, or were well established S427 procyclic cells of *T. b. brucei*.

### Differentiation conditions

Trypanosomes were harvested from infected mice by cardiac puncture and placed directly into differentiation medium (SDM-79+ 6 mM cis aconitate) at a cell density of  $2 \times 10^6$  parasites/ml (Brun and Schonemberger, 1979; Turner et al., 1988; Matthews and Gull, 1994b). Where the effect of particular differentiation triggers was under examination, mouse blood was harvested into syringes and flasks pre-warmed to 37°C. The total harvesting procedures, from mouse to 'trigger-specific' culture condition was routinely of the order of less than one minute. Where parasite protein was to be prepared, cells were purified over DEAE cellulose according to the method of Lanham and Godfrey

(1970) using PSG (488 mg/l  $\text{NaH}_2\text{PO}_4$ , 2.55 g/l NaCl, 8.808 g/l  $\text{Na}_2\text{HPO}_4$ , 15g/l D-glucose, pH 7.8), warmed to 37°C. Proteins were prepared as previously described (Laemmli, 1970; Matthews et al., 1995).

### Immunofluorescence and western blotting

For immunofluorescence,  $2 \times 10^6$  of parasites were harvested, concentrated in a microcentrifuge at 1,000 g, spread onto microscope slides and briefly left to air dry. The cells were then fixed in 100% methanol for 30 minutes to 24 hours at -20°C and slides processed for immunofluorescence as previously described (Sherwin et al., 1987). For VSG analysis a rabbit-polyclonal antiserum specific for GUTat 7.2 was used (gift of Dr C. M. R. Turner, University of Glasgow, Scotland); procyclin specific antibodies (Roditi et al., 1989) were obtained from Cedar Lane Laboratories, Canada; CAP5.5 specific rat polyclonal antiserum was a kind gift from Dr R. Gerke Bonet (University of Manchester). Where DNA synthesis was being assayed, 50  $\mu\text{M}$  5'-bromo-2'-deoxy uridine (BrdU) and 50  $\mu\text{M}$  2'-deoxycytidine was included in the culture medium. Cells were thereafter harvested, washed once with SDM-79 without BrdU and then spread onto microscope slides. Slides were then processed for BrdU incorporation as described previously, using anti-BrdU antibody (BioCell; Switzerland; Woodward and Gull, 1990). In all cases parasite DNA was stained with DAPI and slides were mounted in Mowiol (Harlow Chemical Co., Harlow, UK) containing phenylene diamine (1 mg/ml) as an anti-fading agent. Cell images were captured using a Hamamatsu SIT camera linked to a Zeiss Axioscope microscope, and processed using Improvision cytometry software and Adobe Photoshop 3.0. Kinetoplast-posterior measurements were derived from measuring at least 100 cells at each time point.

Western blotting was performed as described by Matthews et al. (1995), with antibody binding being visualised by enhanced chemiluminescence (ECL; Amersham International plc).

### Differentiation commitment experiments

Cells were harvested from a mouse and placed into SDM-79 at 27°C. Cis aconitate was then added to the cell culture to a final concentration of 6 mM, thereby defining  $t=0$ . At timed intervals thereafter, duplicate cell samples were harvested by centrifugation in a microfuge at 1,000 g for 1 minute and then washed twice with either SDM-79 without cis aconitate, or in SDM-79 containing cis aconitate to 6 mM. The respective samples were then resuspended in SDM-79 either with or without cis aconitate and plated into 24 well plates. At  $t=14$  hours, all wells were harvested and air dried smears prepared. Of these, half were fixed in 75% ethanol and half fixed in 100% methanol. The slides were then processed to assay BrdU incorporation (for the ethanol fixed slides) or VSG/procyclin (for the methanol fixed slides). The experiment was performed in duplicate using separate stumpy cell populations on different days. At each time point, 300-500 cells were counted for each parameter.

## RESULTS

### Dissection of the influence of cis aconitate and temperature reduction upon differentiation and the cell cycle

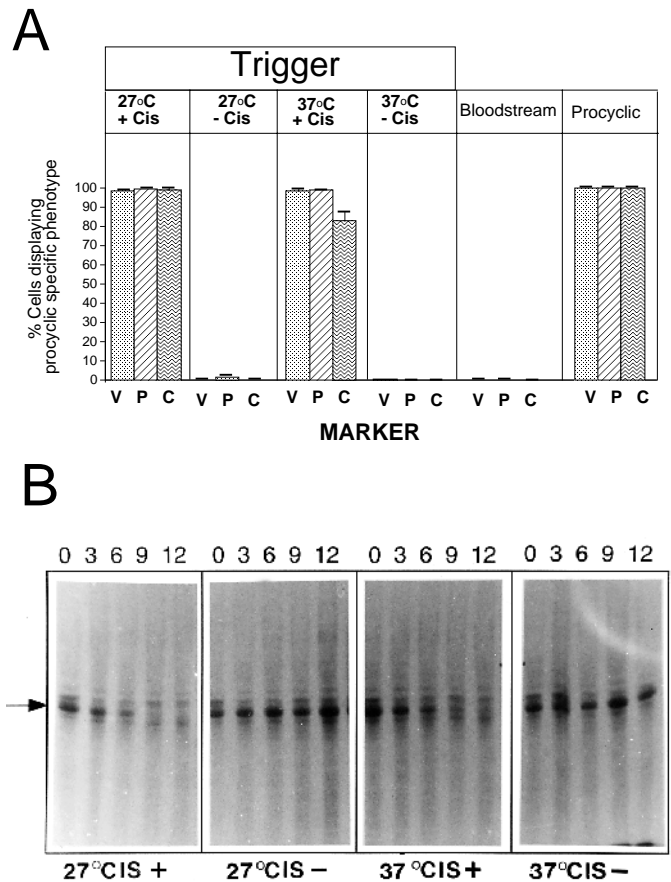
Detailed analyses have been performed previously regarding the requirements for efficient transformation of trypanosomes through to tsetse fly midgut procyclic forms. This differentiation can be induced *in vitro* and occurs most efficiently in the presence of two key triggers: the presence of Krebs cycle intermediates (cis aconitate/citrate) and a temperature reduction from 37°C to 27°C (Brun and Schonemberger, 1981; Overath et al., 1983, 1986; Simpson et al., 1985; Czichos et

al., 1986). Previous investigation by others (Czichos et al., 1986) and our own unpublished experiments have demonstrated the very minor influence of citrate in stimulating the differentiation process. Therefore, we have focused on the action of cis aconitate and temperature drop alone as stimuli of differentiation.

Previous experiments have exploited the reproducible synchrony of differentiation of bloodstream stumpy form cells to procyclic forms cells in order to map the temporal hierarchy of events in the differentiation pathway (Matthews and Gull, 1994b; Matthews et al., 1995). This has been assisted by the availability of a panel of antibodies and morphological markers which define particular events of the differentiation process. Importantly, the expression of these markers is temporally regulated, with defined events occurring both early (expression of the insect stage specific coat protein, procyclin/PARP; loss of the bloodstream specific variable surface glycoprotein, VSG) and at later stages through differentiation (repositioning of the kinetoplast, expression of a procyclic specific cytoskeleton associated protein CAP5.5, progress through DNA synthesis). This availability of markers which are temporally regulated and expressed synchronously in the population has allowed a detailed investigation of the cytological response of stumpy-form trypanosomes to the combined or independent action of the inducers of differentiation. Fig. 1A summarises the differentiation response of stumpy form parasites when induced to differentiate in the presence or absence of cis aconitate and at 27°C or 37°C. Under these conditions we found that the key trigger for all differentiation events was the presence of cis aconitate. Regardless of whether cells were at 27°C or 37°C, the addition of cis aconitate stimulated the cells to express not only early differentiation markers, but also to progress through all subsequent events for which we have markers. Thus, cells at both 27°C and 37°C not only shed the VSG coat and gained procyclin but they also expressed the cytoskeleton associated protein CAP 5.5 which is normally induced 8-10 hours through differentiation. The temporal kinetics of these events was also maintained; VSG loss occurred between 3 and 6 hours through differentiation (Figs 1B, 2A) and procyclin gain was detectable as early as 2-3 hours regardless of whether the cells were cultured at 37°C or the temperature was dropped to 27°C (Fig. 2B). The fluorescence assay of surface expression of procyclin was also reflective of the quantitative level of total procyclin protein within the cells. In comparison to trypanosomes in which both differentiation triggers were present, there was a very low level of detectable procyclin protein in cells exposed to temperature drop alone, even on long over-exposures of western blots (Fig. 2C).

### Cell cycle re-entry requires a prolonged temperature drop

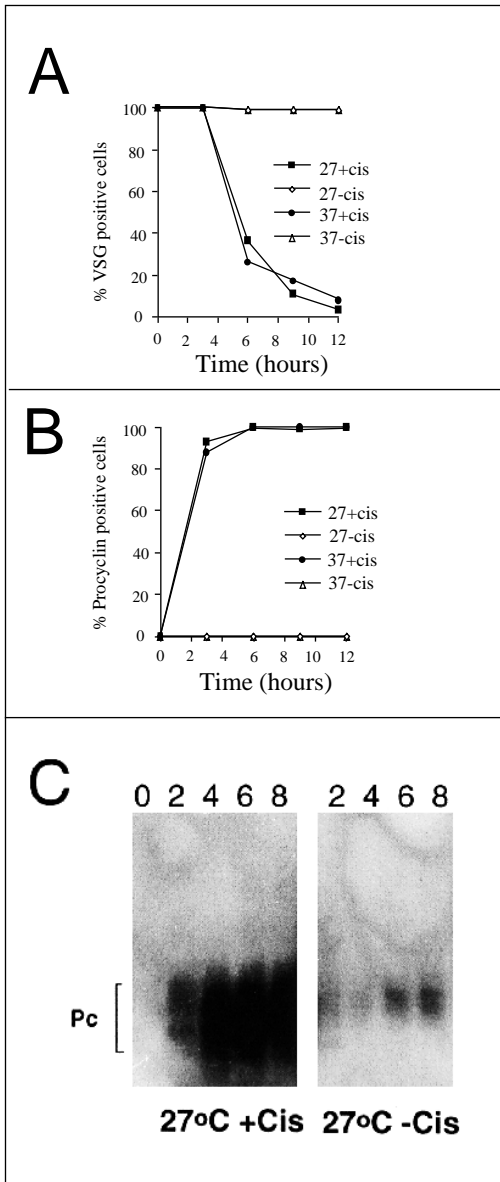
Coincident with the differentiation events associated with progression through to procyclic forms, stumpy cells undergo re-entry into a proliferative cell cycle. We, therefore, employed the earliest available marker of cell cycle progression (entry into DNA synthesis) to determine the requirements for escape from division-arrest for the stumpy cell (Fig. 3A). In this case and in contrast to the differentiation events, progression of the cells through DNA synthesis and efficient establishment of a proliferative procyclic cell population was dependent upon the combination of cis aconitate and a drop in temperature from 37°C



**Fig. 1.** (A) Summary of the response of stumpy form trypanosomes to exposure to the triggers of differentiation either in isolation or in combination. V, cells which have successfully shed the VSG coat; P, cells which have expressed the procyclic specific surface coat, procyclin; C, cells which have expressed the procyclic specific cytoskeleton associated protein, CAP 5.5. Cells were assessed by IFAT at  $t=+12$  hours. Bars represent the s.e. (B) Coomassie stained gel of protein samples prepared at 3 hour time intervals after stumpy-form cells have been induced with either trigger of differentiation. The VSG is arrowed.

to 27°C. Where cells were maintained at 37°C (but in the presence of cis aconitate), there was a variable but significant proportion of cell death (the cell number decreased by between 20% and 60% in the first 12 hours). For the remaining viable cell population, although there was successful expression of all differentiation events for which we have markers, there was a failure to re-enter into DNA synthesis (13.5% of cells had incorporated BrdU at +14 hours, in contrast to 98% of cells at 27°C; Fig. 3A). As the cells continued to be maintained at 37°C, however, this small proportion increased until the majority of cells had progressed through DNA synthesis, and established a proliferative procyclic population (Fig. 3A, right hand panel).

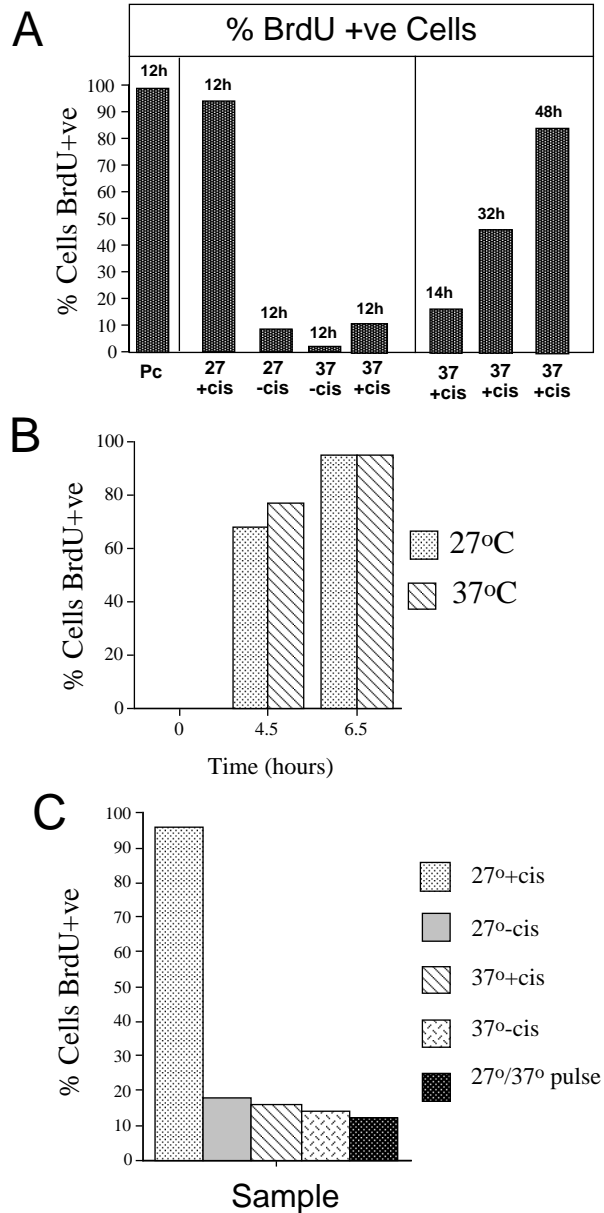
We were interested to investigate the basis of the failure to rapidly re-enter a proliferative cell cycle (as assessed by BrdU incorporation) for the stumpy cell population at 37°C since this represents a physiological condition under which cell-cycle progression can be uncoupled from progression through transformation. It is also closely analogous to the phenomenon of abortive transformation, in which asynchronous monomorphic



**Fig. 2.** Surface antigen and protein expression under different differentiation conditions. Surface antigen expression was determined by IFAT for (A) VSG and (B) procyclin at time intervals during differentiation. (C) Total cellular protein of stumpy cells 0, 2, 4, 6 and 8 hours after being placed at 27°C into SDM-79 either in the presence (left hand panel) or absence (right hand panel) of cis aconitate and probed with an antibody specific for procyclin.

cell differentiation at 37°C results in the generation of non-proliferating morphologically procyclic-like forms (Czichos et al., 1986; Overath et al., 1986). In particular we wished to distinguish whether the phenomenon represented a failure to trigger the cell cycle re-entry of stumpy cells, or more simply, a failure to successfully progress through DNA synthesis.

Initially, we asked whether the failure of transforming cells to undergo DNA synthesis at 37°C was a consequence of their development of a temperature-sensitive procyclic cell cycle? Therefore, the sensitivity of the S-phase of procyclic cells to elevated temperature was assessed. Initially a procyclic popu-



**Fig. 3.** The effect of differentiation triggers on cell cycle re-entry. (A) BrdU incorporation of cultures in the presence or absence of cis aconitate and at 27°C or 37°C. Numbers above the bars indicate the time at which the sample was prepared. The right hand panel shows the outgrowth of proliferative procyclic forms when parasites are maintained at 37°C in the presence of cis aconitate. (B) BrdU incorporation into procyclic cells in logarithmic growth. Flecked bars represent cells growth at 27°C; Hatched bars represent cells at 37°C. (C) Successful entry into DNA synthesis only occurs in the presence of both cis aconitate and prolonged temperature reduction; exposure of cells to 27°C for 20 minutes (27/37 pulse) does not precipitate entry into DNA synthesis.

lation in logarithmic growth was incubated at 37°C for a period of 4 hours, thereby allowing cells already committed to progress through DNA synthesis to complete this process. After this time, the nucleotide analogue BrdU was added to the culture and its incorporation into parasite DNA over the following 6.5 hours assayed by immunofluorescence using

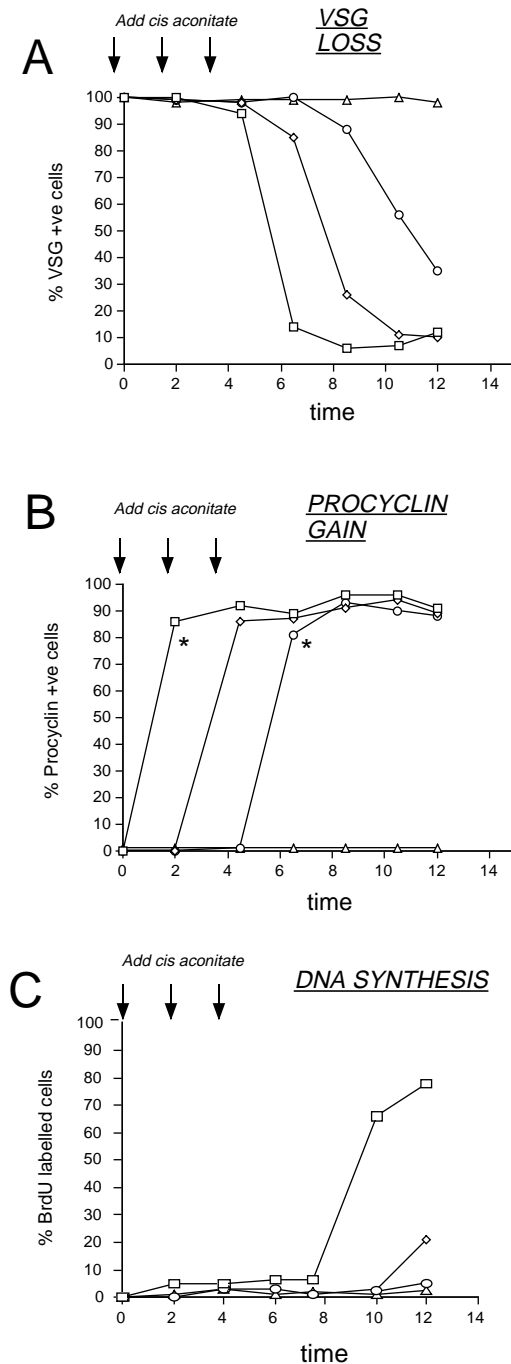
anti-BrdU antibody. Fig. 3B demonstrates that under these conditions procyclic cells were able to continue to initiate and progress through DNA synthesis whether at either 27°C or 37°C. Furthermore, regardless of the incubation temperature, the kinetics of progression through S-phase in the population was not markedly altered, at least within the context of a single cell-cycle. Clearly, the procyclic cell-cycle was not exquisitely sensitive to elevated temperature and bloodstream cells transforming at 37°C are not restricted by a temperature dependent procyclic cell-cycle checkpoint.

We next asked whether successful re-entry into DNA synthesis for stumpy cells required stimulation by a dynamic temperature change. This possibility was assessed by exposing stumpy cells at 37°C in the presence of cis aconitate to a transient temperature drop to 27°C. It is important to note in these experiments that parasites were harvested using needles and syringes pre-warmed to 37°C and that they were placed immediately into differentiation conditions, without purification from blood. In this way we were able to ensure that our harvesting procedures took less than 1 minute, such that control parasite samples would not be exposed to even a very transient reduction in temperature. The cells were then either maintained at 37°C, incubated at 27°C, or were incubated at 27°C for 20 minutes and then returned to 37°C. Fig. 3C shows that under these conditions, cells successfully progressed through DNA synthesis when maintained at 27°C, but did not do so either when at 37°C or when briefly exposed to temperature reduction to 27°C. This indicates that under conditions of abortive transformation, re-entry into the cell cycle is not triggered either by a dynamic change in temperature or by a brief triggering at lower temperature. Instead, cells clearly require to be retained at the lower temperature in order to promote successful survival, transformation and cell cycle re-entry.

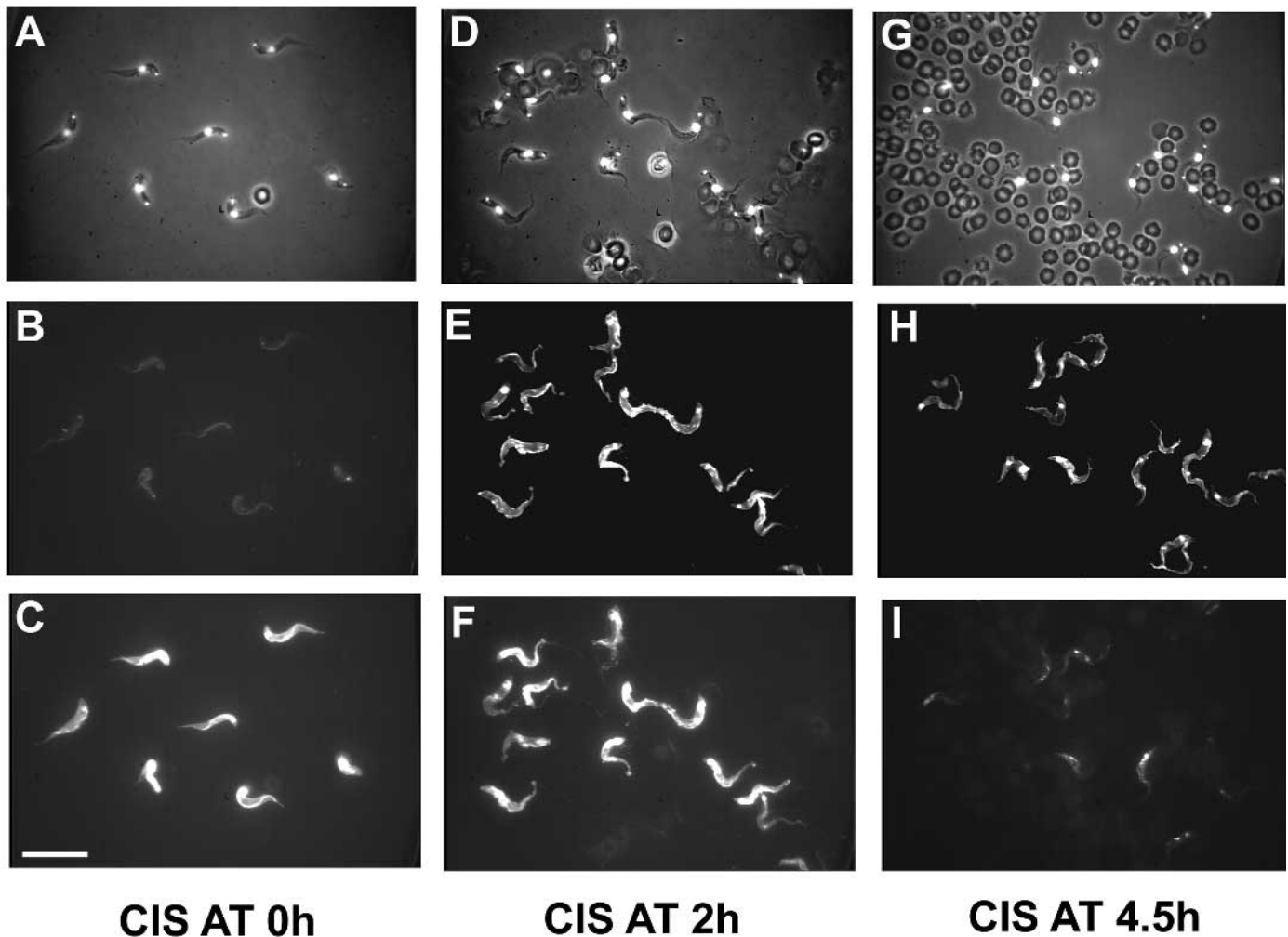
#### Cis aconitate sets the clock for differentiation

Having demonstrated that the expression of all differentiation events for which we have markers are dependent upon the presence of cis aconitate, we investigated whether temperature drop would influence the temporal kinetics of their expression. In particular, we wished to determine whether prior exposure to temperature drop would prime cells for differentiation, or whether instead the initiation and relative timing of differentiation events was absolutely dependent upon the addition of cis aconitate. Therefore stumpy form parasites were harvested, incubated at 27°C and then, at various time periods thereafter, cis aconitate was added to the culture medium. Fig. 4 shows the kinetics of procyclin appearance and the loss of VSG after cis aconitate addition at either 0, 2 or 4.5 hours after incubation at 27°C. In each case, VSG loss occurs synchronously between 4 and 6 hours after this triggering (Fig. 4A) and procyclin gain occurs precisely 2 hours after the addition of cis aconitate (Fig. 4B). Thus, at 6 hours after the start of the experiment, cells exposed to cis aconitate at 0h were VSG<sup>-</sup>/procyclin<sup>+</sup>, cells exposed to cis aconitate at +2 hours were VSG<sup>+</sup>/procyclin<sup>+</sup> and cells exposed to cis aconitate at +4.5 hours were VSG<sup>+</sup>/procyclin<sup>+/-</sup> (Fig. 5). This temporal preservation is not merely restricted to stage regulated surface antigens. During differentiation there is a morphological restructuring of the trypanosome cell, a consequence of which is the repositioning of the parasite's mitochondrial genome, the kinetoplast (Brown et al., 1973; Steiger, 1973). In bloodstream form trypanosomes this organelle lies close to the

posterior end of the cells. However, between 6 and 12 hours through the differentiation process, the kinetoplast is repositioned to mid way between the cell posterior and cell nucleus (Matthews et al., 1995). The spatial distance between the cell posterior and kinetoplast therefore provides a rapid and conve-



**Fig. 4.** Offsetting the moment of cis aconitate addition with respect to reducing the temperature to 27°C, off-sets the initiation of differentiation. Cis aconitate was added to the cell samples at either 0 hours (open squares), after 2 hours (open diamonds), after 4.5 hours (open circles) or never (open triangles). Cells were assayed by immunofluorescence for either (A) VSG loss (B) procyclin gain or (C) entry in DNA synthesis. An asterisk indicates a weak, though clearly positive staining with the procyclin antibody.



**Fig. 5.** Cells exposed to cis aconitate either at 0 hours (A,B,C), 2 hours (D,E,F), or 4.5 hours (G,H,I) after temperature reduction to 27°C. (A,D,G) Phase contrast images of cells counterstained with the DNA intercalating dye DAPI. (B,E,H) The same cells labelled with VSG-GUTat 7.2 specific polyclonal antiserum. (C,F,I) The same samples labelled with antibodies against procyclin. The staining with procyclin in I is positive though weak, as expected for procyclin appearance 1.5 hours after triggering (Matthews and Gull, 1994b). Bar, 25  $\mu$ m.

nient measure of the extent of morphological restructuring of the trypanosome cell during differentiation.

The kinetoplast-posterior dimension for 100 cells was determined at 10.5 hours after the cells were placed into *in vitro* culture at 27°C. This time point was chosen to permit clearest visualisation of the temporal differences between the samples, given that kinetoplast repositioning occurs between 6 hours and 12 hours after the initiation of transformation. Fig. 6 demonstrates that the relative extent of repositioning exactly reflects the degree of morphological change that would be expected for each cell sample if the moment of cis aconitate addition defined the initiation of differentiation. Those cells to which cis aconitate was added at 0 hours show a kinetoplast-posterior distance of approximately 4  $\mu$ m (representing nearly full repositioning), whereas cells exposed to cis aconitate at +4.5 hours have a kinetoplast-posterior dimension similar to bloodstream cells (approximately 1.5  $\mu$ m). Intermediate between these two, cells to which cis aconitate was added after 2 hours show incomplete repositioning (approximately 3  $\mu$ m).

In a final experiment, the extent to which cells re-entered

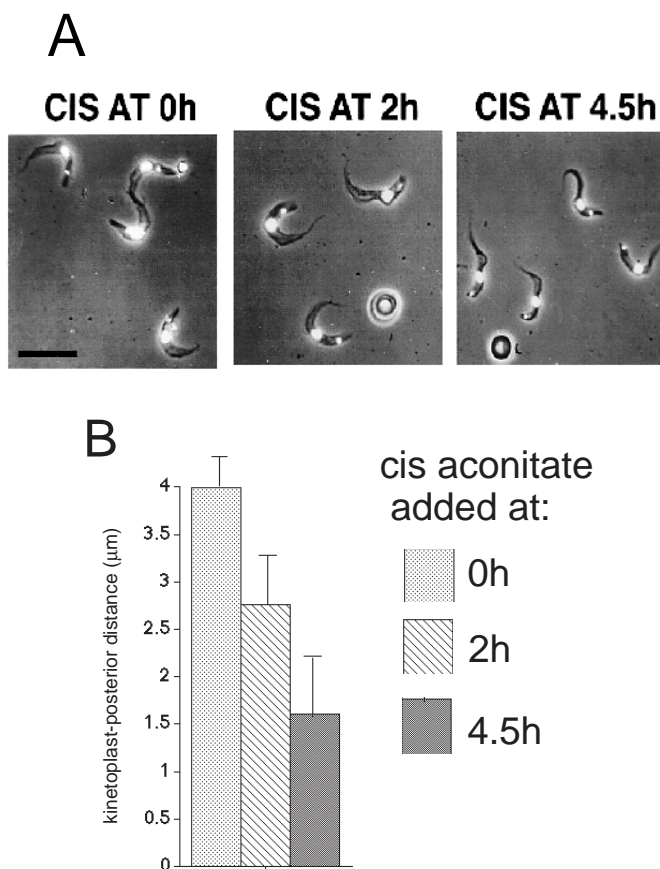
into a proliferative cell cycle after the addition of cis aconitate was measured by following the incorporation of BrdU for each cell sample (Fig. 4C). In this case, the re-entry of the cells into DNA synthesis was also retarded to the exact extent to which the addition of cis aconitate to the cell samples was delayed. Thus, both the differentiation and cell cycle clock is initiated at the moment of cis aconitate addition and thereafter, the order and timing of events is preserved.

#### **The commitment to differentiation and cell cycle re-entry are coincident processes.**

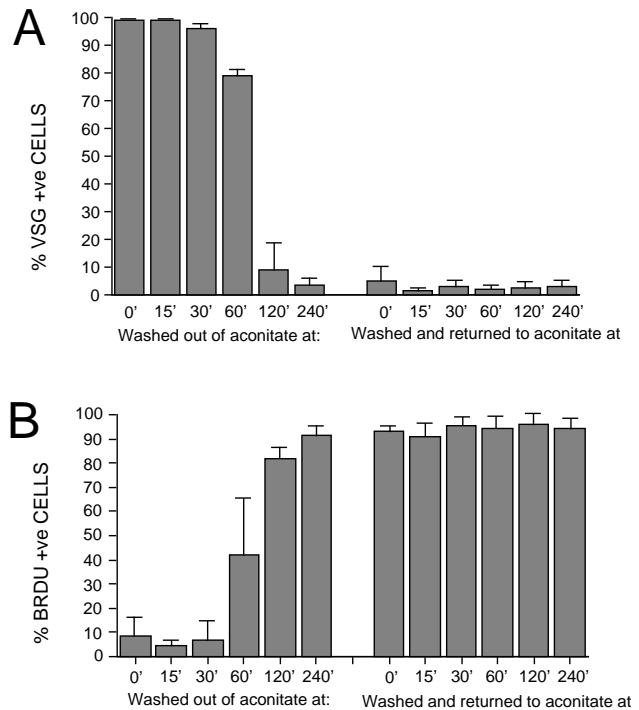
Once bloodstream trypanosomes have initiated differentiation to procyclic forms they progress toward a point of irreversible commitment, when continued differentiation becomes independent of cis aconitate/temperature drop (Czichos et al., 1986; Ehlers et al., 1987). We have previously crudely mapped this commitment point for stumpy-cell differentiation events and found that it occurs before 4 hours through differentiation (Matthews and Gull, 1994b). It has also been shown that it is possible to uncouple DNA synthesis from coincident differen-



tiation events by using cell-cycle inhibitors, and thereby demonstrate that division and differentiation are not absolutely co-dependent, at least in later stages of the process (Ghiotto et al., 1979; Markos et al., 1989; Matthews and Gull, 1994b). We wished to exploit our observation that cis aconitate was the key differentiation trigger in order to map with precision the commitment point for the differentiation pathway and to determine whether this was coincident with the point at which the cells commit to re-entry into a proliferative cell cycle. Therefore bloodstream stumpy cells were incubated at 27°C in the presence of cis aconitate and, at intervals, washed and returned to identical conditions, but lacking cis aconitate. Control samples were treated in the same manner but were returned after washing to medium containing cis aconitate. Cells were then scored after 14 hours in order to determine whether they had lost the VSG coat and progressed through DNA synthesis. Fig. 7 shows the results of this analysis. Cells exposed to cis aconitate for less than 1 hour were found not to shed the VSG coat, and only the small proportion (approximately 10%) of remaining proliferative slender cells in the bloodstream population progressed through DNA synthesis. In contrast, if exposed to cis aconitate for greater than 1 hour the great



**Fig. 6.** Kinetoplast repositioning is delayed by delaying the addition of cis aconitate. Cells exposed to cis aconitate at 0 hours, 2 hours or 4.5 hours were assayed at 10.5 hours for the distance between the kinetoplast and cell posterior. The upper panels show phase-contrast images counterstained with DAPI to reveal the cell nucleus and kinetoplast. Bar, 20 µm. The lower panel shows the kinetoplast-posterior measurement for 100 cells for each sample. The bars represent the s.e.



**Fig. 7.** Commitment to differentiation and cell cycle re-entry. Cells exposed to cis aconitate for varying time periods were washed and returned to cis aconitate-free medium (left hand series), or were returned to medium containing cis aconitate (right hand series). After 14 hours cells were assayed to determine their possession of a VSG coat (A) or incorporation of BrdU (B). For both parameters, commitment occurred between 1 and 2 after exposure to differentiation. Error bars represent the s.e.

majority of the cells both lost their surface coat and underwent DNA synthesis (and thereafter progressed into a proliferative cell cycle). The morphology of the cells was also modified such that cells had the appearance of fully differentiated procyclic forms (data not shown). Together this indicates that the commitment point for both DNA synthesis and progress through the differentiation pathway are coincident and occur after 1-2 hours exposure to cis aconitate.

## DISCUSSION

The life cycle of the African trypanosome entails progression through a series of apparently irreversible differentiation steps. These differentiation events are a fundamental requirement for the parasite, which is exposed to vastly differing environments as it traverses both within and between the mammal host and tsetse-fly vector. Each life cycle transition step is closely associated with alternations in the proliferation status of the parasite, with non-dividing forms apparently representing cells which are well adapted to, and irreversibly committed to progression to the next life cycle stage (Vickerman, 1985; Mottram, 1994; Matthews and Gull, 1994a). Here we have investigated the earliest events of differentiation as parasites progress from the mammalian bloodstream to the midgut of their insect vector. This transformation involves major devel-

omental changes (surface antigen exchange, mitochondrial elaboration, structural re-organisation) and, concomitant with this, re-entry into a proliferative cell cycle. Our results have indicated that the cell cycle and differentiation events are processes that are intimately intertwined, but that are separable under defined physiological conditions. Furthermore, by detailed analysis of the synchronous entry into both these pathways we have mapped the point at which progress through the differentiation pathway becomes independent of the combined triggers for the process, and have thereby mapped the commitment point for differentiation.

There is a long history of elegant experiments which have allowed the experimental conditions under which trypanosomes initiate efficient differentiation to their procyclic form *in vitro* to be determined (Brun and Schonberger, 1981; Overath et al., 1983, 1986; Simpson et al., 1985; Czichos et al., 1986). Such studies have been restricted, however, by the asynchrony of differentiation when initiated by laboratory adapted monomorphic trypanosomes. These lines have been derived by continual passage of bloodstream parasites through mice, and have lost the ability to generate division-arrested stumpy forms. The transformation kinetics of such lines is, therefore, complicated by the complex counterbalance between continued growth as the bloodstream form and differentiation to the procyclic form (Pays et al., 1993; Matthews and Gull, 1994b). With the establishment of a highly synchronous and reproducible differentiation system (Zeigelbauer et al., 1990), it has now been possible to study in detail the influence of the differentiation triggers in isolation and to determine their effect upon a panel of precisely mapped stage-regulated differentiation markers. Furthermore, the synchrony of differentiation when initiated with division-arrested stumpy bloodstream cells has allowed the expression of such markers to be followed at both the population and single-cell level.

Analysis of the independent effects of differentiation triggers demonstrated that the expression of all available differentiation markers was absolutely dependent upon the presence of *cis* aconitate. Therefore, whether at 27°C or 37°C the cells not only became antigenically procyclic (VSG<sup>-</sup>, procyclin<sup>+</sup>), but they also completed the complex morphological changes associated with this differentiation step. The kinetics of these processes was also unaffected. VSG coat shedding, one of the most temporally reproducible events during synchronous differentiation (Pays et al., 1993; Matthews and Gull, 1994b), occurred precisely between 3 and 6 hours after *cis* aconitate addition, regardless of the differentiation temperature.

In contrast to these differentiation events, re-entry into a proliferative cell cycle for the stumpy form was only triggered by the combined action of both *cis* aconitate and reduction in temperature to 27°C. This closely corresponds in many respects to previous observations for the asynchronous differentiation of monomorphic cells. In a comprehensive analysis of the metabolic effects of the differentiation triggers, Overath et al. (1986) found that in the absence of temperature drop, most cells formed procyclic-like forms (on the basis of proline oxidation and cellular morphology), but that these cells failed to establish a proliferative procyclic population. This phenomenon was termed abortive transformation. In contrast to our findings, however, the proliferative population of cells that eventually outgrew under abortive conditions remained as bloodstream-form cells rather than as the procyclic cells that

we observed. This difference may be due to the contrasting degree of commitment to differentiation to the procyclic form exhibited by monomorphic and stumpy bloodstream cells. Both dividing slender and monomorphic cells represent an uncommitted cell type, which is capable of either continued growth in the bloodstream, or transformation to the procyclic form. In contrast, the non-dividing stumpy cell is already irreversibly committed to differentiation to procyclic cells or, failing this, death in the bloodstream. Therefore, abortive transformation with stumpy cell populations may select for cells able to differentiate to procyclic forms and divide at 37°C, whereas in monomorphic cells the emphasis of selection may be biased toward continued growth as the bloodstream form.

In order to investigate the basis of abortive transformation in more detail we determined whether exposing cells to a short pulse at low temperature would be sufficient to stimulate them to commit to entry into DNA synthesis. In fact, we found that this short exposure to temperature reduction did not precipitate cell cycle re-entry, but that instead cells required to be maintained for prolonged periods at the lower temperature for successful DNA synthesis. This indicates that the cell cycle re-entry in these organisms was sufficiently sensitive to temperature for their progress into DNA synthesis to fail. The basis of this failure is unknown, but it is not due to an exquisite temperature sensitivity of the procyclic cell cycle. Established procyclic cells successfully progressed through S-phase with similar kinetics at both 27°C and 37°C, at least over the duration of 1 cell cycle. This is supportive of a previous brief report by Honigberg and Gabre (1972). Therefore the failure of differentiating cells to undergo DNA replication at 37°C may be due to one of two possibilities. Firstly, it has been suggested that a failure to undergo full metabolic differentiation at elevated temperature might prevent successful establishment of a proliferative cell population (cells at 37°C fail to oxidise 2-oxoglutarate; Overath et al., 1986). Alternatively, the contrasting sensitivity of the procyclic and differentiating cells to temperature may indicate that the underlying controls of the differentiation cell-cycle are modified from those of the established procyclic cell (Matthews et al., 1995). Regardless of the mechanism involved, these observations upon abortive transformation extend our previous demonstration that it is possible to use inhibitors of DNA synthesis to uncouple cell cycle progression from both kinetoplast repositioning and CAP5.5 expression. Clearly, even in the absence of artificial drug blockade (Ghiotto et al., 1979; Markos et al., 1989; Matthews and Gull 1994b), the differentiation pathway and re-entry into a proliferative cell cycle are separable pathways.

We were interested whether off-setting the addition of the triggers for transformation would result in either premature expression, or an altered order of differentiation events following the addition of *cis* aconitate. The striking result of this analysis was that delaying the addition of *cis* aconitate precisely delayed the initiation of differentiation, but that once initiated, the order and timing of subsequent antigenic and structural changes was preserved. Such analyses emphasise the exquisite importance of *cis* aconitate in stimulating trypanosome differentiation *in vitro*, and indeed, exogenous *cis*-aconitate is equilibrated within 15 minutes between medium and cells in the monomorphic bloodstream forms of *T. brucei* MITat 1.4 while citrate, *trans*-aconitate, 2-oxoglutarate or succinate are not taken up significantly (A. Markos and P.



Overath, unpublished observations). Despite this, it seems unlikely that this molecule is the essential trigger *in vivo*; a number of studies have analysed the influence of this molecule upon differentiation in the tsetse fly and no effect has been observed (e.g. Overath et al., 1986). It may be, therefore, that a distinct factor or combination of factors in the fly midgut are instead able to efficiently stimulate the initiation of differentiation. Regardless of the identity of such a molecule our observations demonstrate that the addition of cis aconitate alone marks the moment of initiation of differentiation *in vitro*. This provides an invaluable system with which to dissect the control of differentiation in a defined experimental environment.

The control of differentiation events for many eukaryotic cell types involves an initially reversible phase which is followed by progression through a point of irreversible commitment to differentiation. Once this threshold has been passed, the cells become locked into the cascade of events which result in the fully differentiated state. Previous analyses of this commitment of monomorphic cells at the population level indicated that the repression in VSG synthesis and coat loss occurred after approximately 30 hours exposure to differentiation triggers (Czichos et al. 1986; Ehlers et al., 1987). In the experiments described in this study we have exploited the observation that cis-aconitate is the key differentiation trigger to determine that the commitment point for both progress through the differentiation pathway and for DNA synthesis are coincident, at 1-2 hours after triggering. This time period is some time after the already well-characterised events that are known to control the gene expression of several stage-regulated genes. For example, both the repression of VSG gene expression, and the activation of procyclin gene transcription occurs very shortly after the cells are exposed to differentiation triggers (Van Hamme et al., 1995; Dorn et al., 1991). Therefore, it is apparent that although particular molecular events can occur very early on, these are not necessarily transduced unchecked into a phenotypic response. Instead, cells require passage through a point of commitment, after which they are locked into the cellular changes required for differentiation to viable procyclic forms.

The strict temporal control of differentiation once the cells are committed to the process predicts the existence of a co-ordinated underlying control pathway in which particular events occur in a specific and regulated order. The possession of such a hierarchical differentiation programme has obvious advantages to the trypanosome. When undergoing transition from the blood to the tsetse midgut there are profound changes in cell antigenicity, cell shape and cell proliferation controls. By regulating the timing and ordering of these events with respect to one another, catastrophic conflicts within the cell are avoided and the ability to generate a viable differentiated procyclic cell is maximised. The unravelling of the molecular control events underlying these highly regulated processes are the essential next steps in understanding the basis of life cycle progression in the African trypanosome. Defining the crucial initiation and commitment points for such processes has provided the necessary platform on which to base these investigations.

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