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Repression and reactivation of the variant surface glycoprotein gene in *Trypanosoma brucei*

Bernhard Ehlers*, Joachim Czichos+ and Peter Overath

Max-Planck-Institut für Biologie, Corrensstrasse 38, D-7400 Tübingen, FRG

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Rapid repression of variant surface glycoprotein (VSG) synthesis is an early event during the in vitro transformation of *Trypanosoma brucei* from coated bloodstream forms to uncoated procyclic cells. Repression occurs at the transcriptional level and is triggered by the combined action of two signals: a reduction in temperature from 37 to 27 °C and the addition of the citric acid cycle intermediates citrate and *cis*-aconitate. It is shown that synthesis of VSG mRNA can be reactivated up to 8 h after triggering differentiation by releasing either one or both of the signals. After 30 h repression is irreversible. The results suggest that transformation of bloodstream forms to procyclic cells proceeds through a reversible phase to an irreversible committed state. A reversible repression of VSG mRNA synthesis is also observed upon inhibition of protein synthesis in bloodstream forms at 37 °C.

Variant surface glycoprotein; Reversible synthesis; Differentiation; mRNA synthesis; Protein synthesis inhibition; (*Trypanosoma brucei*)

1. INTRODUCTION

During its life cycle, *Trypanosoma brucei* proceeds through a series of differentiation steps each of which is considered to be unidirectional and irreversible. Nevertheless, the transition between two consecutive developmental stages may involve an initial, reversible phase and a subsequent, irreversible phase. This question is addressed here for the differentiation of bloodstream forms to procyclic cells, a process also called transformation, which normally takes place in the midgut of the tsetse fly but can also be performed in vitro.

Essentially synchronous transformation in vitro can be initiated by the combined action of two signals: a reduction in temperature from 37 to 27°C and the addition of the citric acid cycle intermediates, citrate and *cis*-aconitate (CCA) [1–4]. A rapid event observed upon triggering differentiation is the repression of the synthesis of the variant surface glycoprotein (VSG) which covers the surface of bloodstream forms but is absent in the arising procyclic cells. Repression is caused by the disappearance of VSG mRNA occurring with a half-time of $t_{1/2}$ = 1–1.5 h. This loss of VSG mRNA is attributed to the combination of two effects: an immediate 25-fold reduction in the rate of synthesis and transcript labilization from a half-life of $t_{1/2}$ = 4–5 h in bloodstream forms to $t_{1/2}$ = 1–1.5 h in transforming cells [5]. After 1 day synthesis of VSG mRNA or VSG can no longer be

Correspondence address: P. Overath, Max-Planck-Institut für Biologie, Corrensstrasse 38, D-7400 Tübingen, FRG

* Present address: Institut für Pathologie, Hindenburgdamm 30, D-1000 Berlin 45, Germany

+ Present address: Denagen GmbH, Im Neuenheimer Feld 517, D-6900 Heidelberg, FRG

Abbreviations: VSG, variant surface glycoprotein; CCA, citrate/*cis*-aconitate

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detected. Release of the surface coat into the medium, formation of mitochondrial enzymes and the typical change in cellular morphology occur within 24–72 h after triggering transformation [2,6–9].

In a previous study [4] we briefly noted that repression of VSG synthesis during transformation was initially reversible. The further analysis of this observation is reported here. It is shown that during the early phase of transformation expression of the VSG gene can be reactivated after release of either one or both of the transformation signals. After about 24 h the cells are irreversibly committed to differentiation and VSG gene transcription cannot be reactivated. Furthermore, studies with different inhibitors of protein synthesis indicate that protein synthesis is required for VSG gene expression.

2. MATERIALS AND METHODS

Transformation of the variant clone MITat 1.4 (117) of *T. brucei* was performed as described previously [2,4,5,9]. Labeling of cells with [^{35}S]methionine and immunoprecipitation of VSG have been described [4]. [α-^{32}P]dATP labeling by nick translation in vitro of plasmid TcV-117.5, which is specific for VSG 117 mRNA [10], and hybridization to total trypanosomal RNA, labeling of RNA in vitro with [^{3}H]adenine, isolation of RNA and quantitation of ^{3}H-labeled VSG 117 mRNA, β-tubulin mRNA and VSG 118 mRNA (for estimation of background) by hybridization of total RNA to filter-bound plasmids TcV-117.5, pBT102 and TcV-118.2, respectively, were performed as described by Ehlers et al. [5].

3. RESULTS

3.1. *Reactivation of variant surface glycoprotein mRNA synthesis during transformation*

Bloodstream forms of the *T. brucei* variant clone MITat 1.4 (117) were grown overnight at 37°C in modified MEM medium + 15% inactivated fetal bovine serum and differentiation to procyclic cells was initiated by the addition of CCA and a temperature reduction to 27°C. Fig.1 (●—●) shows the decrease in the level of VSG-specific mRNA (panel b) and the corresponding

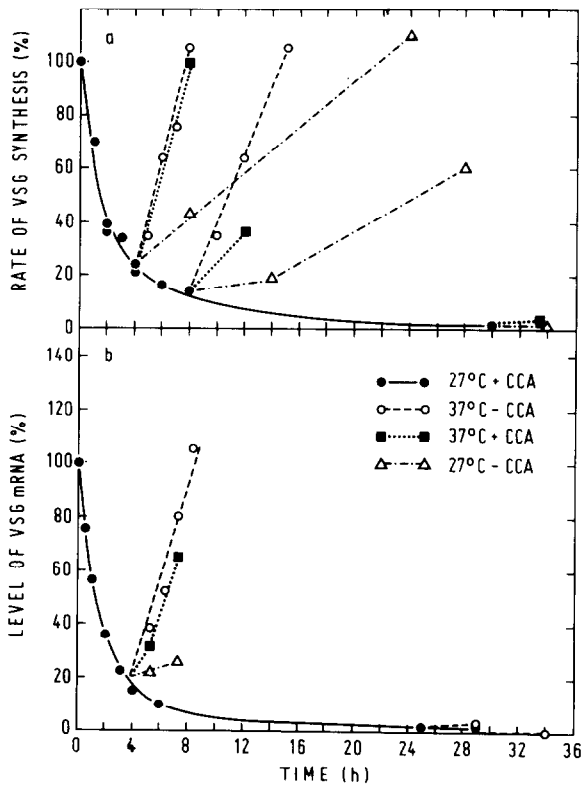

Fig.1. Reactivation of VSG gene expression during transformation. Bloodstream forms of variant clone MITat 1.4 were cultivated overnight at 37°C. Transformation was triggered at time 0 h by the addition of CCA and temperature reduction to 27°C (●—●). At 4, 8 and 30 h (a) or 4 and 25 h (b) cells from culture aliquots were collected by centrifugation, resuspended in fresh medium and incubated at 37°C – CCA (○---○), 37°C + CCA (■ ··· ■) or 27°C – CCA (△ ----- △). (a) At various times the rate of VSG synthesis was determined by labeling cells with [^{35}S]methionine and subsequent immunoprecipitation. (b) The relative level of VSG mRNA was estimated by immobilizing total RNA on filters and probing with ^{32}P-labeled plasmid TcV-117.5 DNA.

decrease in the rate of VSG synthesis (panel a). After an initial exponential decay with a half-time of $t_{1/2}$ = 1–1.5 h, the level of VSG mRNA dropped to a scarcely detectable level after 24 h. The change in morphology from bloodstream forms to procyclic cells and the release of the surface coat occurred with similar kinetics to those described before [2,4,5], i.e. the cells retained the morphology of coated bloodstream forms for

≤ 12 h and thereafter differentiated to dividing procyclic cells (generation time 24 h).

Reversibility of VSG gene expression was first studied 4–8 h after triggering transformation by resuspending cells in fresh medium without CCA at 37°C, i.e. by the simultaneous release of both transformation signals. At this time the rate of VSG synthesis and the level of VSG mRNA in the population had declined to 10–20% of that in bloodstream forms. As is evident from fig.1 (○---○) the synthesis of VSG mRNA and of VSG resumed without detectable lag reaching the level of bloodstream forms within 2–3 h. In addition, labeling experiments of RNA with [^{3}H]adenine demonstrated that VSG mRNA synthesis rapidly returned to the rate typical for bloodstream forms growing at 37°C; a steady state was not reached within 4–5 h of labeling, suggesting that VSG mRNA had a similar half-life ($t_{1/2}$ = 4.5 h) to that of bloodstream forms [5]. Upon further incubation at 37°C the cells retained their surface coat and multiplied as bloodstream forms. Therefore, repression of VSG synthesis at the transcriptional level is initially reversible if both transformation signals are released.

We have previously demonstrated that synchronous transformation of bloodstream forms to dividing procyclic cells is not achieved when only one of the differentiation signals is administered (see [4,9] for details). Thus, rapid repression of VSG synthesis is observed neither when CCA is added to bloodstream form cultures at 37°C nor when the cultures are transferred to 27°C without addition of the citric acid cycle intermediates. Remarkably, a corresponding behavior was observed when only one of the signals was released after initiation of transformation. In cultures shifted back to 37°C with CCA (fig.1, ■···■) or resuspended in fresh medium at 27°C without CCA (fig.1, △ ----- △) synthesis of VSG mRNA and VSG was reinitiated, albeit at a slower rate under the latter conditions. Upon further incubation at 37°C + CCA the cells underwent the abortive transformation to non-dividing procyclic-like cells described in [9] while at 27°C in the absence of CCA they retained their coat and the morphology of bloodstream forms. Therefore, expression of the VSG gene can also be reactivated when only one of the signals is released.

The reversible p ase is followed by an irreversible state in which the cells are committed to differentiation. As shown in fig.1 release of either one or both of the signals 24 h after triggering transformation did not lead to reactivation of VSG gene expression. Under all three conditions (27°C + CCA, 37°C − CCA and 27°C − CCA) the cells proceeded to shed their coat and acquired procyclic morphology. However, growth of procyclic cells occurred only in the cultures incubated at 27°C.

Throughout all experiments expression of β-tubulin mRNA served as a control. As expected [5], no significant changes in the level of this mRNA were observed.

3.2. *Reactivation of variant surface glycoprotein mRNA synthesis after inhibition of protein synthesis*

Inhibition of protein synthesis in bloodstream forms growing at 37°C results in the rapid shut-off of VSG mRNA synthesis and transcript labilization from a half-life of 4.5 to 0.75 h. This effect is

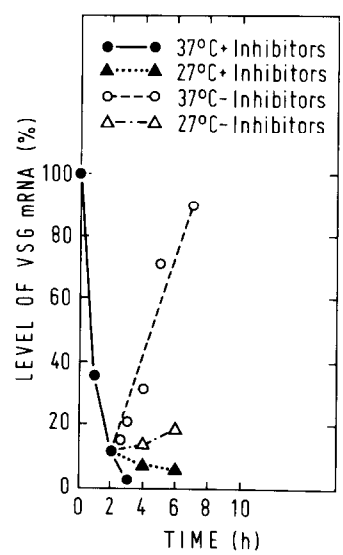

Fig.2. Reactivation of VSG gene expression after inhibition of protein synthesis. Bloodstream forms were grown overnight at 37°C. At time 0 h cycloheximide (50 μg/ml) and puromycin (25 μg/ml) were added to the cultures (●——●). After 2 h cells were centrifuged and resuspended in fresh medium at 37°C (○---○) or 27°C (△ ----- △) in the absence of inhibitors or at 37°C (●——●) or 27°C (▲ ··· ▲) in the presence of inhibitors. At the indicated times the level of VSG mRNA was determined.

specific for VSG gene expression because the syntheses of poly(A)$^+$ RNA, β-tubulin mRNA and the mini-exon-derived RNA remain unaffected [5]. Fig.2 (●——●) shows the decline in level of VSG mRNA after addition of cycloheximide (50 μg/ml) and puromycin (25 μg/ml) to a bloodstream form culture growing at 37°C. Under these conditions the rate of protein synthesis was reduced to below 10% within 15 min. After 2 h the cells were centrifuged and resuspended in fresh medium at 37°C (○---○) or 27°C (△ ----- △) in the absence of inhibitors or at 37°C (●——●) or 27°C (▲ ··· ▲) in their presence. At 37°C and in the absence of inhibitors immediate resumption of mRNA synthesis and VSG synthesis (not shown) was observed. 5 h later the VSG mRNA level was close to that in untreated bloodstream forms. A much slower increase was observed at 27°C in the absence of inhibitors while in their presence a further decline occurred independently of the temperature. Cells released from the temporary inhibition of protein synthesis upon further cultivation at 37 or 27°C retained their bloodstream form morphology and their surface coat but multiplied only at 37°C. Therefore, repression of VSG mRNA synthesis caused by inhibition of protein synthesis is not suf-

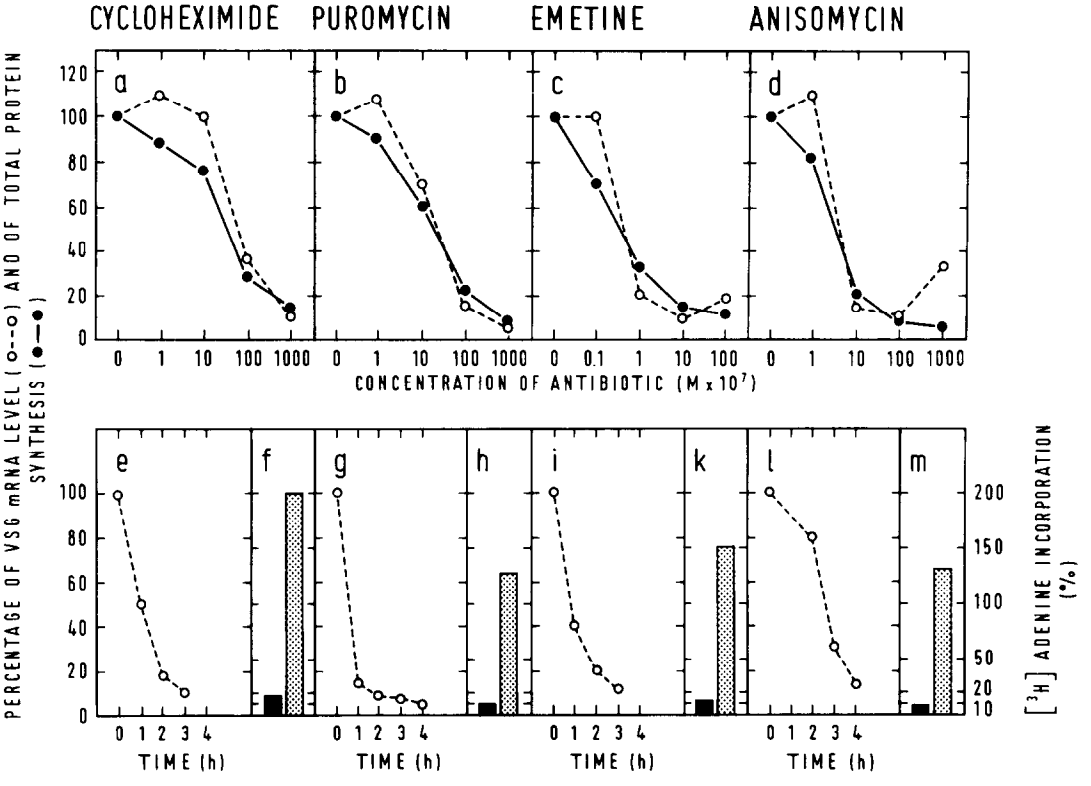

Fig.3. Effect of various inhibitors of protein synthesis on VSG gene expression. (a–d) Cultures of bloodstream forms grown overnight at 37°C were supplemented with cycloheximide (a), puromycin (b), emetine (c) or anisomycin (d) at the indicated concentrations. After 5 min [^{35}S]methionine was added and incorporation into proteins was estimated 3 h later by trichloroacetic acid precipitation (●——●). At the same time, cells were harvested for the determination of the VSG mRNA level (○---○). (e–m) Bloodstream forms were cultured at 37°C in the presence of cycloheximide (10^{-4} M; e,f), puromycin (10^{-4} M; g,h), emetine (10^{-5} M; i,k) and anisomycin (10^{-4} M; l,m). At various times after addition of the antibiotics the VSG mRNA level was determined. Also, aliquots of the cultures were labeled with [^{3}H]adenine after addition of the antibiotics. RNA was isolated 5 h later and the incorporation of label into VSG mRNA (solid bar) and β-tubulin mRNA (dotted bar) was determined (f,h,k,m). Data are expressed as percent of incorporation in an untreated control.

ficient to stimulate subsequent differentiation to procyclic cells at 27°C in the absence of Krebs cycle intermediates.

The relationship between the level of VSG mRNA and the rate of total protein synthesis has been analyzed further by the experiments depicted in fig.3. Panels a–d demonstrate that the decline in the VSG mRNA level (○---○) occurred in parallel to the extent of inhibition of protein synthesis (●——●) and was independent of the type of inhibitor used. The kinetics of VSG mRNA decrease was also similar (fig.3e,g,i,l). Furthermore, labeling of RNA by addition of [^{3}H]adenine after addition of the inhibitors resulted only in minor incorporation into VSG mRNA showing the inhibition of transcription of this gene (fig.3f,h,k,m, solid bars). In contrast, synthesis of β-tubulin mRNA varied between 120 and 200% (dotted bars).

4. DISCUSSION

Repression of the VSG gene and labilization of VSG mRNA triggered by the reduction in temperature and the addition of citric acid cycle intermediates is initially reversible but becomes irreversible after about 1 day (fig.1). In the reversible phase, repression requires the presence of both differentiation signals because the release of either one allows resumption of VSG mRNA synthesis. Within the limited temporal resolution of the experiments transcription is reinitiated without detectable lag and the stability of these transcripts appears to be similar to that in bloodstream forms. An equally prompt resumption of VSG mRNA synthesis occurs after a transient inhibition of protein synthesis by cycloheximide and puromycin (fig.2). Furthermore, fig.3 demonstrates that the decline in VSG mRNA occurs in proportion to the inhibition of protein synthesis with four different inhibitors all of which interfere with polypeptide chain elongation [11]. Therefore, transcription of the VSG gene appears to be dependent on ongoing protein synthesis.

The intracellular factors regulating VSG gene transcription are unknown. We have previously argued [5] that the immediacy of repression of VSG mRNA synthesis during transformation or upon inhibition of protein synthesis suggests regulation by rapid changes in the concentration of low molecular mass effectors or by a short-lived positive regulatory protein. The ready resumption of VSG gene transcription upon release of one or both of the transformation signals or upon recovery from inhibition of protein synthesis most likely involves a rapid readjustment in the concentration of these putative regulatory factors.

About 1 day after triggering transformation VSG mRNA synthesis cannot be reactivated and further differentiation proceeds irrespective of the presence of the Krebs cycle intermediates. One possible explanation for this observation is that at this time the expression-linked copy of the VSG gene becomes permanently unavailable for transcription because of a change in chromatin organization. Indeed, this gene is highly sensitive to DNase I in nuclei isolated from bloodstream forms as first shown for a different VSG gene by Pays et al. [12] and relatively resistant in nuclei obtained from established procyclic cells (Ehlers, B., unpublished). Unexpectedly, DNA in nuclei from transforming cells appeared to be much more accessible to endogenous nucleases prohibiting the isolation of intact nuclear DNA. Therefore, the question as to what time during differentiation the VSG gene becomes DNase-resistant remains open.

The transition from a reversible to an irreversible state is a phenomenon encountered in other differentiation systems. For example, glial progenitor cells from rat optic nerve differentiate in vitro to oligodendrocytes if cultured in serum-free medium and into astrocytes if cultured in fetal bovine serum. The choice of the developmental pathway taken by the bipotential glial progenitor cells in culture is reversible for 1–2 days and then becomes fixed [13]. Likewise, hormones, such as epidermal growth factor or somatomedins have to be present for prolonged times to stimulate DNA replication of responsive cells. In contrast, exposure of 3T3 cells to platelet-derived growth factor is required for only 30 min for the cells to become competent to replicate their DNA and divide (review [14]). In the trypanosome system further progress in the unraveling of the molecular mechanism of the early effects observed during transformation depends on the development of a suitable in vitro system for VSG gene transcription while there appears to be no easy access to the study of the complex late effect at this time.

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