The EMBO Journal Vol.2 No.10 pp.1721 – 1728, 1983

Repression of glycoprotein synthesis and release of surface coat during transformation of *Trypanosoma brucei*

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Communicated by P.Overath
Received on 8 June 1983; revised on 25 July 1983

The biosynthesis of the variant surface glycoprotein (VSG) and its release from the surface of *Trypanosoma brucei* 427 variant clone MITat 1.4 (117) during *in vitro* transformation of bloodstream trypomastigotes to procyclic trypomastigotes was investigated. After transfer to the transformation medium at 27°C, VSG synthesis is repressed with a half-time, $t_{1/2}$ = 30 min. Concomitantly VSG-specific mRNA is lost suggesting that repression operates at the transcriptional level. The expression-linked extra gene copy, which codes for VSG, is retained during and after completion of transformation. After repression of VSG synthesis, surface VSG is shed from the cells into the culture medium. During release part of VSG (apparent mol. wt. 61 000) is proteolytically cleaved to a product (apparent mol. wt. 51 000) which represents the N-terminal domain of the protein as judged by the absence of the carbohydrate moiety normally linked to the C terminus.
Key words: Trypanosoma brucei/transformation/surface coat/glycoprotein synthesis/expression-linked extra gene copy

Introduction

The life cycle of the unicellular parasite *Trypanosoma brucei* in the mammalian host and the tsetse fly proceeds through a series of morphological states which are accompanied by profound alterations in ultrastructure and metabolism (Vickerman and Preston, 1976). Particularly distinctive are changes in the surface architecture. In the mammalian host, these flagellated cells are completely covered by a dense coat (Vickerman and Luckins, 1969), which consists of a single glycoprotein characteristic for an individual trypanosome clone (Cross, 1975). Each clone can give rise to variants in which gene rearrangements lead to the expression of structurally and immunologically distinct glycoproteins at the cell surface (Borst and Cross, 1982). Upon ingestion by the vector, trypanosomes transform in the insect midgut into so-called procyclic forms which lack the glycoprotein coat and are not infective for mammals (Seed, 1964; Brown *et al.*, 1973). After multiplication in the gut as procyclic forms, further development and invasion of the salivary glands culminates in the formation of metacyclic trypanosomes which have re-acquired the surface coat and the ability to infect mammals.

Transformation of bloodstream trypanosomes to procyclic forms is the only developmental step in the life cycle which can be readily performed *in vitro*. Procyclic cells from *in vitro* cultures when ingested by tsetse flies mature into infective metacyclic trypanosomes (Evans, 1979; Schöni *et al.*, 1982).

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The *in vitro* system has been used to investigate the time course of various morphological and biochemical parameters throughout the transformation process. In particular, it has been shown by electron microscopic and immunofluorescent techniques that the surface coat is lost within the first 24 – 72 h in culture (Barry and Vickerman, 1979; Ghiotto *et al.*, 1979; Bienen *et al.*, 1981).

This paper presents an attempt to define in detail the relationship between glycoprotein expression and the loss of the surface coat during the *in vitro* transformation process. Bloodstream forms, initially fully coated, rapidly and completely repress glycoprotein synthesis at the transcriptional level. The surface coat is released into the culture medium during morphological transformation. For the variant clone used in this investigation the extra gene copy coding for the glycoprotein is retained during and after completion of transformation.

Results

Transformation protocol

Application of recent methods for *in vitro* cultivation of bloodstream (Brun *et al.*, 1981) and procyclic (culture) forms (Brun and Schönenberger, 1979, 1981) enables the synchronous transformation of the monomorphic *T. brucei* stock 427, variant clone MITat 1.4 (117) (Figure 1a). Trypanosomes harvested from mouse blood were inoculated into medium B above a feeder layer of *Microtus montanus* embryo fibroblasts (MEF) at 37°C (Brun *et al.*, 1981) and supplemented with citric acid cycle intermediates (citrate plus *cis*-aconitate, CCA, cf., Brun and Schönenberger, 1981; Simpson *et al.*, 1980). Under these conditions, cells retained the morphology of bloodstream trypomastigotes and the cell count doubled. Transformation was initiated at t = 0 h by transfer of the cells to medium SDM-79 (Brun and Schönenberger, 1979) plus CCA above the same MEF-feeder layer at 27°C. Exponential growth either resumed immediately (Figure 1a) or after a lag time of 12 – 18 h. Morphological transformation occurred within the first 48 h (see below). Thus, under optimized conditions, this monomorphic variant clone transforms as readily as pleomorphic populations of trypanosomes (Barry and Vickerman, 1979; Ghiotto *et al.*, 1979; Bienen *et al.*, 1980). The transformation protocol described in Figure 1a was used for all experiments throughout this paper and provides a reference time scale for developmental changes during transformation. Within the experimental error of the experiments these changes occurred with similar kinetics whether a lag in cell division after the medium shift was apparent or not.

Repression of VSG synthesis

The expression of VSG during transformation was investigated by *in vivo* labeling of proteins with L-[^{35}S]-methionine (cf., numbers with arrows in Figure 1a). Total trypanosomal proteins labeled at the indicated time points for 40 min were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The autoradiograph in Figure 1b

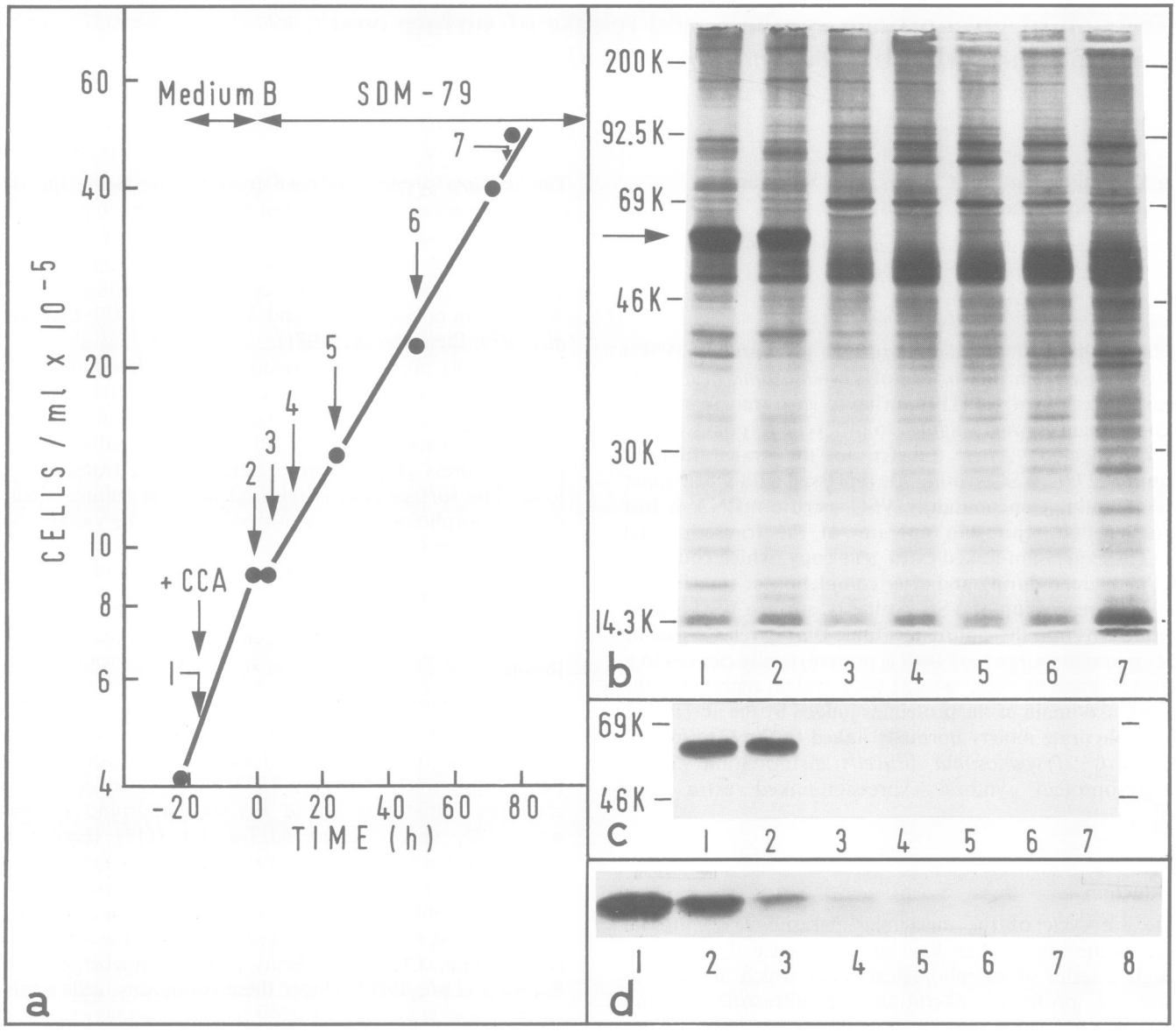

Fig. 1. Cell growth during transformation and labeling with L-[^{35}S]methionine. **(a)** Growth of variant 117 first in medium B (−23 to 0 h) and then in medium SDM-79 (0−77 h). The time of addition of citrate/*cis*-aconitate (CCA) is indicated. No. 1−7 refer to time points where samples were taken for biosynthetically labeling the cells with L-[^{35}S]methionine. **(b)** Radioautograph of cellular proteins after separation by SDS-PAGE (50 000 c.p.m./lane). Locations of mol. wt. standards and VSG (arrow) are indicated. **(c)** Section of an autoradiograph of a similar gel where equal aliquots of immunoprecipitates from cells containing 50 000 c.p.m. label were applied to each lane. Numbers 1−7 below the lanes in **(b)** and **(c)** refer to the time points in part **(a)**. **(d)** Time course of repression of VSG synthesis during the first 5 h after transfer of cells to medium SDM-79. In this experiment cells were labeled for only 15 min at 37°C. A section of an autoradiograph similar to **(c)** is shown where equal aliquots of immunoprecipitates from cells containing 100 000 c.p.m. label were applied to each lane. Time after transfer to SDM-79: 0 min **(lane 1)**, 30 min **(lane 2)**, 60 min **(lane 3)**, 90 min **(lane 4)**, 120 min **(lane 5)**, 180 min **(lane 6)**, 240 min **(lane 7)** and 300 min **(lane 8)**. The radioactive band was excised and counted after digestion with 90% Protosol (NEN Chemicals). A semi-logarithmic plot of radioactivity against time indicates an exponential decrease in VSG-associated radioactivity ($t_{1/2}$ = 30 min). After this decrease a constant level of residual radioactivity is found (compare **lanes 5−8**) which amounts to 10% of that observed at t = 0 min.

(lane 1) shows a prominent band corresponding to a mol. wt. 61 000 daltons which can be specifically immunoprecipitated with antiserum against purified VSG-117 (Figure 1c, lane 1). VSG is still synthesized in the same proportion to all labeled proteins after the growth of the cells for 17 h in the presence of CCA (lanes 2). Therefore, over this time range, CCA does not affect VSG expression. However, 5 h after transfer to medium SDM-79 (lanes 3 in Figure 1b and c) and at later times (lanes 4−7) there is a total repression of VSG synthesis. The time course of this repression was further investigated (Figure 1d) by pulse-labeling experiments within the first 5 h after the medium shift leading to an estimate for the half-time of repression, $t_{1/2}$ = 30 min, down to a residual level of ~10% (cf., lanes 5−8 in Figure 1d). The residual level of VSG synthesis observed between 2 and 5 h after the medium shift varies from <5% to 20% for independent experiments. One interpretation of this result is that a minor fraction of the cell population represses VSG synthesis with a half-time considerably longer than 30 min.

Retention of the expression-linked extra gene copy and repression of VSG mRNA synthesis

At least two mechanisms can be considered for the repression of variant glycoprotein synthesis. First, clone 117

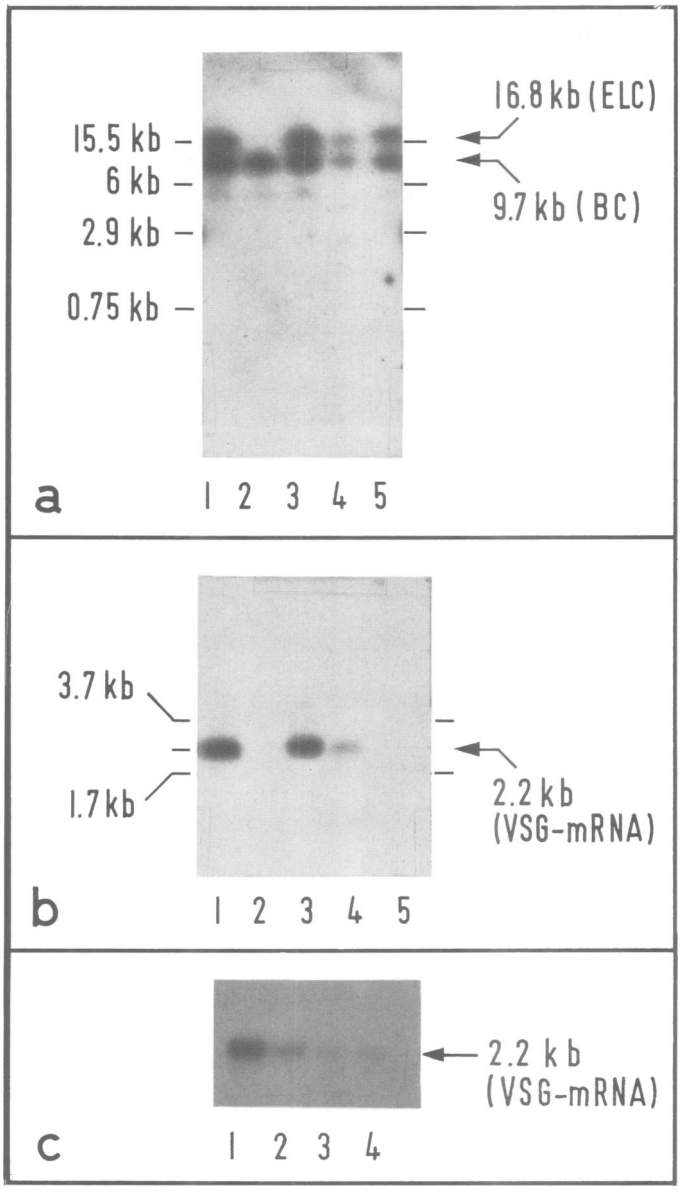

Fig. 2. Retention of the ELC and repression of mRNA synthesis during transformation. **(a)** Hybridization of total trypanosome DNA cleaved by *Hind*III and size-fractionated by agarose electrophoresis with the 900-bp 5′-fragment of the insert of plasmid TcV-117.5: **lane 1**, bloodstream forms of variant 117; **lane 2**, bloodstream forms of variant 118; **lane 3**, procyclic cells harvested at t = 150 h from a transformation experiment (cf. Figure 1a); established procyclic cells grown for ~30 **(lane 4)** or 50 generations **(lane 5)**. Bands referring to the BC and the ELC are indicated. **(b)** Hybridization of the 900-bp 5′-fragment of the insert of plasmid TcV-117.5 to total trypanosome RNA size-fractionated by agarose gel electrophoresis: **lane 1**, bloodstream forms of variant 117; **lane 2**, bloodstream forms of variant 118; **lanes 3–5**, RNA prepared from cells of variant 117 at times 0 h, 9 h and 79 h of a transformation experiment (cf., Figure 1a for time scale). **(c)** same as **(b)** but RNA prepared from cells of variant 117 at times 0 min **(lane 1)**, 30 min **(lane 2)**, 60 min **(lane 3)**, and 120 min **(lane 4)** of a transformation experiment.

belongs to the class of variants in which the duplication and transposition of a so-called basic copy of the VSG-117 gene leads to the appearance of a second copy, the expression-linked extra copy (ELC), which is the one transcribed (Hoeijmakers *et al.*, 1980b; Bernards *et al.*, 1981). In a variant of a different trypanosome strain in which VSG expression is activated by the same mechanism, the ELC was lost after extended growth as procyclic cells (12 subcultures, Pays *et al.*, 1981b). Therefore, the rapid excision of the ELC during transformation and inactivation of existing mRNA could result in the cessation of VSG formation. Alternatively, the ELC could remain in the expression site and VSG synthesis could be turned off by the repression of VSG-specific mRNA synthesis and degradation of the existing mRNA.

The DNA-DNA hybridization experiments in Figure 2a show that, in variant 117, the ELC is retained during and long after completion of transformation. The 5′ end of the cDNA insert in hybrid plasmid TcV-117.5 (van der Ploeg *et al.*, 1982a) which carries the information for the N-terminal part of VSG 117 was hybridized to *Hind*III-digested DNA from bloodstream forms of variants 117 (lane 1) or 118 (lane 2) and to DNA from transformed cells of variant 117 shortly after transformation (lane 3) or after extended growth as procyclic cells (lanes 4 and 5). All DNA preparations give rise to a 9.7-kb fragment characteristic of the basic copy (BC) of the gene (van der Ploeg *et al.*, 1982a). The 16.8-kb fragment characteristic of the ELC of variant 117 is absent in the genome of bloodstream forms of the heterologous variant 118 but present in the genome of bloodstream forms as well as in all three DNA preparations of procyclic forms of variant 117. Thus, excision of the ELC cannot be the cause for the shutdown of VSG synthesis during transformation.

The same DNA probe was hybridized to total RNA of bloodstream forms of variants 117 or 118 (Figure 2b, lanes 1 and 2) and to RNA prepared from cells at times 0 h, 9 h, and 79 h of a transformation experiment (Figure 2b, lanes 3, 4 and 5, see Figure 1a for time scale). The band specific for the mRNA of VSG 117 (2250 nucleotides, Hoeijmakers *et al.*, 1980a) is still present at t = 0 h but essentially absent 9 h after transfer of the cells to medium SDM-79. An independent experiment showed that VSG-specific mRNA decreased by ~90% within the first 2 h after transfer of the cells to medium SDM-79 (cf., Figure 2c). Therefore, the time course of the loss of VSG-specific mRNA is very similar to the kinetics of repression of VSG synthesis. A minor fraction of VSG mRNA remains at least up to 9 h after the medium shift but disappears at later times (compare lanes 4 and 5 in Figure 2b). This residual level of mRNA probably accounts for the corresponding residual level of VSG synthesis mentioned above.

Release of surface coat

The release of surface coat during transformation was investigated at the level of the individual cell by immunofluorescence staining and at the level of the entire population by radioactive labeling experiments.

In the upper series of photographs in Figure 3 surface VSG was specifically stained in formaldehyde-fixed cells by immunofluorescence using rabbit anti-VSG 117 antibody followed by fluorescein isothiocyanate (FITC)-conjugated sheep anti-rabbit globulin. The lower series shows all cells by counter-staining with the dye Evans blue. Six hours after transfer of the cells to medium SDM-79, i.e., at a time when VSG synthesis has already ceased, all cells retain the coat (compare sample at t = 6 h with freshly harvested bloodstream forms at t = −17 h). At later times, between ~6 h and 48 h, >95% of the cells lose the surface antigen; at 72 h <1% of the cells retain the coat (compare upper and lower parts at 14 h, 24 h and 72 h). Thus, there is a progressive decrease in the number of coated cells in the population

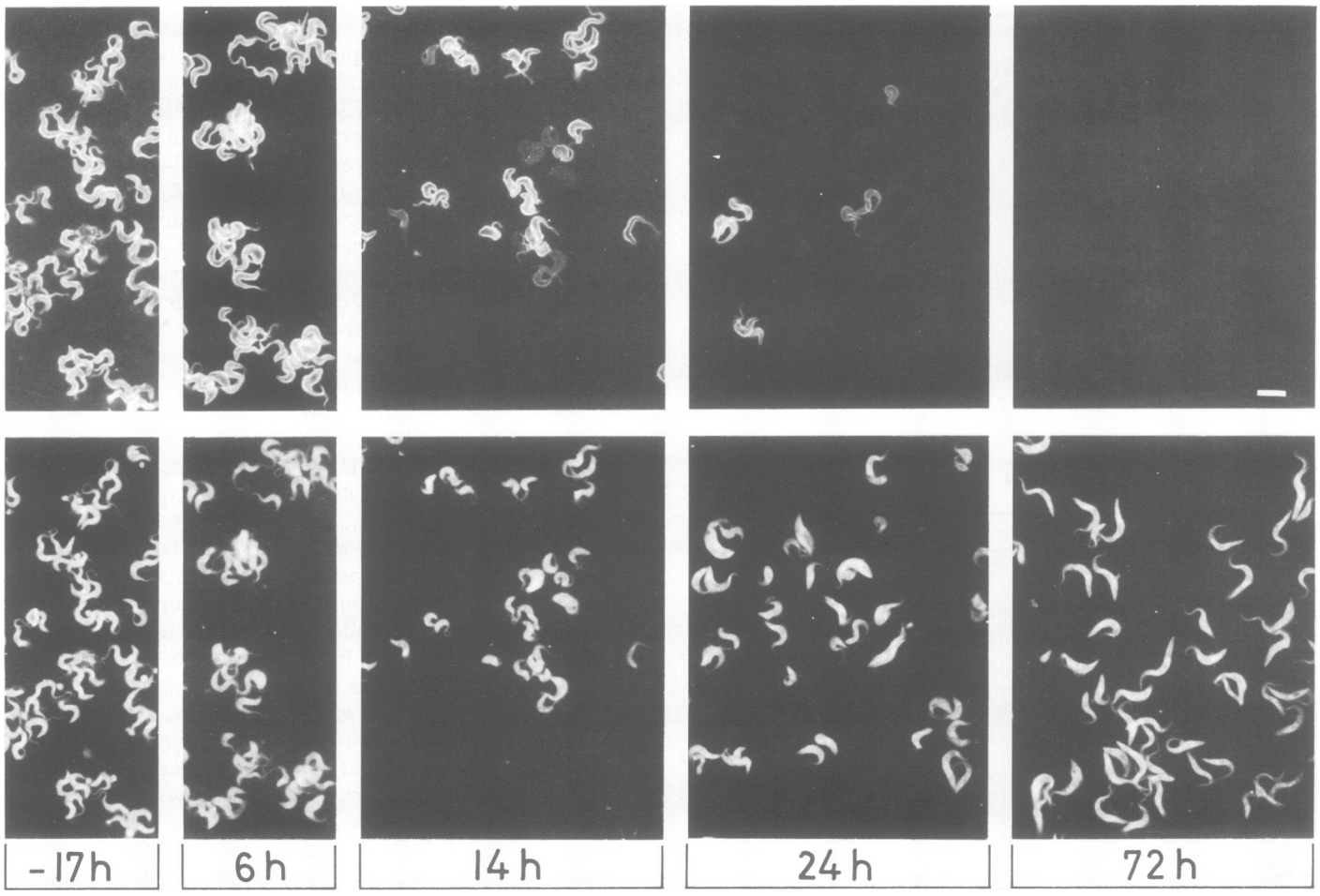

Fig. 3. Release of surface coat. The upper series shows immunofluorescence staining of formaldehyde-fixed cells, using rabbit anti-VSG 117 antibody followed by FITC-conjugated sheep anti-rabbit globulin, the lower series shows the same cells stained with Evans blue. Times refer to the transformation protocol in Figure 1a. Emission was observed with a Leitz I2-filter for FITC-fluorescence and an M2-filter for Evans blue-fluorescence. Bar represents 10 μm.

which is most readily explained by the assumption that VSG release is triggered by a specific signal in each individual cell (see also Barry and Vickerman, 1979; Ghiotto *et al.*, 1979; Bienen *et al.*, 1981). The occurrence of cells in the transforming population which are weakly stained over the entire surface (compare staining intensity on cells in upper parts at 14 h or 24 h) suggests that throughout the release process the distribution of remaining VSG is diffuse. Finally, comparison of the cells in the lower series from left to right demonstrates the increase in size of the cells which typically accompanies the conversion of bloodstream to procyclic forms.

The surface coat is shed into the culture medium (Figure 4). Bloodstream forms were first labeled with radioactive methionine and then subjected to the entire transformation procedure (cf., Figure 1a). At various times, VSG bound to the cells or released into the culture supernatant was analyzed by immunoprecipitation. Radioactivity in immunoprecipitates was either determined directly (b) or by fluorography after separation on SDS-PAGE gels [(a) cells; (c) supernatant]. VSG release starts at $t \sim 6$ h after transfer to medium SDM-79 and is complete around 60 h. As indicated in Figure 4b, the slope of the curve gives an upper estimate of 32 h for the time required for a single cell to release the coat. The cell-associated VSG has the typical mobility of mature, soluble VSG (apparent mol. wt. 61 000, Figure 4a), the culture medium (Figure 4c) contains both this species and a new species (apparent mol. wt. 51 000). The cell-associated VSG in a sample taken at $t = 0$ h can be immunoprecipitated to the same extent both by polyvalent anti-VSG antibody and the so-called cross-reacting antibody which is specific for the carbohydrate moiety linked to the C terminus of VSG (Figure 4e, lanes V and VI, Holder and Cross, 1981). In contrast, the cross-reacting antibody precipitates only the 61-K component from the supernatant for samples taken both at $t = 48$ h and 97 h (Figure 4e, lanes III and IV). This suggests that the 51-K component represents the N-terminal part of the polypeptide chain derived from the 61-K component by proteolytic cleavage. The ratio of the 51-K to 61-K components varied from one to three in different experiments. These two related protein species are the only components detected in the culture supernatant (Figure 4d). Because both appear at the earliest time of coat release and their relative proportion does not change significantly after several days at 27°C in the culture medium (compare lanes 4–8 in Figure 4d or I and II in Figure 4e), it appears unlikely that the 51-K component is derived from VSG after shedding from the cell surface by a serum protease or a protease released by lysis of some trypanosomes in the culture.

Loss of infectivity

The infectivity of the cells during transformation was estimated by injecting 10^6 trypanosomes into the peritoneal

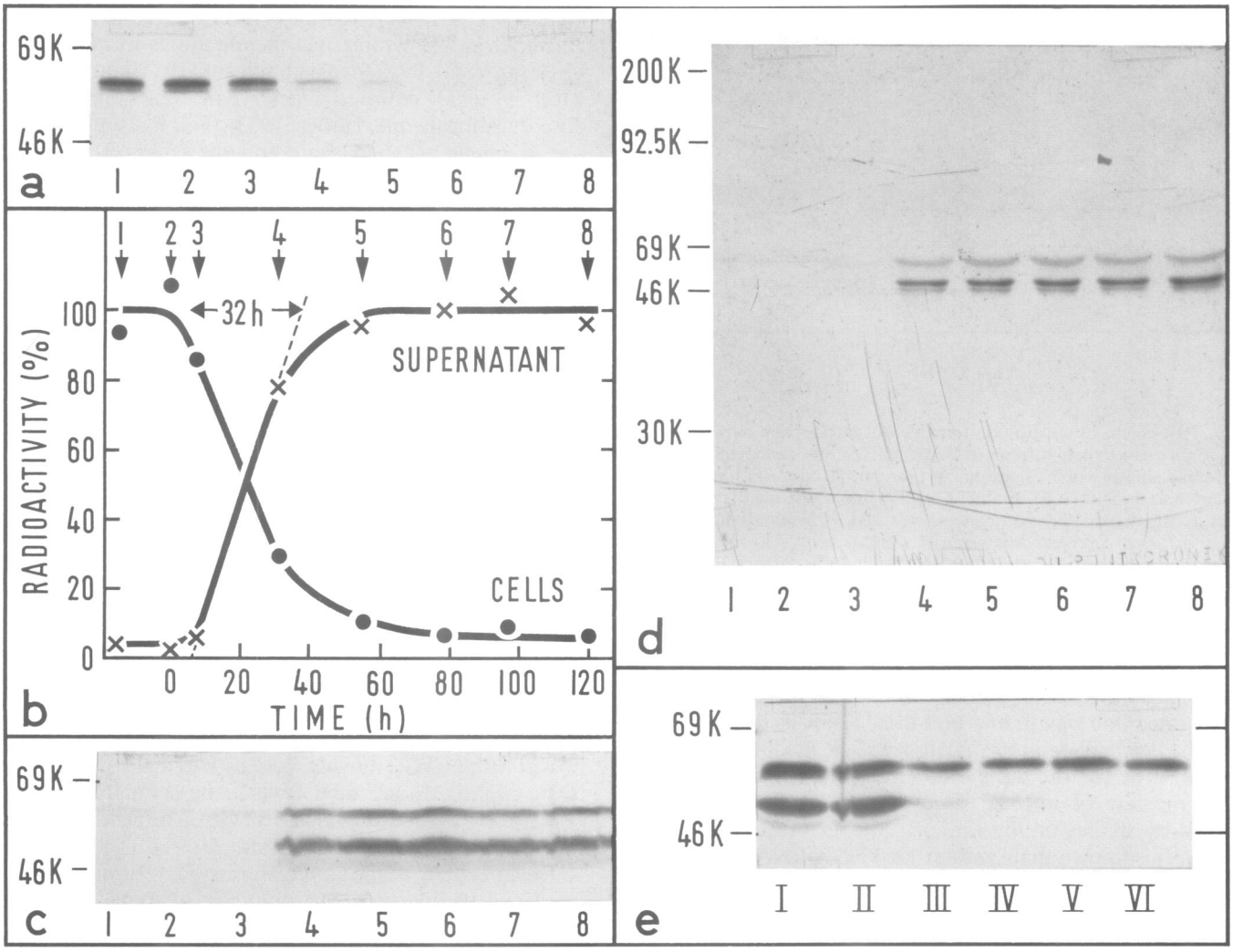

Fig. 4. Release of surface coat. Freshly harvested bloodstream forms were labeled with L-[^{35}S]methionine (cf., Materials and methods) and then subjected to the transformation procedure (cf., Figure 1a for time scale). **(b)** Time course of radioactivity in immunoprecipitates using anti-VSG antibody in cells (●——●) or respective culture supernatants (×——×). Immunoprecipitates from cells at t = 0 h contained only 76% (range 50–100% for different experiments) of the amount of radioactivity obtained from an equivalent amount of culture medium at t = 120 h, i.e., 100% in **part b** refers to 8533 c.p.m. for cells from 100 µl culture and 11 710 c.p.m. for 100 µl culture supernatant. Numbers with arrows refer to sampling times. **(a)** and **(c)** Sections of autoradiographs after separation of immunoprecipitates by SDS-PAGE for cells and supernatant, respectively. Numbers refer to sampling times in **(b)**. **(d)** Autoradiograph from a gel to which aliquots of the culture supernatant were directly applied. Numbers refer to sampling times in **(b)**. **(e)** Section of autoradiograph after separation of immunoprecipitates by SDS-PAGE. Samples were from an independent but otherwise similar experiment. **Lanes I** and **III**: supernatant from sample taken at 48 h. **Lanes II** and **IV**: supernatant from sample taken at 96 h. **Lanes V** and **VI**: cells from sample taken at 0 h. **Lanes I, II** and **V**: polyvalent anti-VSG 117 antiserum. **Lanes III, IV** and **VI**: cross-reacting antibody specific for the carbohydrate moiety linked to the C terminus of VSG.

cavity of mice and counting blood samples 72 h after the injection. The dependence of these blood counts on the incubation time in medium SDM-79 (0–80 h, cf., Figure 1a) can be reasonably well described by a decreasing exponential function with a half time, $t_{1/2}$ ~ 90 min. A small percentage (0.1–0.01%) of the cells remained infective for up to 48 h; infectivity was <0.0001% at t = 73 h.

Discussion

The initial *in vitro* cultivation of the monomorphic variant clone 117 in a medium used for growth of bloodstream forms (Brun *et al.*, 1981) supplemented with citric acid cycle intermediates was found to be instrumental for the subsequent synchronous transformation to procyclic forms. When applied only for a limited period of time (17 h, cf., Figure 1a), the acids do not affect growth, infectivity for mice, VSG synthesis, or the morphology of the bloodstream forms although they cause extensive cell death when incubation under these conditions is continued (compare Simpson *et al.*, 1980). The acids may induce or activate enzymes involved in energy metabolism and may thus prepare the cells for transformation (Brun and Schönenberger, 1981). The transfer of the cells to medium SDM-79 involves changes in the type and concentration of nutrients, a shift from rabbit to fetal calf serum, as well as a decrease in temperature from 37°C to 27°C. It remains unknown which of these alterations provides the signal which triggers transformation.

The time course of several developmental parameters investigated in this and previous publications is summarized in Figure 5. The most immediate change detected so far is the cessation of VSG synthesis. After the medium shift, VSG synthesis falls off with a half time, $t_{1/2}$ = 30 min. In the same

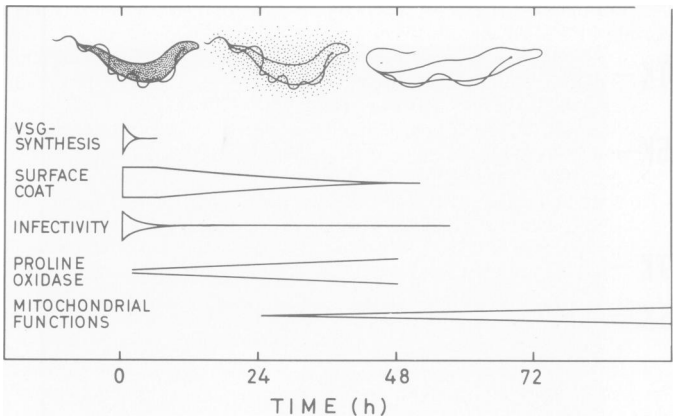

Fig. 5. Time-course of *in vitro* transformation of bloodstream trypomastigotes to procyclic trypomastigotes of *T. brucei*. The time scale refers to the transformation protocol in Figure 1a. At time 0 h, bloodstream forms are transferred to medium SDM-79 at 27°C. At the top of the figure morphological transformation and coat release is indicated. Biosynthesis of the variant surface glycoprotein decreases with a half-time of ~30 min. The cells become non-infective for mice with a half-time of ~90 min. Coat release into the culture medium occurs between 6 h and 60 h. The kinetics of formation of prolin oxidase activity and mitochondrial functions are taken from Evans and Brown (1972) and Bienen *et al.* (1983), respectively.

time range, the level of VSG-specific mRNA decreases at a similar rate. One way to interpret these results is that mRNA synthesis is repressed immediately after the medium change. The half-time for the decline in VSG synthesis would then reflect the rate of mRNA inactivation. This point can be clarified by studies on the rate of turnover of VSG-specific mRNA. It is known that synthesis of VSG mRNA involves a longer precursor which is spliced to yield the mature mRNA (van der Ploeg *et al.*, 1982b; Boothroyd and Cross, 1982). Since the accumulation of mRNA precursors is not observed in our experiments, it appears unlikely that cessation of VSG synthesis is caused by defective mRNA processing.

Repression of mRNA synthesis is clearly not caused by the rapid excision of the ELC of the VSG gene since the ELC is still present after growth as procyclic cells for at least 50 generations. Excision of the ELC has so far only been demonstrated for variant AnTat 1.1 of the AnTAR 1 serodeme after extended growth of the cells as procyclic forms (Pays *et al.*, 1981b). Loss of the ELC is also expected to occur in the tsetse fly since a broad spectrum of variant clones gives rise to the same limited repertoire of metacyclic variant types (Hajduk *et al.*, 1981). Speculations on possible reasons for the different behavior of variants 117 and AnTat 1.1 are premature until more data for other variants become available.

For studies on the regulation of gene expression, the system considered here has two attractive features. Firstly, repression occurs rapidly in at least 90% of the cell population. Secondly, VSG synthesis is a major activity of bloodstream forms since VSG amounts to 7 – 10% of the cellular protein (Cross, 1977). Therefore, VSG-specific mRNA must be one of the most abundant mRNAs in trypanosomes. These properties should be useful in more detailed studies on the mechanism of repression. One obvious question is whether repression of mRNA synthesis abolishes the DNase I hypersensitivity of the ELC (Pays *et al.*, 1981a).

The release of the variant antigen from the cell surface occurs after repression of VSG synthesis and poses a different mechanistic problem. Recent evidence suggests that VSG is anchored in the cytoplasmic membrane as membrane-form VSG (mfVSG) by a hydrophobic moiety (lipid? peptide?) which is quickly removed when trypanosomes are lysed (Cardoso de Almeida and Turner, 1983). This hydrophobic structure is bound via phosphate and the cross-reacting carbohydrate (linkage order undetermined) to the hydroxyl group of an ethanolamine residue which forms an amide linkage to the α-carboxyl group of the C-terminal aspartyl residue of the polypeptide chain (Holder and Cross, 1981; Holder, 1983; Baltz *et al.*, 1982; Allen *et al.*, 1982). Since no special precautions were taken in this study to prevent the removal of the hydrophobic moiety, the species of apparent mol. wt. 61 000 in Figures 1b and c and 4a corresponds to the commonly isolated water-soluble form of VSG (sVSG). The 61-kd component released into the medium during transformation (Figure 4c – e) is also identical to sVSG as judged by mol. wt. and the presence of the cross-reacting carbohydrate. The second component (apparent mol. wt. 51 000, Figure 4c – e) lacks the cross-reacting determinant and, therefore, most likely represents the N-terminal part of VSG. This fragment appears to be similar to a cleavage product produced by incubation of isolated VSG 117 with trypsin (Johnson and Cross, 1979). Release of VSG from the membrane may involve enzymatic conversion of mfVSG to sVSG and at least for variant 117 partial proteolytic cleavage. These hydrolytic enzymes acting as 'clipases' may either be secreted and travel along the surface or they may be localized in the flagellar pocket with mfVSG moving there by lateral diffusion in order to be clipped. Either model would be compatible with the observation that during release VSG still attached to the surface is diffusely distributed (Figure 3).

The infectivity of the culture during transformation was investigated by injecting equal numbers of trypanosomes into mice and counting blood samples after 72 h. The dependence of these blood counts on the time of incubation in medium SDM-79 can be interpreted as a nearly exponential decrease in infectivity in the original culture with a half-time of ~90 min. As indicated in Figure 5, the decrease in infectivity appears to occur after repression of VSG synthesis but precedes coat release. Thus, repression of VSG synthesis or other early events in transformation do not seem to be readily reversible and the cells may proceed *in vivo* to release their coat and thus become subject to lysis by activation of the alternative complement pathway (Tetley *et al.*, 1981). As noted also by previous investigators (Ghiotto *et al.*, 1979; Bienen *et al.*, 1980), a small percentage of cells remains infective at a time when most cells have transformed. Some of these may represent mutants defective in various steps in transformation.

Materials and methods

Trypanosome strains

The variant clones MITat 1.4 (117) and 1.5 (118) of *T. brucei* stock 427 were kindly provided by Dr.G.A.M.Cross (Cross, 1975). This monomorphic stock is not transmissible through the tsetse fly (L.Jenni, personal communication).

Transformation experiments

Citrated blood from infected mice was rapidly cooled in ice, diluted with an equal volume of buffer (TDB; Cross, 1975) and centrifuged for 10 min at 250 *g*. The trypanosome layer above the erythrocyte pellet was directly used to inoculate tissue-culture flasks (area 75 cm^2) containing 20 ml medium B (Brun *et al.*, 1981) supplemented with 15% inactivated rabbit serum, 55 mg Na-pyruvate and 7 mg hypoxanthine per 500 ml at a density of 5 x 10^5 cells/ml.

The flasks contained a nearly confluent feeder layer of irradiated (3300 rad, R.Le Page, personal communication) *Microtus montanus* embryo fibroblasts (MEF, kindly provided by L.Jenni and R.Brun). Each flask was supplemented with a solution (600 μl) containing 100 mM citric acid, 100 mM *cis*-aconic acid, and 200 mM Mg-acetate, pH 7.0 (Brun and Schönenberger, 1981), and incubated at 37°C in 4% CO_2 – 96% air. After 17 h the cultures were cooled on ice and centrifuged at 600 *g*. The trypanosome pellet was suspended in the same volume of SDM-79 (Brun and Schönenberger, 1979) containing 10% inactivated fetal calf serum, 10 mg/l gentamycin, 10 ml/l penicillin-streptomycin (Gibco Bio-Cult, Paisley, Scotland, No. 600–5140) and Krebs cycle intermediates as above and incubated in the previously used fibroblast-containing flasks at 27°C in air.

[^{35}S]Methionine biosynthetic labeling

Trypanosomes were centrifuged in the cold, washed twice in modified MEM (Eagle, 1959) with Earl's salts containing per liter 3 g glucose, 7.14 g HEPES, 10 ml MEM non-essential amino acids (Gibco Bio-Cult, No. 320-1140), 1 g bovine serum albumin (BSA, Sigma Chemical Co. St. Louis, MO No. A4503) but lacking methionine, pH 7.8. After resuspension in this medium, the cells were incubated at 37°C in a shaking water bath.

For the experiment described in Figure 1, 7 x 10^6 cells in 250 μl modified MEM were labeled for 40 min with 30 μCi L-[^{35}S]methionine (1200 Ci/mmol, Amersham, Buchler, Braunschweig, FRG). Aliquots (5 μl) were added to 50 μl BSA (5 mg/ml) and processed for the determination of trichloroacetic acid-precipitable radioactivity as described by Taylor and Cross (1977). Over this time range incorporation was linear. The trypanosomes were collected by centrifugation and stored at –70°C. The frozen pellet was taken up in 200 μl 0.5 mM iodoacetamide/1 mM phenylmethyl sulfonyl fluoride/0.1 mM tosyl-L-lysine chloromethyl ketone in water and aliquots processed for SDS-PAGE and immunoprecipitation.

For pulse-chase experiments (Figure 4), 2 x 10^7 trypanosomes in 1 ml modified MEM were labeled for 20 min at 37°C using 115 μCi L-[^{35}S]-methionine. The parasites were then centrifuged, washed twice in medium B, containing 2 mM L-methionine, resuspended in this medium and used for *in vitro* transformation experiments as described above. At various times, 1.5 ml aliquots were centrifuged, and both pellet and supernatant were stored at –70°C until used for gel electrophoresis and immunoprecipitation.

Polyacrylamide gel electrophoresis, immunoprecipitation, and immunofluorescence

SDS-PAGE was performed as described by Laemmli (1970) using 3% polyacrylamide in the stacking gel and 10% polyacrylamide in the separation gel. The stained and fixed gels were treated for 1 h in Enhance (New England Nuclear, Boston, MA), washed for 1 h in water and dried. Radio-labeled polypeptides were visualized by fluorography at –70°C using a DuPont Cronex 4 X-ray screen film. Radio-labeled protein standards were purchased from Amersham-Buchler, Braunschweig, FRG (Cat. No. CFA626).

Immunoprecipitations were performed according to Kessler (1975) as modified by Holder and Freeman (1982). Antiserum against the purified surface glycoprotein (a kind gift of G.A.M.Cross) was raised in rabbits following the protocol described by Coudrier *et al.* (1981). The cross-reacting antibody was a kind gift of A.A.Holder.

For immunofluorescence, trypanosomes were collected by centrifugation and fixed overnight at 4°C in 1 ml 2% formaldehyde in SDM-79 lacking serum. The fixed cells were washed twice with phosphate buffered saline (PBS), suspended in aqueous 2% BSA, applied to microscope slides, and dried for 1 h at 37°C. The slides were sequentially treated at 20°C with rabbit anti-VSG 117 antiserum (1:100) in PBS (15 min), PBS (15 min), FITC-labeled anti-rabbit globulin (1:400, bioMerieux, Charbonnières-les-Bains, France)/0.0004% Evans blue in PBS (15 min) and PBS (15 min). The slides were then mounted in 50% glycerol in PBS.

Nucleic acid preparations

The cDNA clone TcV-117.5 was kindly provided by P.Borst and P.A.M. Michels (Hoeijmakers *et al.*, 1980a; Bernards *et al.*, 1981). *E. coli* strain HB101 (Boyer and Roullard-Dussoix, 1969) was used for amplification of this plasmid. TcV-117.5 was digested with *Pst*I + *Sal*I and the 900-bp fragment coding for the N-terminal end of VSG 117 was isolated (Boothroyd *et al.*, 1982). The fragment was labeled to a specific activity of ~1 x 10^8 c.p.m./μg with [α-^{32}P]dCTP (3200 Ci/mmol, New England Nuclear, Boston, MA) by nick-translation (Rigby *et al.*, 1977).

Total DNA from *T. brucei* was isolated essentially as described by Bernards *et al.* (1981). 1–1.5 x 10^9 cells were suspended in 0.5 ml 100 mM EDTA/100 mM NaCl/10 mM Tris-Cl, pH 7.5. The cells were lysed by addition of 25 μl 20% SDS and incubated with gentle agitation with 25 μg proteinase K for 30 min at 37°C. The sample was then extracted with 0.5 ml phenol/chloroform/iso-amylalcohol (50:49:1) and twice with 0.5 ml water-saturated diethylether. Dialysis against 10 mM Tris-Cl/1 mM EDTA (TE), pH 7.4 (2 l, 48 h at 4°C, two buffer changes) yielded ~1 ml of solution. The sample was then treated with 2 μl DNase-free RNase (10 mg/ml, see Miniatis *et al.*, 1982) for 15 min at 37°C and then slowly rotated for 60 min at 20°C. The same incubation conditions were used for a second proteinase K treatment (75 μg/ml) in the presence of 0.1% SDS. After two phenol extractions (equilibrated against 100 mM Tris/0.2% 2-mercaptoethanol pH 7.5) and two ether extractions the sample was dialysed for 72 h, as above, against TE buffer containing 10 mM NaN_3 (yield 200–600 μg).

Total RNA from *T. brucei* was isolated by the method of Glisin *et al.* (1974) and Chirgwin *et al.* (1979). Typically, 1–5 x 10^9 cells were lysed under vigorous agitation in 2 ml 2 M 2-mercaptoethanol/4 M guanidinium thiocyanate/0.1 M Tris-Cl pH 7.5. The clear solution was layered above a 1 ml cushion of 5.7 M CsCl/0.1 M EDTA, pH 7.0 and centrifuged at 40 000 r.p.m. and 20°C for 21 h in a Beckman SW60Ti rotor. The RNA sediment was resuspended in 2 ml cold 3 M Na-acetate pH 6.0, kept on ice for 30 min, centrifuged (10 min, 12 000 *g*), and washed with 70% ethanol. The pellet was dissolved in water and stored at –20°C after addition of two volumes of ethanol.

DNA-DNA and RNA-DNA hybridization

DNA-DNA filter hybridization was carried out as described by Southern (1975). DNA (5 μg) was digested with 57 units *Hind*III in 110 mM Tris-Cl pH 7.5, 6 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM NaCl (V = 270 μl, 4 h 37°C). DNA was precipitated with ethanol and 1 μg was electrophoresed in a 0.6% agarose gel (Baby Gel, Model H6, BRL, Inc., Gaithersburg, MD) in 40 mM Tris-acetate/20 mM Na-acetate/5 mM EDTA (pH 8.2). The gel was treated at 20°C sequentially with 0.25 N HCl (2 x 15 min), 0.5 N NaOH-1.5 M NaCl (2 x 15 min) and 1 M NH_4-acetate-0.02 N NaOH (2 x 30 min). DNA was transferred to nitrocellulose filters (Sartorius Membranfilter, Göttingen, FRG, Cat. No. SM 11306) in 20 x SSPE (Miniatis *et al.*, 1982) overnight. Filters were washed in 2 x SSPE for 10 min and baked for 2 h at 80°C. Prehybridization was carried out in a plastic box on a rotary shaker at 65°C for 4 h in 30 ml of a mixture containing 5 x SSPE, 5 x Denhardt's solution, 50 mM Na-phosphate pH 7.0, 0.1% SDS, 10 mM pyrophosphate and 100 μg/ml denatured salmon-sperm DNA. Hybridization was carried out under the same conditions overnight after addition of the heat-denatured probe (500 ng). The filters were successively washed at 65°C with 0.3 x SSPE/0.1% SDS (1 h), 0.5 M Na-phosphate, pH 7.0 (1 h) and 0.3 x SSPE/0.1% SDS (1 h) and autoradiographed.

RNA-DNA hybridization was performed according to McMaster and Carmichael (1977) and Thomas (1980). In brief, 1 μg RNA was treated for 1 h at 50–55°C with 3.6 μl 6 M glyoxal, 8 μl dimethylsulfoxide, 1.6 μl Na-phosphate (100 mM, pH 7.0), total volume 16 μl. 0.5 μg of glyoxylated RNA was separated in a 1.2% agarose gel in 10 mM Na-phosphate pH 7.0. RNA was transferred to nitrocellulose filters in 20 x SSPE and dried for 2 h at 80°C without previous washing with 2 x SSPE. Prehybridization: 4 h at 42°C in 5 x SSPE, 5 x Denhardt's solution, 50 mM Na-phosphate pH 7.0, 50% formamide, 0.1% SDS, and 100 μg/ml denatured salmon sperm DNA. Hybridization: overnight at 42°C in 20 ml 5 x SSPE, 1 x Denhardt's solution, 20 mM Na-phosphate pH 7.0, 50% formamide, 0.1% SDS, 100 μg/ml denatured salmon sperm DNA, and 600 ng radioactive probe. Filters were washed as described above.

Acknowledgements

We thank Dr.G.A.M.Cross (Rockefeller University) and Drs.L.Jenni and R.Brun (Swiss Tropical Institute) for teaching one of us (P.O.) how to grow and handle trypanosomes. We also appreciate the helpful advice of Drs.H.Schwarz, I.Riede, H.Jaeckle and J.K.Wright. This work was supported by the Fond der Chemischen Industrie.

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