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A Gene from the Variant Surface Glycoprotein Expression Site Encodes One of Several Transmembrane Adenylate Cyclases Located on the Flagellum of *Trypanosoma brucei*

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The bloodstream form of *Trypanosoma brucei* contains transcripts of at least four genes showing partial sequence homology to the genes for eucaryotic adenylate and guanylate cyclases (S. Alexandre, P. Paindavoine, P. Tebabi, A. Pays, S. Halleux, M. Steinert, and E. Pays, Mol. Biochem. Parasitol. 43:279–288, 1990). One of these genes, termed ESAG 4, belongs to the polycistronic transcription unit of the variant surface glycoprotein (VSG) gene. Whereas ESAG 4 is transcribed only in the bloodstream form of the parasite, the three other genes, GRESAG 4.1, 4.2, and 4.3, are also expressed in procyclic (insect) forms. These genes differ primarily in a region presumed to encode a large extracellular domain. We show here that ESAG 4-related glycoproteins of about 150 kDa can be found in the trypanosome membrane, that they are detected, by light and electron gold immunocytochemistry, only at the surface of the flagellum, and that the products of at least two of these genes, ESAG 4 and GRESAG 4.1, can complement a *Saccharomyces cerevisiae* mutant for adenylate cyclase. The recombinant cyclases are associated with the yeast membrane fraction and differ with respect to their activation by calcium: while the GRESAG 4.1 and yeast cyclases are inhibited by calcium, the ESAG 4 cyclase is stimulated. ESAG 4 thus most probably encodes the calcium-activated cyclase that has been found to be expressed only in the bloodstream form of *T. brucei* (S. Rolin, S. Halleux, J. Van Sande, J. E. Dumont, E. Pays, and M. Steinert. Exp. Parasitol. 71:350–352, 1990). Our data suggest that the trypanosome cyclases are not properly regulated in yeast cells.

The bloodstream form of *Trypanosoma brucei* is characterized by the presence of a homogeneous layer of a variant surface glycoprotein (VSG). The antigenic variability of this surface layer allows the parasite to escape the immune response of the host. The VSG is no longer synthesized in the procyclic (insect) form of the parasite (for a recent review, see reference 7). The VSG gene is transcribed in a telomeric expression site, together with a battery of additional genes (ESAGs, for expression site-associated genes) (1, 8, 14, 16, 21, 23). Among these, ESAG 4 shares with three related sequences (GRESAGs, for genes related to ESAGs) a 3′-terminal sequence likely to encode the catalytic domain of either adenylate or guanylate cyclase, since this region is homologous to the C-terminal catalytic domain of both the adenylate cyclase of *Saccharomyces cerevisiae* and the membrane form of rat brain guanylate cyclase (2). The N-terminal region of the proteins encoded by the ESAG 4/GRESAG 4 family is more variable than the C-terminal domain (37 to 41% identity in the 900 N-terminal amino acids, compared with 70 to 82% identity in the 350 C-terminal residues). Hydropathy analysis revealed in each case two probable membrane-spanning segments flanking a large N-terminal domain with several N-glycosylation sites, possibly exposed at the external surface of the plasma membrane (2).

In this report, we demonstrate, by yeast complementation, that at least two genes of the ESAG 4 family, one of which is differentially transcribed during the parasite life cycle, encode distinct adenylate cyclases. These enzymes appear to be transmembrane proteins located along the flagellum.

MATERIALS AND METHODS

Trypanosomes. Both bloodstream- and procyclic-form trypanosomes were from the EATRO 1125 stock. Bloodstream forms were either AnTat 1.3A or AnTat 1.1A. Procyclic forms were derived from AnTat 1.1B by in vitro cultivation.

Production of anti-ESAG 4 antibodies. The 5′-terminal 1.25-kb *Hin*dII-*Pvu*II and 3′-terminal 1.52-kb *Eco*RI-*Bgl*II fragments of ESAG 4, isolated from the genomic clones 1ES200.10 and pES200.8, respectively (21), were subcloned into the *Sma*I and *Eco*RI-*Bam*HI sites of plasmid pEX (28). The resulting expression plasmids were termed pEX-ES4 5′ and pEX-ES4 3′, respectively. Expression of the ESAG 4 polypeptides, fused with a 117-kDa Cro–β-galactosidase hybrid protein, was induced by shifting the cell incubation temperature to 42°C after growth at 30°C. The recombinant bacteria were lysed with Nonidet P-40, freeze-thawed, and solubilized in 8 M urea. Lysates were electrophoresed in sodium dodecyl sulfate (SDS)–7.5% polyacrylamide gels, and the ESAG 4 fusion proteins were sliced from the gels and incubated in 50 mM NH_4HCO_3–0.1% SDS at 37°C for 24 h. After dialysis of the supernatants in 50 mM NH_4HCO_3–0.01% SDS, samples were freeze-dried and resuspended in phosphate-buffered saline (PBS). Two rabbits were immunized three times at 3- to 4-week intervals with 100 μg of either the N-terminal or the C-terminal ESAG 4 fusion protein. The immunoglobulins G (IgGs) were affinity purified

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from rabbit sera by DEAE-cellulose chromatography and were pooled for most experiments. Separately, each antibody gave similar Western immunoblot and immunogold pictures.

Immunodetection of ESAG 4-related polypeptides in membrane extracts. Yeast and trypanosome membranes were obtained as described by Heideman et al. (11) and Voorheis and Martin (31), respectively. The membrane proteins were solubilized in 0.15 M NaCl–1% SDS–0.01% Tween 80–1% Triton X-100–1% sodium deoxycholate–1 mM phenylmethylsulfonyl fluoride–1 mM EDTA–0.05 M Tris HCl (pH 7.2). Then 50 μg of protein was electrophoresed in SDS–7.5% polyacrylamide, transferred to nitrocellulose, and incubated with the anti-ESAG 4 IgGs (dilution of 1/6,000) and alkaline phosphatase-conjugated goat anti-rabbit IgGs (Promega Biotec) as primary and secondary antibodies, respectively.

Immunodetection of ESAG 4-related polypeptides at the cell surface. Live trypanosomes were incubated for 30 min with 5 mg of primary antibody per ml in 5% bovine serum albumin–4% serum in PBS, washed, and then incubated for 60 min with the secondary antibody. After washing, the trypanosomes were applied to slides and fixed with a buffered 9% formaldehyde–45% acetone solution for 2 min. Alternatively, trypanosomes were fixed for 2 h in 2% formaldehyde (freshly prepared from paraformaldehyde dissolved in PBS) with 0.05% added glutaraldehyde, washed in PBS, and applied to either glass (light microcopy) or plastic (electron microscopy) slides. At this stage, drying of the slides in an oven ensured good adhesion of the cells during the following incubations and washes. Slides were then incubated in 0.1 M glycine in 50% ethanol before overnight reaction with the primary antibody (anti-AnTat 1.1A or anti-ESAG 4; 1 mg/ml). After three washes in PBS with 0.1% Tween 20, the cells were incubated for 4 h in goat anti-rabbit IgG bound to gold particles (AuroProbe LM GAR or EM

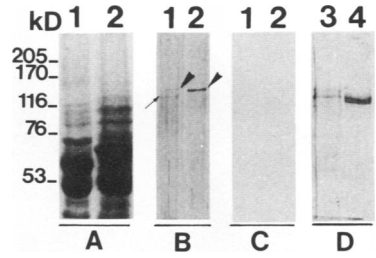


FIG. 1. Detection of ESAG 4 and GRESAG 4 proteins in trypanosome membrane extracts. Shown are the Coomassie staining patterns (A) and the immunodetection profiles (B and C) with the anti-ESAG 4 and anti-β-galactosidase antibodies, respectively (lane 1, bloodstream forms; lane 2, procyclic forms). The arrow and arrowheads point to presumptive ESAG 4 and GRESAG 4-related polypeptides, respectively. (D) Effect of tunicamycin on the apparent molecular weight of the specific polypeptides. Bloodstream forms were incubated at 37°C with 0.5 μg of tunicamycin per ml for 0 h (lane 3) or 7 h (lane 4).

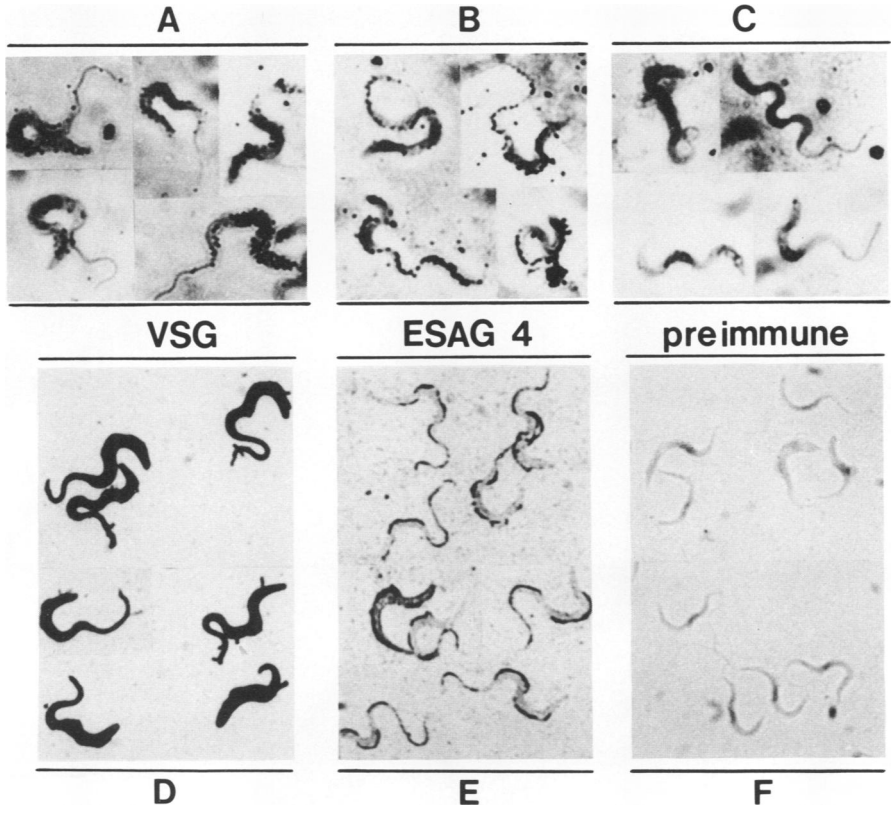


FIG. 2. Light microscope localization of ESAG 4-related polypeptides at the surface of trypanosome bloodstream forms. Live (A to C) or fixed (D to E) AnTat 1.1A trypanosomes were incubated with rabbit IgGs raised against the VSG, ESAG 4-specific polypeptides, or preimmune rabbit serum, as indicated. Antibodies bound to the cell surface were revealed by anti-rabbit IgGs labelled with 5-nm gold particles and silver enhancement. Virtually 100% of the cells reacted as in the examples shown, although in some cases the ESAG 4 antibodies did not label the whole length of the flagellum.

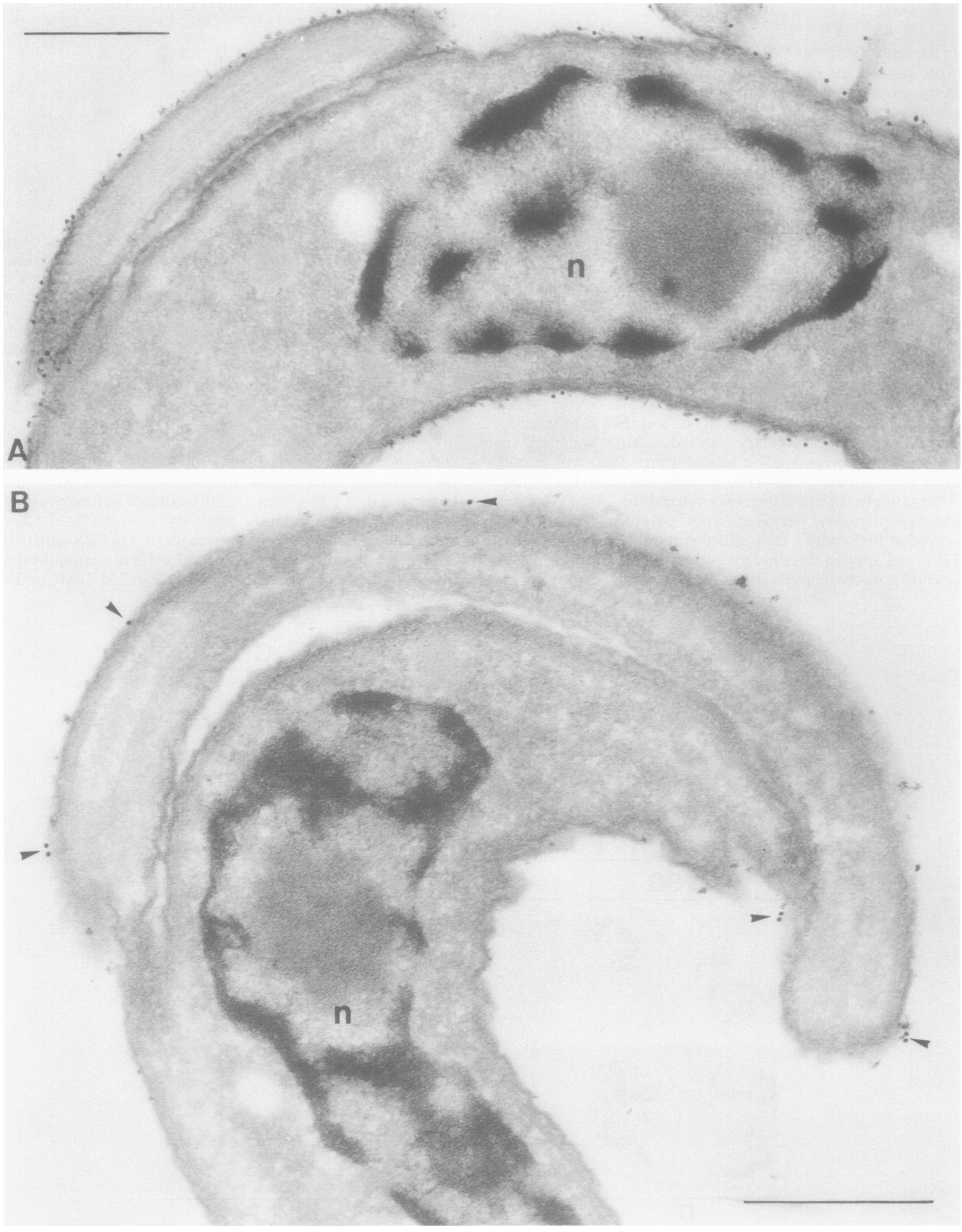
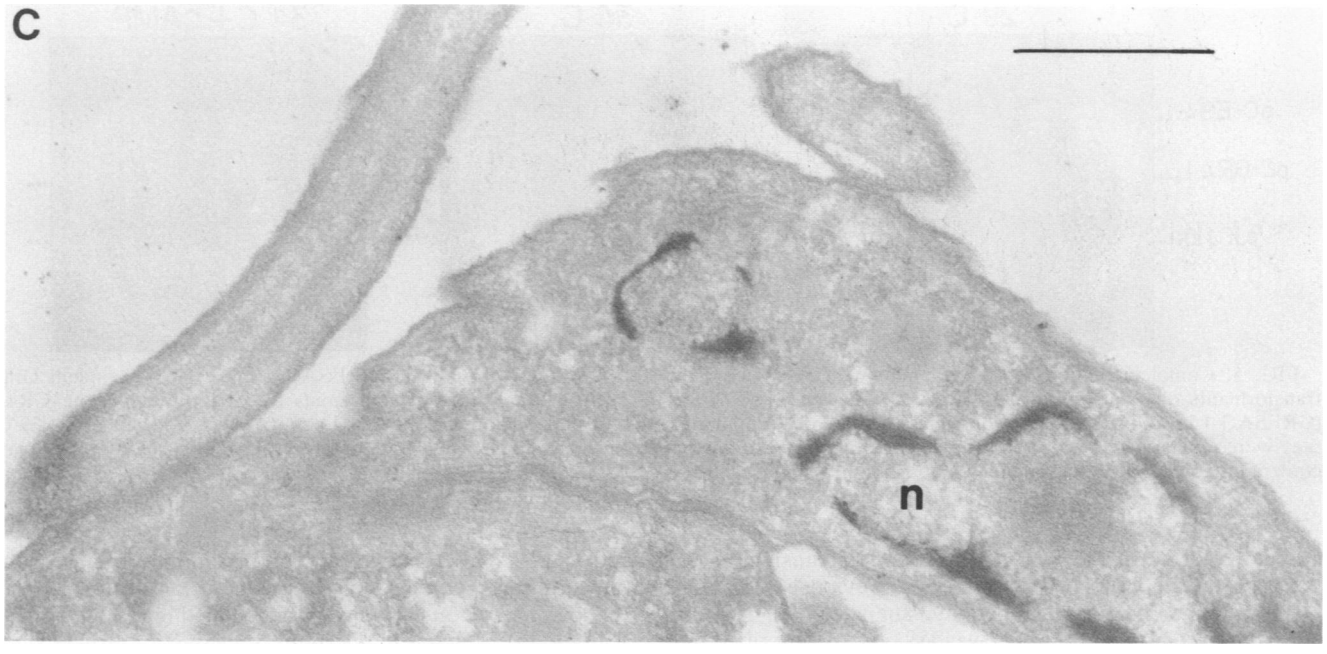


FIG. 3. Electron microscope localization of ESAG 4-related polypeptides at the surface of trypanosome bloodstream forms. Antibodies bound to fixed AnTat 1.1A bloodstream forms were revealed by 15-nm gold particles coated with anti-IgGs. (A) The whole cell surface, including the flagellum, is labelled with anti-AnTat 1.1A VSG antibody. (B) Only the flagellum surface appears to be labelled with the anti-ESAG 4 antibody (arrowheads). The labelling density shown is typical of that observed at the surface of all flagellar sections. (C) The labelling is absent when the primary antibody is omitted. n, nucleus; bars, 0.5 μm.



GAR G15 from Amersham; dilution of 1/40 or 1/50, respectively). For light microscopy, silver enhancement was carried out with the Amersham Intense kit according to the manufacturer's instructions. For electron microscopy, trypanosomes were fixed for 1 h in 2% OsO_4 in cacodylate buffer (pH 7.2), dehydrated in ethanol, and embedded in situ in Epon. Ultrathin sections made parallel to the cell layer were stained with uranyl acetate and lead citrate before examination in an AEI6B electron microscope at 60 kV.

Complementation of the *cyr1* mutant of *S. cerevisiae*. ESAG 4 was reconstituted from the separate 5′ and 3′ halves. Plasmid pEX-ES4 3′ was digested by *Eco*RI and ligated with an 8-kb *Eco*RI fragment from the genomic clone 1ES200.10, which contains the 5′ half of ESAG 4 and flanking region (21). The resulting recombinant, pEX-ES4tot, contains the complete ESAG 4 open reading frame except for the last 290 bp. To generate pC-ES4, a 3.3-kb *Mlu*I-*Bgl*II fragment was isolated from pEX-ES4tot, blunt ended, and cloned into the *Asp* 718 site of the *S. cerevisiae* plasmid pJCJ10. pJCJ10 is derived from pJCJ1 (12), which contains the arginase gene as well as part of the yeast 2 μm plasmid and *URA3* marker in pBR322. pJCJ10 was obtained by removal of the arginase gene between its transcription promoter and terminator, and insertion of an *Asp* 718-*Nco*I-*Bam*HI polylinker in its place. To obtain pC-GR4.1, a 4.1-kb *Eco*RI-*Hpa*I fragment of GRESAG 4.1 was isolated from a full-sized cDNA clone (2)

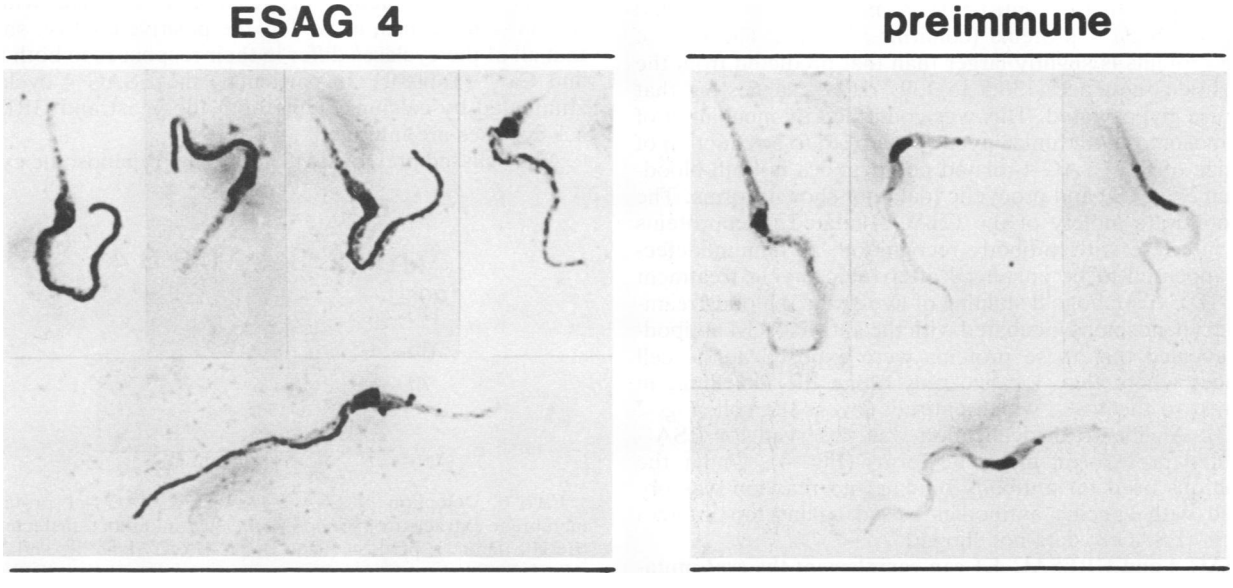


FIG. 4. Localization of ESAG 4-related polypeptides at the surface of trypanosome procyclic forms. Anti-ESAG 4 antibody bound to fixed procyclic forms was revealed by 5-nm gold particles coated with anti-IgG as described for Fig. 2.

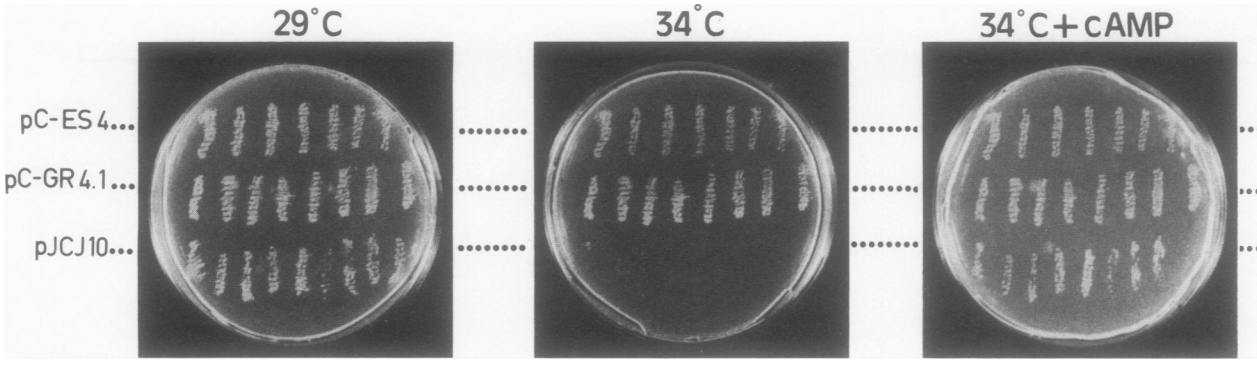


FIG. 5. Complementation of the *cyr1* mutant of *S. cerevisiae* by the *T. brucei* ESAG 4 and GRESAG 4.1 genes. Independent Ura$^+$ transformants obtained following T50-3A (*MATα leu2 his3 trp1 ura3 cyr1-2*) transfection with plasmids pC-ES4 (ESAG 4), pC-GR4.1 (GRESAG 4.1), and pJCJ10 (control) were streaked onto minimum supplemented medium lacking uracil. After 2 days of incubation at 29°C, they were replica plated onto the supplemented plates lacking uracil and incubated at 29 and 34°C for 3 days. As a control, a replica plate containing 1 mM cAMP was incubated at 34°C.

and cloned into pJCJ10 as described above. Transfection in yeast cells was performed as described previously (13).

Assays of adenylate cyclase activity. The adenylate cyclase activity of membrane fractions was assayed as described by Casperson et al. (5), with 50 to 100 μg of membrane proteins (17).

RESULTS

ESAG 4-related polypeptides in trypanosome membranes. ESAG 4 was cloned in pEX, an *E. coli* expression vector, and antibodies were raised in rabbits against the ESAG 4–β-galactosidase fusion protein. These antibodies recognized proteins with an apparent molecular size of 150 kDa in the membrane fraction of both bloodstream and procyclic forms of *T. brucei* and of about 140 kDa in bloodstream forms only (Fig. 1B). Nothing was detected in the membrane fraction by antibodies raised against the β-galactosidase moiety (Fig. 1C) or in the cytosolic fraction by antibodies directed against ESAG 4 (data not shown). As the ESAG 4 protein is likely to be absent from procyclic forms (2), it would appear that the anti-ESAG 4 antibodies cross-reacted with GRESAG 4 proteins (confirmed below). The size of these proteins is slightly larger than that predicted from the nucleotide sequence (136.5 to 139.7 kDa), suggesting that they are glycosylated. This was confirmed by incubation of trypanosomes with tunicamycin, which led to a reduction of the size of the ESAG 4-related polypeptides in both bloodstream (Fig. 1D) and procyclic (data not shown) forms. The carbohydrate moiety of the ESAG 4-related glycoproteins may interfere with antibody recognition, as immunodetection appeared to be enhanced after tunicamycin treatment (Fig. 1D). Immunogold staining of live or fixed bloodstream-form trypanosomes incubated with the anti-ESAG 4 antibodies revealed that these proteins were exposed at the cell surface, where they localize only along the flagellum, in contrast to the VSG, which entirely covers the cell (Fig. 2 and 3). An identical localization was observed for ESAG 4-related proteins in procyclic forms (Fig. 4). Under the conditions used for antibody binding, no reaction was observed with specific antibodies raised against an internal protein (ESAG 8; data not shown).

ESAG 4 and GRESAG 4.1 can complement the *cyr1* mutation of *S. cerevisiae*. To test whether the ESAG 4/GRESAG 4 gene family encodes adenylate cyclases, we cloned ESAG 4 and GRESAG 4.1 under the control of the arginase gene transcription promoter in a yeast expression vector, pJCJ10 (Fig. 5). These constructs were introduced into *S. cerevisiae* T50-3A (15), which is temperature sensitive for growth since it contains the *cyr1-2* allele encoding a thermolabile adenylate cyclase (20). The nonrecombinant plasmid pJCJ10 was used as a negative control, while plasmid YRp7-ADC1-CYR1, containing the *S. cerevisiae* adenylate cyclase gene controlled by the yeast alcohol dehydrogenase I promoter (35), was used as a positive control. In contrast to the negative control, the two constructs with trypanosome genes were able to rescue the mutation at the nonpermissive temperature (Fig. 5), as was the positive yeast control (35; data not shown). As expected, addition of cyclic AMP (cAMP) to the medium restored cell growth of the negative control under nonpermissive conditions (Fig. 5).

Characterization of the trypanosome adenylate cyclases expressed in *S. cerevisiae*. In accordance with the complementation data, the anti-ESAG 4 antibodies recognized specific polypeptides in the membrane fraction of the recombinant yeasts (Fig. 6). Assays of adenylate cyclase activity of the membrane fraction of yeast cells rescued with the trypanosome genes, as well as the positive control, showed that all of the cyclases differ in their responses to both Mn^{2+} and Ca^{2+} (Table 1). In particular, the ESAG 4 cyclase is stimulated by calcium, while both the yeast and GRESAG 4.1 cyclases are inhibited.

As discussed previously (23, 27), the trypanosome cyclase

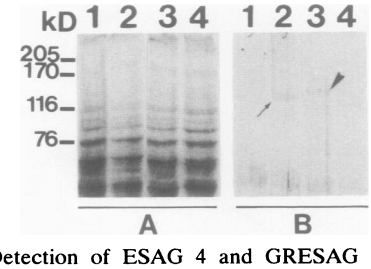


FIG. 6. Detection of ESAG 4 and GRESAG 4.1 proteins in membrane extracts from *S. cerevisiae cyr1* mutants transfected with pJCJ10 (lane 1), pC-ES4 (lane 2), pC-GR4.1 (lane 3), and YRp7-ADC1-CYR1 (lane 4). (A) Coomassie staining patterns; (B) immunodetection profile with the anti-ESAG 4 antibody. Symbols are as for Fig. 1.

TABLE 1. Adenylate cyclase activities of recombinant yeast membrane extracts[a]

Plasmid	Adenylate cyclase activity (U; mean ± SE)				Relative activity		
	Mn	Mg	Ca + Mg	Ras + Mg	Mn/Mg	Ca + Mg/Mg	Ras + Mg/Mg
pJCJ10	<0.1	ND	ND	ND			
YRp7-ADC1-CYR1	32.5 ± 4.2	4.9 ± 0.9	3.3 ± 0.9	9.7 ± 0.7	6.6	0.7	2.0
pC-ES4	46.1 ± 2.6	11.2 ± 0.9	25.2 ± 1.2	11.6 ± 1.0	4.1	2.3	1.0
pC-GR4.1	84.4 ± 4.5	6.7 ± 1.9	6.0 ± 1.0	ND	12.6	0.9	ND

[a] Units of activity are defined as picomoles of cAMP synthesized per minute per milligram of protein. Values are for 6 to 10 independent determinations. ND, not determined. The cation concentrations (2.5 mM $MnCl_2$ or 5 mM $MgCl_2$, with or without 0.7 mM $CaCl_2$) were present in the optimal range for the different cyclases tested (data not shown). The experiments involving Ras were conducted in the presence of GppNp according to Kataoka et al. (15), with 8 μg of p21 Ras from Harvey sarcoma virus and 5 mM $MgCl_2$.

genes appear to lack the region which, in *S. cerevisiae*, is responsible for interaction with Ras. To determine whether the ESAG 4 and GRESAG 4.1 activities can be regulated in yeast cells, the complemented yeast cells were subjected to nitrogen starvation and heat shock. Under starvation conditions or in stationary phase, yeast cells appear to enter a G_0 state similar to the one described for mammalian cells. In this state, the cells are more refractory to heat shock and persist in a quiescent form for extended periods without significant loss of viability. This is achieved through regulation of cAMP levels (for a review, see reference 4). As shown in Table 2, the ESAG 4- and GRESAG 4.1-complemented yeast cells, in contrast to the controls, did not survive heat shock or prolonged starvation, suggesting that the trypanosome cyclases are not properly regulated. However, similar results were obtained following overexpression of the yeast adenylate cyclase gene cloned in plasmid YRp7-ADC1-CYR1 (Table 2). Although not directly comparable with the data obtained on the trypanosome genes (the transcription promoters and the replication origins of the expression plasmids are different), these latter results suggest that the phenotype of the ESAG 4- and GRESAG 4.1-complemented yeast cells may be due to overexpression of the cyclase. To differentiate between these possibilities, the adenylate cyclase activity of membranes from complemented yeast cells was directly tested for stimulation by Ras in vitro. As shown in Table 1, this activity was not stimulated by incubation in the presence of the p21 Ras protein from murine Harvey sarcoma virus, whereas the yeast enzyme did appear to be stimulated.

TABLE 2. Viability of different *S. cerevisiae* strains under nitrogen starvation and heat shock[a]

Strain	% Survival			
	30-min heat shock[b]			Stationary phase, 136 h[c]
	16 h	40 h	64 h	
Σ1278b (wild type)	0.1	98	98	94
T50-3A(pJCJ10)	0.2	96	95	86
T50-3A(pC-ES4)	0.5	0.3	0.1	0.01
T50-3A(pC-GR4.1)	0.3	0.6	0.2	0.02
T50-3A(YRp7-ADC1-CYR1)	0.2	0.5	0.4	0.1

[a] The five strains indicated were inoculated from fresh cultures into YNB medium (13) poor in nitrogen [1 mM $(NH_4)_2SO_4$], supplemented with the required auxotrophic supplements without uracil or tryptophan, inoculated at 5×10^5 cells per ml, and grown at 29°C.

[b] After 16 h (log phase), 40 h (early stationary phase), and 64 h, aliquots of the cultures were subjected to a 30-min heat shock at 50°C and briefly sonicated. Aliquots were plated on rich medium (YPD [13]), and the number of viable cells was determined relative to the number in an untreated sample.

[c] After 136 h, cell viability (without heat shock) was determined relative to the number of viable cells after 40 h.

DISCUSSION

By complementation of a yeast mutant, we show that at least two distinct adenylate cyclase genes are present in *T. brucei*. These genes, ESAG 4 and GRESAG 4.1, differ primarily in a region believed to encode a large extracellular domain. As ESAG 4 belongs to the VSG gene transcription unit and is transcribed only in the bloodstream form (2), the ESAG 4-encoded cyclase activity must be restricted to that stage. Interestingly, a calcium-activated adenylate cyclase has been found to be restricted to the bloodstream form (24, 31, 32), and the ESAG 4 cyclase is stimulated by calcium in the membranes of recombinant yeast cells. This finding strongly suggests that ESAG 4 encodes the bloodstream-specific calcium-activated adenylate cyclase.

The trypanosome cyclases do not seem to be properly regulated in yeast cells. This observation is in accordance with the fact that the trypanosome cyclase genes differ from that of *S. cerevisiae* except for the region encoding the catalytic domain of the enzyme (2, 21). In particular, the trypanosome cyclases are devoid of the leucine-rich domain known to be involved in activation by Ras in yeast cells (23). It is interesting to note that a gene encoding such a leucine-rich domain (ESAG 8) is located close to ESAG 4 in the VSG gene expression site (23, 27). This finding raises the possibility that a Ras-interacting domain, able to activate the ESAG 4 cyclase, is encoded by a gene distinct from, but coordinately expressed with, ESAG 4 (23, 27).

The ESAG/GRESAG 4 cyclases are localized along the surface of the flagellum. This observation is in accordance with previous results (19, 34) indicating that the adenylate cyclase activity of *T. brucei* was restricted to a subfraction of the plasma membrane. Together with other observations on the presence of low-density lipoprotein (6) and transferrin (26) receptors in the flagellar pocket and on the flagellum of *T. brucei* bloodstream forms, this fact suggests that, in addition to its role in cell motility, the flagellum may also function as a specialized sensory structure. How such receptors escape recognition by the immune system of the host is not clear. However, since different treatments leading to the release of the VSG also provoke a stimulation of adenylate cyclase activity (25, 30), it is possible that the cyclases of bloodstream forms are shielded by the VSG coat.

As GRESAG 4.1 is transcribed in both bloodstream and procyclic forms, it can be assumed that the corresponding cyclase is present in both forms. Since at least two other genes from the same family are also transcribed in both forms (GRESAG 4.2 and 4.3 [2]), *T. brucei* may contain at

least three to four different adenylate cyclases, depending on the stage in the parasite life cycle. The presence of several adenylate cyclases in a protozoan raises the question of their respective functions, as *S. cerevisiae*, another unicellular organism, appears to contain only a single type of adenylate cyclase (15). On the basis of analogy with the different membrane forms of guanylate cyclase in higher eucaryotes (9), it may be hypothesized that the trypanosome cyclases differ in their responses to specific ligands which interact with the variable extracellular domain. This complexity may be tentatively linked to the variety of environmental conditions to which the parasite is exposed during its life cycle. Although the role of adenylate cyclase in trypanosomatids has not been precisely defined, it appears to be linked to different stages of transformation and proliferation (10, 18, 22, 29, 33). The ESAG 4 cyclase in particular could be involved in one of these steps. The topological relationship between the ESAG 4 and VSG genes may reflect a functional interaction between the two proteins. A correlation has indeed been found between VSG release and adenylate cyclase stimulation: either the release of the entire VSG by mild acidic treatment of the cells or removal of the N-terminal domain of the VSG by trypsin leads to a strong activation of adenylate cyclase (25). This activation is specific to the bloodstream form, since the same treatments do not activate adenylate cyclase in procyclic forms (25). Moreover, a transient activation of adenylate cyclase has been observed paralleling VSG release in the early phase of in vitro differentiation from bloodstream to procyclic forms (25). According to the apparent linkage between VSG release and bloodstream-specific cyclase activation, one may speculate that the ESAG 4 cyclase is inactivated by the VSG and that the triggering of its activity following VSG release would induce a cellular commitment to differentiation into procyclic forms. A similar role, but for other stages of differentiation, could be envisaged for the other cyclases.

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