Influence of growth conditions on RNA levels in relation to activity of core metabolic enzymes in the parasitic protists *Trypanosoma brucei* and *Trichomonas vaginalis*

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Levels of mRNAs encoding metabolic enzymes and their cellular activities were measured on continuous culture samples of the parasitic protists *Trypanosoma brucei* and *Trichomonas vaginalis*. The organisms were grown in chemostats at varying growth rates under glucose limitation or in the presence of excess glucose (EG), resulting in extensive adaptation of the cellular activities of glycolytic enzymes. rRNA and mRNA for β-tubulin were monitored as controls. In *Trypanosoma brucei* levels of all RNAs showed a biphasic dependence on growth rate (= dilution rate $D$), with a sharp increase at higher $D$ values. Cellular RNA levels of *Trichomonas vaginalis* rate-limited by glucose decreased slightly with increasing $D$. In EG-grown cells the opposite trend was observed. Equal levels for both carbon regimes were observed at intermediate $D$ values. In both species the ratio between rRNA and mRNA encoding β-tubulin was constant, independent of the carbon regime. mRNA encoding metabolic enzymes showed varying degrees of correlation with rRNA and β-tubulin mRNA. In contrast, there was little to no correlation between mRNA levels and the activities of the enzymes they encode, even though only one of these is allosterically regulated. The data indicate that RNA levels in *Trypanosoma brucei* and *Trichomonas vaginalis* are determined by growth rate and in the latter species by the availability of the carbon and energy source. Rates of synthesis of metabolic enzymes are most likely regulated at the post-transcriptional level.

**Keywords**: mRNA levels, energy metabolism, parasitic protists, glycolysis, chemostat

**INTRODUCTION**

Growing organisms have to balance a variety of requirements to maintain functional homeostasis and add biomass. This necessitates a coordinated regulation of all cellular processes so that the available energy and resources are optimally distributed. In every organism a considerable amount of energy is devoted to biosynthetic processes. Changes in the physiology of cells, whether cell-cycle-dependent or induced by environmental changes, are often brought about by varying levels of the relevant mRNAs (Pardee, 1994; Roeder, 1996). mRNA has high turnover rates in comparison to structural and metabolic proteins, because long half-lives would lengthen response times and could result in unnecessarily large quantities of protein being synthesized. In prokaryotic, yeast and mammalian cells control over transcription and translation has been well documented (de Haro et al., 1996; Kunzler et al., 1996; McCarthy & Brimacombe, 1994; Roeder, 1996). Far less information is available on the regulation of these processes in protists. Only in trypanosomes has the regulation of transcription and translation been studied in detail (Vanhamme & Pays, 1995). The present study explores the regulation of mRNA levels in relation to the
expression of the proteins they encode in two parasites, *Trypanosoma brucei* and *Trichomonas vaginalis*.

The procyclic form of *Trypanosoma brucei* resides in the gut of the tsetse fly. In contrast to the bloodstream form, it does not rely solely on glycolysis for energy generation, but also has a partially functional mitochondrion (Opperdoes, 1987; Tielens & Van Hellemond, 1998). The major end-products when growing on glucose are acetate and succinate (Ter Kuile, 1997). Part of the glycolytic pathway is compartmentalized in glycosomes (Clayton & Michels, 1996; Opperdoes, 1987). *Trichomonas vaginalis* does not possess mitochondria but contains hydrogenosomes, organelles involved in the generation of molecular hydrogen under anaerobic conditions (Müller, 1988, 1996). Its energy metabolism is also fermentative, even in the presence of oxygen. The end-products of glucose metabolism are lactate, acetate, glycerol, CO₂, and H₂ in ratios dependent on culture conditions (Ter Kuile, 1996). It is a parasite of the human genito-urinary tract and as such is subjected to long-term environmental variations due to the menstrual cycle (Fouts & Kraus, 1980).

Chemostats can be used to study adaptation of organisms able to grow in suspension cultures to varying growth rates and environmental conditions (Veldkamp, 1976). Our previous chemostat studies have shown that both *Trypanosoma brucei* and *Trichomonas vaginalis* adapt the activities of their glucose transporter and most glycolytic enzymes to growth rate and availability of the carbon and energy source (Ter Kuile, 1994a, b; 1996, 1997; Ter Kuile & Opperdoes, 1992a, b). The mechanisms by which this adaptation occurs are not understood. Regulation of unchanged amounts of enzyme by low-molecular-mass effectors is not likely because in both species only one of the enzymes of the glucose catabolic pathway, pyruvate kinase (PK), is known to be regulated this way (Mertens et al., 1992; Van Schafting et al., 1985). Enzyme recruitment is more probable. In that case either rates of transcription have to be adjusted, yielding a change in mRNA levels, or rates of translation have to be adjusted. In the latter case there will be no correlation between steady-state mRNA levels and cellular enzyme activities. Enzyme turnover cannot be a regulatory mechanism in *Trypanosoma brucei* because most enzymes are stable over a 2 d period (Clayton, 1988). Turnover rates of glycolytic enzymes have not been measured in *Trichomonas vaginalis*, but in the yeast *Saccharomyces cerevisiae* they are low compared to those of other enzymes (Werner-Washburne et al., 1993).

Regulation of transcription in *Trypanosoma brucei* has mostly been studied with respect to expression of the variable surface glycoprotein (VSG) and procyclic repetitive acidic protein (PARP). The data on these systems suggest an important role for post-transcriptional control (Vanhamme & Pays, 1995). The same conclusion was drawn for regulation of hexose transporter mRNA (Bringaud & Baltz, 1993; Hotz et al., 1995). Several properties set the transcription machinery of *Trichomonas vaginalis* apart from that of most eukaryotic cells. It has an unusual DNA-dependent RNA polymerase II and does not have TATA boxes upstream from the ORFs (Quon et al., 1994, 1996). Therefore, the mechanisms controlling rates of transcription and thus gene expression may differ from those found in the more extensively studied eukaryotes (Liston & Johnson, 1998).

The question addressed in this study is how *Trypanosoma brucei* and *Trichomonas vaginalis* regulate mRNA and rRNA levels under different growth conditions and to what extent expression of metabolic enzymes is controlled by mRNA levels. Levels of the small subunit rRNA (*Trypanosoma brucei*) and the 16S rRNA (*Trichomonas vaginalis*) and mRNA for β-tubulin and enzymes involved in energy metabolism were measured and the latter are correlated to the cellular activities of the corresponding enzymes.

**METHODS**

**Growth conditions and sampling methods.** Single-stage flow-controlled chemostats (Ter Kuile, 1994a, b; Ter Kuile & Opperdoes, 1991) were used to adapt *Trichomonas vaginalis* (strain NIH-C1, ATCC 30001) and *Trypanosoma brucei* brucei 427 procyclic trypanosomes to different growth rates. Growth rates can be set experimentally to any value below the maximum growth rate of the organism by changing the dilution rate (D) of the chemostat (Veldkamp, 1976). After rapid initial growth, one component of the medium becomes exhausted and only its supply in the fresh medium being pumped in enables further growth. After about three volume changes, rates of supply and consumption become equal and a steady-state, at which the growth rate equals D, is reached. The nutrient whose concentration limits growth rates is called the rate-limiting substrate. In this study it was either the carbon and energy source, glucose, or a component of the serum, in which case glucose was supplied in excess concentrations.

The driving gas was N₂ (99.99 % pure) to maintain anaerobic conditions for *Trichomonas vaginalis* and air for *Trypanosoma brucei*. The pH was controlled at 6.40 (± 0.02) for *Trichomonas vaginalis* and 6.90 (± 0.02) for *Trypanosoma brucei* and the growth temperatures were 37 and 27.5°C, respectively. For *Trichomonas vaginalis* the phosphate/trypotide/yeast extract medium of Diamond (1957) was used without agar and without maltose. Instead, 5-1 or 35 mM glucose and 10 or 6.25 % (v/v) horse serum were added for "limiting glucose" (LG) and "excess glucose" (EG) conditions, respectively. The medium for *Trypanosoma brucei* was the glucose-based SDM79 (Brun & Schonenberger, 1979) with a glucose concentration of 5 (LG) or 40 mM (EG) and lowered amino acid levels. Foetal bovine serum made up 10 (LG) or 7.5 % (EG) of the final volume. The establishment of a steady-state was assumed when the culture had been growing under constant conditions for at least five doubling times and the cell density, monitored by counting cells in a haemacytometer, had remained unchanged for at least one doubling time. For each carbon regime, LG or EG, five steady-states were obtained at D values between 1 and 4.5 d⁻¹ for *Trichomonas vaginalis* and between 0.15 and 1 d⁻¹ for *Trypanosoma brucei*. Thus a total of 10 steady-states were evaluated for each species.

At each steady-state the following sampling programme was conducted: (1) Five 1 ml samples of rapidly filtered culture
fluid were stored at -20°C for later determination of the glucose content. (2) A 20 ml sample was rapidly distributed in 1 ml aliquots over 18 microfuge tubes which were centrifuged for 30 s at 13,000 g. The supernatant was removed and the cell pellets were stored at -20°C. These samples were used for measurements of the cellular composition and enzyme activities. (3) A 90 ml sample was taken for the isolation of total RNA content was measured at 260 nm to prevent RNA degradation. Total RNA content was washed cells in a 4 M guanidinium isothiocyanate solution and centrifuging it through a layer of a CsCl solution for 23 h in an ultracentrifuge (Chirgwin et al., 1979). The resulting pellet consisted of RNA of high purity and with minimal degradation. The RNA was dissolved by a boiling 0.1% SDS (dodecyl pyrocarbonate) solution and centrifuging it through a layer of a 5-7 M CsCl solution for 23 h in an ultracentrifuge (Chirgwin et al., 1979). The resulting pellet consisted of RNA of high purity and with minimal degradation. The RNA was dissolved in 0.1% SDS and stored in aliquots at -80°C. All standard procedures were observed to prevent RNA degradation. Total RNA content was determined by measuring A260/280.

**Northern blotting.** Northern blotting was performed using the NorthernMax Blotting Kit (Ambion) according to the instructions of the manufacturer. Blots of 10 lanes, containing a sample from each steady-state, were transferred to BrightStar-Plus positively charged nylon membrane (Ambion) and cross-linked by UV light in the case of *Trichomonas vaginalis*, steady-state levels of 16S rRNA and mRNA encoding inorganic pyrophosphate-dependent phosphofructokinase (PPiPFK), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), malate dehydrogenase (MDH), malic enzyme, adenylate kinase (AK) and β-tubulin were measured using radiolabelled specific probes (36 nt) based on the corresponding genes (Table 1). Using a similar approach for *Trypanosoma brucei*, the rRNA and mRNAs for the 60 kDa mitochondrial heat-shock protein (HSP60), β-tubulin, hexokinase (HK), phosphoglucomutase (PGI), aldolase, triosephosphate isomerase (TIM), glycerol-3-phosphate dehydrogenase, G-3-PDH, GAPDH and PK were measured. The amounts of hybridized probe were estimated from the radioactivity determined using the phosphor imaging method. Its extreme dynamic range yielded accurate results for RNAs present at widely different levels. In preliminary experiments it was ascertained that all measurements, including those of rRNA, were in the linear range. Cell number, rather than protein, was used as normalizing factor. However, the variation in protein per cell was small for both species (data not shown). The values obtained are relative, but a reasonable degree of quantification was achieved because for any given mRNA, samples from all steady-states were measured simultaneously on a single blot. Four blots were prepared for each species and used several times after stripping the hybridized probe by immersion in boiling 0.1% SDS (diethyl pyrocarbonate-treated). The procedure was performed twice for each RNA on different blots and, although absolute numbers differed depending on the specific activity of the probe and exposure time, the observed trends were always very similar.

**Enzyme measurements.** The specific activities of HK (Bergmeyer, 1974), PGI (Misset & Oppedoe, 1984), PPiPFK (Mertens et al., 1989), aldolase, TIM, G-3-PDH and GAPDH (Misset & Oppedoe, 1984), MDH (Hrdy et al., 1993), malic enzyme (Avilán & García, 1994) and PK (Misset et al., 1987) were measured as described previously (Ter Kuile, 1996, 1999).
RESULTS

In *Trypanosoma brucei* all cellular RNA levels showed a biphasic dependence on growth rate (= D) independently of whether glucose was rate-limiting (LG cells) or present in excess (EG cells) (Fig. 1). Below half the maximum growth rate there was hardly an increase in rRNA or total RNA, but at higher D values a steep increase in RNA levels was observed. Only HSP60 mRNA showed higher levels in EG cells than in LG cells (Fig. 1c). The mRNAs encoding PGI, aldolase and GAPDH (not shown) exhibited the same biphasic dependence on D, also without a difference between LG and EG cells.

Cellular levels of mRNA encoding PPiPFK, GAPDH,
Regulation of RNA levels in protists

6.15 ± 0.58 µg per 10^6 cells, independent of culture conditions.

Cellular levels of the different RNAs were very strongly correlated in Trypanosoma brucei (R^2 > 0.9), independent of the carbon regime (Fig. 3). mRNA levels of all core metabolic enzymes measured, including PGI, aldolase and GAPDH (not shown), were a linear function of the cellular rRNA content. Slightly more variation was observed with the mRNAs for the non-energy-related β-tubulin and HSP60, but they were a linear function of rRNA levels as well. Less strong, but still significant, was the correlation between the different metabolic mRNAs of Trichomonas vaginalis and its 16S rRNA (Fig. 4). The highest correlation coefficients were found for GAPDH, PPiPFK and β-tubulin. Considerably less correlation was found between mRNA levels of MDH, AK and LDH and 16S rRNA.

There was no consistent relationship between the mRNA level and the cellular activity of the glycolytic enzymes of Trypanosoma brucei (Fig. 5). HK activity increased with increasing mRNA, but much less in LG than in EG cells. The opposite trend was observed for PGI, LG cells having lower activities at higher mRNA levels. Only EG cells had significant PK activities, which increased as a function of their mRNA. LG cells had similar mRNA levels, but barely detectable PK activities. In the cases of aldolase, TIM and G-3-PDH there was no meaningful trend. Aldolase activities were higher in EG cells than in LG cells. There was no difference for TIM, and G-3-PDH activities were higher in LG cells.

In Trichomonas vaginalis the relationship between steady-state levels of mRNA and the cellular activities of the enzymes they encode was random as well (Fig. 6). The cellular activity of PPiPFK varied by a factor of 6, but highest and lowest mRNA levels differed by a factor of only 1.5. Levels of mRNA encoding GAPDH were lower in EG cells than in their LG counterparts and so too were enzyme activities. However, even if the one outlier point corresponding to LG at high D is ignored, the correlation between mRNA levels and enzyme activity remains minimal. Variations in MDH activity far exceeded those of mRNA levels. Activities and mRNA levels correlated positively in LG cells, but in EG cells the trend was reversed. AK activities and mRNA levels of LG cells varied little, but in EG cells they seemed to have a more or less constant ratio. In the context of the observations on the relationship between mRNA and activity for other enzymes, this may be entirely stochastic.

**Fig. 3. Relative abundance of different mRNAs as a function of rRNA relative abundance in Trypanosoma brucei.**

(a) HK (□), TIM (●), G-3-PDH (○), PK (▲). (b) β-Tubulin (■), HSP60 (●).

The trends for 16S rRNA of Trichomonas vaginalis and the mRNA encoding β-tubulin were very similar to those observed for the mRNAs encoding the metabolic enzymes of this species; decreasing with increasing D under LG conditions and increasing under EG conditions (Fig. 2c). Even the slopes of the curves were the same. In these cases highest levels were measured in LG organisms and only at the highest D were equal levels detected in EG cells. Total RNA content was MDH and AK decreased slightly with increasing growth rate in LG-grown Trichomonas vaginalis (Fig. 2a). The opposite trend was observed in EG cells (Fig. 2b). The differences between the highest and lowest values never exceeded a factor of 2. The curves for each of the different mRNAs for LG and EG cells intersected at approximately half the maximum growth rate, thus at this growth rate mRNA levels were equal for both carbon regimes. There were minor differences in the trends for the four mRNAs shown. In LG cultures levels of mRNA encoding GAPDH and MDH decreased most while that of AK changed little. In EG-grown organisms AK mRNA increased more than the other mRNAs. The mRNA encoding malic enzyme was dependent on growth rate in a manner similar to that found for the mRNA of PPiPFK (not shown).

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relevant genes. Growth rate determines cellular RNA levels in both *Trypanosoma brucei* and *Trichomonas vaginalis*, but in a different manner in the two species. The good correlation between the levels of different mRNAs but not between mRNA and product indicates that transcription is regulated in both species by intrinsic factors. None of the major proteins of procyclic *Trypanosoma brucei*, including metabolic enzymes, shows detectable degradation over a 2-d period (Clayton, 1988). In general, metabolic enzymes have low turnover rates (less than 2% h⁻¹) and, of the enzymes measured in this study, only PK of *Trypanosoma brucei* is regulated by a low-molecular-mass effector (van Schaftingen *et al.*, 1985, 1987). Hence, regulation of the overall rates of protein synthesis occurs at the post-transcriptional level. This argues against, but does not entirely rule out, specific control of transcription rates of each of the genes examined. A similar relationship between growth rate and mRNA levels of specific genes, but not between mRNA and specific activity of the corresponding enzyme, was found in two species of yeast, *Kluyveromyces lactis* and *Kluyveromyces marxianus* (Hoekstra *et al.*, 1994), and in *Escherichia coli* (Pease & Wolf, 1994). In rat liver levels of mRNA encoding PFK and its specific activity decreased simultaneously (Casado *et al.*, 1993). However, the regulation of collagenases in humans could not be explained solely by transcriptional regulation (Vincenti *et al.*, 1996).

The observed biphasic dependence of mRNA levels on growth rate in *Trypanosoma brucei* indicates a metabolic switch at half maximal growth rate. A switch in the metabolism, revealed by increased efficiency at

![Graphs showing the correlation between 16S rRNA and different types of mRNA in LG cells (□) and EG cells (○) of *Trichomonas vaginalis*.](image-url)
higher growth rates, was also observed in Leishmania donovani (Ter Kuile & Opperdoes, 1992a, b) and Trichomonas vaginalis (Ter Kuile, 1994a, b). Both above and below the switch point, transcription must be tightly regulated because levels are more or less constant at low growth rates and increase in a very coordinated manner at high rates. Surprisingly, the energetic state of the cells, which were either limited by the carbon and energy source or had access to larger quantities than could be used, did not affect RNA levels.

The cellular activities of the core metabolic enzymes of Trypanosoma brucei can differ by a factor of 2–40 depending on culture conditions (Ter Kuile, 1997). All activities seem precisely adjusted to maintain a more or less constant ratio of the major end-products, acetate and succinate. There was no group of enzymes changing activities in parallel comparable to that found in Trichomonas vaginalis (Ter Kuile, 1996, 1997). The lack of correlation between steady-state mRNA and enzyme levels indicates that this regulatory process does not involve changes in mRNA levels. Absence of a direct relationship between mRNA and product has been documented extensively for the PARP or procyclin surface-coating proteins of procyclic trypanosomes (Biebinger et al., 1996; Hehl et al., 1994; Hotz et al., 1997; Hug et al., 1993).

In Trichomonas vaginalis the steady-state level of mRNAs and 16S rRNA is determined both by the growth rate and by the availability of the carbon source. It seems, therefore, that in contrast to what was observed in Trypanosoma brucei, the energetic state of the cell influences RNA levels in Trichomonas vaginalis. The observation that the trends were similar for rRNAs and mRNAs encoding proteins that serve different purposes.
in the cell indicates that in this species a general mechanism regulates steady-state mRNA levels. Possibly the organism balances the metabolic expenses of DNA, RNA and protein synthesis. In such a case there is no need for direct crosstalk between RNA polymerase II and DNA polymerase, energy or substrate availability accounting for the rate limitation of both. Limitation of macromolecular synthesis at submaximal growth rates has been documented in prokaryotes and was explained as a mechanism of the cell to protect itself against environmental fluctuations (Koch, 1997). Rates of translation can then be regulated by the requirements of the cell through mechanisms directly influencing that process.

The observed dependence of RNA levels in *Trichomonas vaginalis* on growth rate can furthermore be analysed by regarding steady-state levels as the balance of transcription and degradation. If RNA half-lives are much shorter than doubling times and rates of transcription are independent of growth rate, RNA will be somewhat diluted by newly synthesized cellular substances at the highest growth rates. Therefore, steady-state levels will decrease slightly with increasing growth rates. This was the trend observed in LG cultures of *Trichomonas vaginalis*. Lower turnover rates would cause steeper decreases of the mRNA levels at increasing $D$ values. The opposite trend can be expected when the basic components of the RNA, and not the energy for synthesis, are limiting. This may be the case in EG-grown *Trichomonas vaginalis* cells which are limited by a component of the horse serum. The biphasic dependence on growth rate suggests that RNA levels in *Trypanosoma brucei* cannot be explained by such simple mechanisms. This notion is confirmed by the excellent correlation between the different RNA levels. Therefore, the data on *Trypanosoma brucei* suggest the presence of a mechanism coordinating macromolecular synthesis.

**Regulation of transcription**

The above discussion of the effect of growth rate on mRNA levels suggests that rates of transcription of individual genes are not regulated individually in either procyclic *Trypanosoma brucei* or *Trichomonas vaginalis*. In this respect both species differ from most eukaryotes. Most studies on transcription in *Trypanosoma brucei* have centred around the VSG/PARP-encoding genes that are transcribed by a polymerase that resembles RNA polymerase I in many respects (Janz et al., 1994; Rudenko et al., 1990; Zomerdijk et al., 1993). The stage-specific transcription of these genes has been documented extensively (Rudenko et al., 1994). Similarly, aldolase mRNA levels were sixfold higher in bloodstream *Trypanosoma brucei* than in the procyclic form (Clayton, 1985). Large differences in levels of mRNA encoding tubulin were found between life-cycle stages of *Trypanosoma cruzi* (Urmenyi et al., 1992). This suggests that while gene transcription may be developmentally regulated, further regulation of ex-
pression at each stage in the life cycle occurs post-transcriptionally.

Regulation of transcription in *Trypanosoma brucei* probably differs from most other eukaryotes examined for another reason: no TATA boxes were found upstream of the coding region of the seven genes specifically examined for their presence (Quon et al., 1994) and none have been discovered in *Trichomonas vaginalis* since (Liston & Johnson, 1998). The RNA polymerase II of *Trichomonas vaginalis* strongly differs from homologues in other species in the C-terminal domain (Quon et al., 1996). Reversible phosphorylation of the C-terminal domain is thought to play a role in the regulation of gene expression (Dahmus, 1995; Kang & Dahmus, 1995). Therefore, it is not likely that this particular mechanism of regulation by reversible phosphorylation occurs in *Trichomonas vaginalis*. This in turn fits well with the data from this study, emphasizing the role of post-transcriptional regulation.

The importance of translational control in the regulation of gene expression has long been recognized (Altmann & Trachsel, 1993). Mechanisms regulating translation can act at the level of eukaryotic initiation factor 2 (Samuel, 1993; Wek, 1994), the 3' untranslated region (3' UTR) (Decker & Parker, 1995) or the poly-A tail (Pain, 1996). The 3' UTRs of several proteins that are stage-specifically regulated in *Trypanosoma brucei* strongly influence the expression of a reporter gene (Hotz et al., 1995, 1997). Mechanisms involving solely the 3' UTR would imply different mRNAs under different physiological conditions and are therefore not probable. mRNAs with shorter 3' UTRs are more susceptible to translational regulation by varying poly-A tail length (Tanguay & Gallie, 1996). Hence, the length of the 3' UTR may affect the extent to which the poly-A tail can stimulate translation.

**Conclusions**

The main conclusion from this study is that mRNA levels cannot be used as a measure of expression of the corresponding gene in the case of glycolytic enzymes of *Trypanosoma brucei* and *Trichomonas vaginalis*. Considerable regulation of expression must occur at the post-transcriptional, and probably the translational, level. The general validity of such a statement remains to be verified. Comparison of the physiology of *Trypanosoma brucei* and *Trichomonas vaginalis* suggests that *Trypanosoma brucei* exerts a tighter control over its cellular processes than *Trichomonas vaginalis*. RNA levels correlate better, suggesting central regulation of RNA synthesis, and the ratios of the metabolic end-products are more constant (Ter Kuile, 1996, 1997), indicating more effective control of its energy metabolism.

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