Survival of *Trypanosoma brucei* in the Tsetse Fly Is Enhanced by the Expression of Specific Forms of Procyclin

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Abstract. African trypanosomes are not passively transmitted, but they undergo several rounds of differentiation and proliferation within their intermediate host, the tsetse fly. At each stage, the survival and successful replication of the parasites improve their chances of continuing the life cycle, but little is known about specific molecules that contribute to these processes. Procyclins are the major surface glycoproteins of the insect forms of *Trypanosoma brucei.* Six genes encode proteins with extensive glutamic acid–proline dipeptide repeats (EP in the single-letter amino acid code), and two genes encode proteins with an internal pentapeptide repeat (GPEET). To study the function of procyclins, we have generated mutants that have no EP genes and only one copy of GPEET. This last gene could not be replaced by EP procyclins, and could only be deleted once a second GPEET copy was introduced into another locus. The EP knockouts are morphologically indistinguishable from the parental strain, but their ability to establish a heavy infection in the insect midgut is severely compromised; this phenotype can be reversed by the reintroduction of a single, highly expressed EP gene. These results suggest that the two types of procyclin have different roles, and that the EP form, while not required in culture, is important for survival in the fly.

Two tropical diseases, human sleeping sickness and nagana in domestic animals, are caused by the protozoon *Trypanosoma brucei*, which is transmitted by tsetse flies. The spread of the parasite is strictly dependent on the insect vector, and consequently, these diseases are restricted to sub-Saharan Africa between the latitudes 14°N and 29°S. When trypanosomes are taken up by the insect during a blood meal from an infected animal, it is by no means certain that their progeny will complete the cycle that allows transmission to a new host. Bloodstream forms lose infectivity for the mammalian host within 24 h in the fly midgut (6), while new transmissable parasites only appear in the salivary glands after a lag of 3 wk or more, and then only in a few percent of infected flies (48). There are several hurdles to be overcome before further transmission can take place. The first prerequisite for successful transmission is that bloodstream forms must differentiate into procyclic forms in the midgut, become established, and proliferate. The majority of infections do not proceed beyond this stage, yet for the cycle to be completed, the parasites have to migrate to the fly salivary glands, where they differentiate further into epimastigote forms and subsequently into mature metacyclic forms that are capable of initiating a fresh infection when they are transmitted to a new mammalian host. A number of parameters may influence the efficiency of parasite transmission. The strain of trypanosome, the species of tsetse fly, the sex of the fly, and the presence of rickettsiae-like organisms in the midgut cells have all been implicated (reviewed in 28). In addition, two types of activity have been identified in tsetse flies: one is trypanocidal and kills procyclic forms in the gut, while the second stimulates parasite maturation in the mouthparts. Specific sugars such as glucosamine (27), or lectins such as Con A or WGA (28), can modulate either the establishment of infections by procyclic forms or the production of mature salivary gland forms, leading to the proposal that the tsetse fly factors are themselves lectins.

As trypanosomes cycle between mammals and the tsetse fly, they alternately express two types of surface coats. Bloodstream forms are covered by a dense layer of variant surface glycoproteins (VSG)[1] that shields underlying membrane proteins and prevents lysis of the parasites by serum components (for reviews see 12 and 33). The antigenic variation of bloodstream forms and their consequent evasion of the host immune response are caused by the

1. *Abbreviations used in this paper*: UTR, untranslated region; VSG, variant surface glycoproteins.

consecutive expression of potentially as many as 1,000 different VSG genes. When bloodstream form trypanosomes differentiate to procyclic forms, however, the parasite surface is completely remodeled. VSG synthesis is repressed (32), and the coat is shed as it is progressively replaced by a new, invariant coat composed of procyclins (37, 50). Procyclins are also known as procyclic acidic repetitive proteins (29). They are also detected on epimastigote forms of the parasite (34), but in turn are lost when metacyclic forms activate the expression of a specific subset of VSG genes in preparation for transfer to the mammalian host.

In marked contrast to the VSG genes, there is only a small number of procyclin genes. These have been mapped in detail, and representative members of each type have been sequenced (for reviews see 10, 17, 38). The procyclin promoters (5, 43) and mRNA processing signals (20, 21, 41, 47), which differ from those of higher eukaryotes, have also been characterized. Depending on the strain of *T. brucei*, there are six or seven genes that encode proteins with extensive glutamic acid–proline repeats (EP forms; 13, 24, 29, 36, 39). Two further genes, which are indistinguishable from each other, encode proteins containing several pentapeptide repeats followed by three dipeptide repeats (GPEET forms; 31). There are three EP forms of procyclin that are closely related to each other over their entire coding regions, differing principally in the length of the dipeptide repeats and the presence or absence of an N-linked glycosylation site (Fig. 1). The precursor of the GPEET form has the same highly conserved signal peptide and hydrophobic COOH-terminal peptides as the EP forms, but apart from these, there are only two small stretches of identity between the mature proteins. EP procyclins have been isolated from different strains of *T. brucei* (8, 14, 35), and it has been estimated that there are ~6 million molecules per cell. A battery of mAbs have been mapped to defined epitopes, including the dipeptide repeat itself (35). No antibodies specific for GPEET were available, however, and although the mRNA could be detected (31), it was not certain whether it was translated, since the protein could not be detected using procedures that were designed to purify procyclins on the basis of their negative charge at low pH (8) or the presence of a glycolipid anchor (14).

Although a decade has elapsed since the production of the first mAbs against procyclins (34) and the cloning of the genes (29, 36), their function has remained unresolved. By analogy with the VSG coat of bloodstream forms, procyclins might protect the parasites from the insect immune response or from lytic enzymes. The fact that procyclins are also largely resistant to several proteases (14) would confer obvious advantages in the digestive tract of the fly. In support of this hypothesis, *T. congolense*, another species of trypanosome that is transmitted by the tsetse, expresses an unrelated set of procyclins known as GARPs (for glutamic acid/alanine–rich proteins; 1, 2), which are also protease resistant. It has also been proposed that different domains of procyclins might be the targets for the tsetse factor(s) that might bind to either the N-linked carbohydrate moieties or to sugar residues in the glycolipid anchor (28).

With the advent of stable transfection systems for trypanosomes and an expanding repertoire of selectable mark-

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          1                                      ⇓
EP 1    MAPRSLYLLAVLLFSANLFAGVGFAAAAEGPEDKGLTKGGKGKGEKGTKV
EP 2    MAPRSLYLLAVLLFSANLFAGVGFAAAAEGPEDKGLTKGGKGKGEKeTKV
EP 3    MAPRSLYLLAVLLFSANLFAGVGFAAAAEGPEDKGLTKGGKGKGgGTKV
GPEET   MAPRSLYLLAiLLFSANLFAGVGFAAAAdesasnnivKGGKGK.......

          51     *
EP 1    gaddTNGTDPDPEPEPEPEPEPEPEPEPEPEPEPEPEPEPEPEPEPEPEPEP
EP 2    qdev........EPEPEPEPEPEPEPEPEPEPEPEPEPEPEPEPEPEPEPEP
EP 3    sdddTNGTDPDPEPEPEPEPEPEPEPEPEPEPEPEPEPEPEPEPEPEPEPEP
GPEET   eredgpeepeetgpeetgpeetgpeetgpeetgpeetgpeet........

          101                                   ⇓
EP 1    EPEPEPEPEPEPEPEPEPEPEPGAATLKSVALPFAIAAAALVAAF
EP 2    EPEPEP........EPEPEPGAATLKSVALPFAIAAVGLVAAF
EP 3    ..............EPEPEPGAATLKSVALPFAIAAVGLVAAF
GPEET   ..............EPEPEPGAATLKSVALPFAvAAAALVAAF
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Figure 1. Alignment of EP and GPEET procyclin precursor sequences obtained from the following sources: EP1 (29), EP2 (39), EP3 (36), and GPEET (31). N-linked glycosylation sites in EP1 and EP3 are marked by an asterisk. Processing sites are marked by arrows: the NH$_2$-terminal cleavage site of an EP form was determined by direct protein sequencing (35). The site of GPI anchor addition was deduced from amino acid composition analysis of purified procyclins (8). Underlined capital letters denote amino acids conserved in all four polypeptides, and lowercase letters denote amino acids that diverge from the consensus sequence.

ers, it has now become feasible to study the function of procyclins by creating deletion mutants by homologous recombination. Using this approach, we have been able to show that the EP and GPEET forms of procyclin are functionally distinct, and that the EP form, while not essential in culture, plays an important role in the fly.

Materials and Methods

Culture and Stable Transformation of Trypanosomes

T. brucei 427 (11) and all derivatives were cultured at 27°C in SDM-79 containing 5% heat-inactivated FBS (7). Transfections were performed as described (46), using 5 µg plasmid digested with the appropriate restriction enzymes to release the insert. G418 (46), phleomycin (22), and hygromycin (26) have been used previously to select stable transformants of *T. brucei*. Nourseothricin (obtained from Prof. U. Gräfe, Institut für Naturstoff-Forschung, Jena, Germany) and puromycin (Sigma, Buchs, Switzerland) were used for the first time in this study. The initial selection of nourseothricin-resistant cells was carried out with 150 µg · ml^{-1} increasing to a final concentration of 500 µg · ml^{-1}. Puromycin was used at an initial concentration of 1 µg · ml^{-1} increasing to a final concentration of 5 µg · ml^{-1}. No detectable cross-resistance to these five antibiotics was observed. Trypanosome clones were obtained by limiting dilution in SDM-79 supplemented with 20% FBS.

Construction of Recombinant Plasmids

Four constructs (pKON, pKOP, pKOH, and pKOS) were designed to delete tandemly linked procyclin genes. Each construct consists of four modules: a pBluescript backbone, locus-specific 5′ flanking sequences together with the procyclin promoter and 5′ untranslated region (UTR) of the procyclin α gene, an antibiotic-resistance gene, and 3′ flanking sequences. The four constructs are shown schematically in Fig. 2 *A*.

Pro A/B locus–specific 5′ flanking sequences, promoter, and 5′ UTR: a 900-bp fragment flanked by the KpnI and HindIII sites was subcloned from the plasmid pGARP-neo (18). *ProC* locus–specific 5′ flanking sequences, including the procyclin promoter and 5′ UTR were amplified by PCR from the plasmid pCP1 (24) using the primers Pro C and PCH. All constructs contained the same 3′ flanking sequences consisting of the last 19 bp of the procyclin β gene, the intergenic region, and the first 426 bp of PAG 1 (25). The appropriate fragment was amplified from the plasmid pAP4 (24) using the primers KO1 and KO2. All antibiotic-resistance

genes were cloned as HindIII/BamHI cassettes. The neomycin-resistance gene (Neo) was amplified from pSV2neo (45) with the primers Neo1 and Neo2. The phleomycin-resistance gene (Phleo) was derived in two steps from the plasmid pHD63 (kindly provided by Christine Clayton, Zentrum für Molekulare Biologie, Heidelberg, Germany). The plasmid was first linearized with NcoI and treated with Klenow to generate blunt ends. The coding region was excised with StuI, cloned into the EcoRV site of pBluescript, and subsequently transferred using the HindIII and BamHI sites from the polylinker. The hygromycin-resistance gene (Hyg) was subcloned from the plasmid pBS HYG A (16). The plasmid was digested with XbaI, and the ends were repaired with Klenow. After digestion with BamHI, the coding region was inserted between the EcoRV and BamHI sites of pBS, and then subcloned as a HindIII/BamHI fragment to give the plasmid pKOH. The streptothricin acetyltransferase gene (SAT-1), which confers resistance to nouseothricin, was amplified from the plasmid pLEX SAT (23) using the primers SAT-1H and SAT-1B, and was cloned via the synthetic HindIII and BamHI sites. Replacement of Hyg in pKOH with SAT-1 gave rise to pKOS; the substitution of Neo for Hyg produced the *Pro C* locus–specific construct pKOCN.

The plasmid pKOSα, which was designed to replace the first procyclin gene in the *Pro C* locus, was constructed by replacing the 3′ flanking sequence of pKOS with a BamHI/XbaI fragment extending from nucleotide 165 in the 3′ UTR of the procyclin α gene (Δ164) to a PvuII site in the intergenic region (18, 41).

Reexpression of procyclin genes was achieved by using a bicistronic cassette derived from the *Pro A* locus. The construction of the original plasmid pGAPRONE will be described in detail elsewhere (16a). The plasmid pEPΔ164-PUR is shown schematically in Fig. 5. EP1, EP2, and GPEET forms of procyclin were amplified from the plasmids pAP2, pAP4, and pCP1, respectively (24), using the universal procyclin primers ABC-H and ABC-B, and cloned as HindIII/BamHI fragments. The puromycin resistance gene was derived from pVN3.1 (16). The coding region was excised with NotI and NcoI, and the ends were repaired by treatment with Klenow. The fragment was subsequently cloned between the EcoRV and SmaI sites in pBS(KS⁺). A clone containing the insert in the correct orientation was then used to remodel the 5′ HindIII site into an NheI site and the 3′ BamHI site into a ClaI site. In both cases, this was achieved by cleavage with the appropriate enzyme, treatment with Klenow, and religation.

Primers: Pro C:CTGTCGACTTGCCGCGTAAC
         PCH:GT<u>AAGCTT</u>GTGAATTTTACTT
         KO1:TA<u>GGATCC</u>ATTCGTATGGTTTTGTC
         KO2:TA<u>TCTAGA</u>GGGCAGTGCAGT
         Neo1:CG<u>CAAGCTT</u>ATGATTGAACAAGATGGA
         Neo2:TAA<u>GGATCCT</u>CAGAAGAACTCGTC
         SAT-1H:GC<u>AAGCTT</u>ATGAAGATTTCG
         SAT-1B:AT<u>GGATCCT</u>TAGGCGTCATC
         ABC-H:TA<u>AAGCTT</u>ATGGCACCTCGTT
         ABC-B:CC<u>GGGATCC</u>GCTTAGAATG

The synthetic restriction sites used in cloning are underlined.

Isolation of DNA and RNA; Southern and Northern Blot Analyses

DNA and RNA were isolated as described (37, 49). Northern and Southern blot analyses were performed using standard procedures. Probes corresponding precisely to the EP and GPEET coding regions were amplified as described for the constructs pEPΔ164-PUR and pGPEETΔ164-PUR. A longer probe, EP*, including the entire 5′ UTR and 91 bp of the 3′ UTR, was amplified from pAP2 using the primers SPU (GCT-CACGCGCCTTCGAGTT) and TO4 (24). A tubulin genomic clone, 3B, contains tandemly linked copies of α- and β-tubulin (42). Signals were quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Antibodies and Western Blots

The procyclin-specific mAb TBRP1/247 was generously provided by Terry W. Pearson (University of Victoria, Victoria, Canada). This mAb has previously been shown to recognize EP dipeptide repeats (35). Polyclonal anti-GPEET antibodies (K1) were raised in rabbits using a synthetic peptide, (GPEET)₃C, coupled to KLH (Affiniti Research Products Limited, Nottingham, UK). Western blots were performed as described previously (19), using K1 antiserum at a dilution of 1:1,000.

Double-labeling Immunofluorescence

Parasites were washed twice with PBS and were fixed in suspension with PBS containing 3% paraformaldehyde and 0.05% glutaraldehyde for 15 min at room temperature. During fixation, they were allowed to settle down onto poly-lysine–coated (100 μg/ml) glass coverslips. The coverslips were subsequently incubated in blocking buffer (PBS/0.5%BSA/50 mM lysine) for 1 h. Antibody incubations were performed in blocking buffer in the following order: (*a*) anti-EP mAb 247 (1:200); (*b*) goat anti–mouse FITC (1:100; Cappel Laboratories, Cochranville, PA); (*c*) polyclonal rabbit anti-GPEET (1:200); and (*d*) goat anti–rabbit Texas red (1:100; Becton Dickinson Immunocytometry Systems, Mountain View, CA). Antibodies were applied for 40 min at 24°C in a humid chamber. After labeling, coverslips were washed extensively 6 times for 5 min each in PBS. They were then mounted onto glass slides using a mixture of gelvatol/glycerol and viewed using a Laborlux fluorescence microscope (E. Leitz, Inc., Rockleigh, NJ).

Transmission Electron Microscopy

Trypanosomes were prefixed by the addition of 3% paraformaldehyde to the medium, and were washed three times in 100 mM sodium phosphate buffer, pH 7.2, containing 3% paraformaldehyde at 4°C. Cells were then resuspended in 2% glutaraldehyde in 0.1 M cacodylate buffer (Fluka Chemie AG, Buchs, Switzerland), pH 7.3, for 4 h at room temperature. After washing in cacodylate buffer, they were treated with 2% osmium tetroxide in veronal acetate buffer, pH 7.4, for 1 h at 4°C, followed by buffer rinses. They were then treated with 0.25% tannic acid (Mallinkrodt, St. Louis, MO) in 0.05 M cacodylate buffer for 30 min, followed by washing with 1% Na_2SO_4 in 0.1 M cacodylate for 10 min, and were then incubated in 1% uranyl acetate in veronal acetate buffer for 1 h at room temperature (44). The parasites were dehydrated through a graded series of ethanol (70–95–100%) and embedded in Epon 812 resin (Fluka Chemie). After polymerizing the resin at 65°C for 48 h, ultrathin sections were cut with a diamond knife using an ultramicrotome (Reichert Jung, Austria, Vienna) and the grids were stained with lead citrate and uranyl acetate. All preparations were observed using a transmission electron microscope (model 600; Philips Technologies, Cheshire, CT) operating at 60 kV.

Infection of Tsetse Flies and Determination of Midgut Infection Rates

Pupae of *Glossina moritans centralis* were obtained from the tsetse unit of the International Livestock Research Institute (Nairobi, Kenya). The pupae were kept at 27°C until emergence. Teneral flies were collected over a period of 4 d before they were offered a first blood meal by membrane feeding. The meal consisted of washed horse RBCs in SDM-79 culture medium (7) and procyclic forms of *T. brucei* 427 or cloned derivatives. The infectious meal was prepared in the following way: defibrinated horse blood (TCS Biologicals, Buckingham, UK) was centrifuged at 800 *g* for 15 min, and the pelleted RBCs were washed three times in an equal volume of serum-free SDM-79. Procyclic trypanosomes were grown for one passage in medium without the antibiotics used for selection. They were pelleted by centrifugation (800 *g* for 10 min) and suspended in SDM-79 containing 20% heat-inactivated FBS at a density of 5×10^6 ml^{-1}. Teneral flies were infected by artificial feeding on a silicone membrane on two consecutive days (days 0 and 1). The blood, membrane, and all other materials used for feeding were sterile. Flies that did not take a blood meal on at least one occasion were excluded from the experiment. The flies were subsequently fed on horse blood three times per week. On days 12–14, the flies were killed with ether, and the midguts were removed and examined for the presence of trypanosomes. Infections were scored as qualitatively as light, intermediate, or heavy. Weak was defined as a midgut that revealed ~1 trypanosome per field in 20 fields, and where none of the fields contained more than 5 trypanosomes. An infection was scored as heavy if the average per field was 100–200 parasites, and if the field with the highest trypanosome density contained >300 parasites. An infection was scored as intermediate if it could not be placed in either the weak or the heavy group. An exact determination of the number of trypanosomes contained in each midgut would have been much too time consuming and not feasible for the several hundred flies used in each experiment. Infection rates were calculated taking the surviving flies as 100%. Means and standard deviations were calculated for each group for heavy infections and total infections.
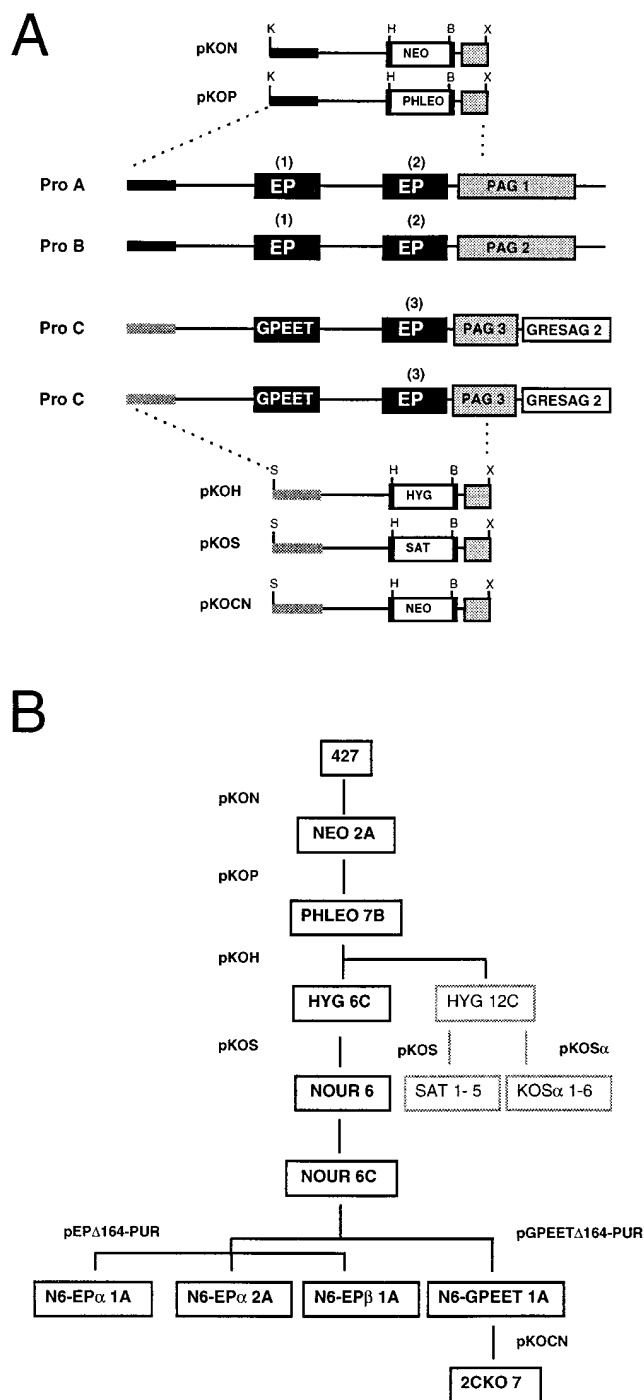
Results

Deletion of Tandemly Linked Procyclin Genes by Homologous Recombination

There are four procyclin expression sites in *T. brucei* 427: *Pro A*, *Pro B*, and two copies of *Pro C* (Fig. 2 *A*). Four constructs were designed in such a way that a pair of procyclin genes would be deleted simultaneously and replaced by a selectable marker. Fig. 2 *B* shows the pedigree of clones that were generated by sequential transformation with plasmids conferring resistance to neomycin or G418 (pKON), phleomycin (pKOP), hygromycin (pKOH), and nourseothricin or streptothricin (pKOS). Deletion mutants were named according to their newly acquired antibiotic resistance, followed by the clone number and a letter denoting the procyclin locus that had been replaced (e.g., Phleo 2B). A minimum of three independent clones was analyzed after each transformation.

Theoretically, the two constructs pKON and pKOP were capable of integrating into either the *Pro A* or the *Pro B* locus, but the three clones analyzed after transformation with pKON had all deleted the procyclin genes from *Pro A* (see clone Neo 2A in Fig. 3 *A*). Northern blot analysis also indicated that most of the transcripts most likely originate from this locus, since removing two genes was sufficient to reduce the steady-state levels of procyclin mRNA to 31% of the wild type (Fig. 3 *B*). Neo 2A trypanosomes were then transfected with the plasmid pKOP and cultured with both G418 and phleomycin to select transformants with deletions in the *Pro B* locus and to eliminate transformants in which the phleomycin-resistance gene had merely replaced the neomycin-resistance gene in the *Pro A* locus. After demonstrating that the procyclin genes had been deleted from the *Pro B* locus (Fig. 3 *A*, *Phleo 7B*), resulting in a further reduction in mRNA to 12% of the control (Fig. 3 *B*), this clone was transfected with the *Pro C*–specific construct pKOH. One of the hygromycin-resistant clones, Hyg 6C, was in turn transfected with pKOS to delete the last two procyclin genes from the second *Pro C* locus. The final set of clones that was obtained (Fig. 2 *B*, *Nour 1-6*) was selected in the presence of all four antibiotics.

Figure 2. (*A*) Schematic depiction of the four procyclin loci in *T. brucei* strain 427 together with the constructs used to knock out paired procyclin α and β genes by homologous recombination. The numbers in brackets above the EP procyclin genes refer to the polypeptides in Fig. 1. In each case, the procyclin genes are at the start of a polycistronic transcription unit that contains at least one additional gene (3, 4, 25). *PAG*, procyclin-associated gene; *GRESAG*, gene related to expression site associated gene 2 (ESAG 2). Homologous recombination was targeted by locus-specific sequences upstream of the promoters. Integration downstream of the procyclin genes occurred via a common sequence within the 5′ UTR of all three PAGs (25) without affecting the open reading frames. Before electroporation, the plasmids pKON and pKOP were digested with KpnI (*K*) and Xba I (*X*); pKOH, pKOS, and pKOSα (see Fig. 3 *C*) were digested with SalI (*S*) and XbaI. Additional sites: HindIII (*H*) and BamHI (*B*). The black and grey bars depict locus-specific sequences upstream of the promoters. At least 4 kb upstream of the transcription start site is conserved between the *Pro A* and *B* loci. The two copies of the *Pro C* locus have 640 bp in common with the other two loci, including the promoter, but have unrelated sequences further upstream (9, 40). (*B*) Lineage of trypanosome clones obtained from *T. brucei 427*. Deletion mutants are named after the antibiotic used for selection, followed by a specific clone number and a letter denoting the locus where integration occurred. The plasmids pEPΔ164-PUR and pGPEET Δ164-PUR are described in the Materials and Methods, and the former is shown schematically in Fig. 5. Clones beginning with the designation N6-EP are derivatives of Nour 6C in which a single copy of an EP procyclin gene has been reintroduced into the *Pro A* locus. N6-GPEET cells have one endogenous copy of GPEET in the *Pro C* locus and a second copy in the *Pro A* locus (see text and Fig. 6).

Retention of One Copy of a GPEET Procyclin Gene

Southern blot analysis of the nourseothricin-resistant clone Nour 6 revealed a fragment of ~12 kb that still hybridized with a procyclin probe, albeit extremely weakly, under stringent conditions (data not shown). To exclude that we were dealing with a mixed population in which a minority of cells had acquired resistance but had somehow retained the last procyclin locus, Nour 6 cells were again cloned by limiting dilution. Three daughter clones were examined; all three showed the same pattern of hybridization as the parental clone. More significantly, when RNA was isolated from these cells, procyclin transcripts could clearly be detected at 5–6% of the wild-type level (see Nour 6C in Fig. 3 *B*), which was comparable to the level in Hyg 6C cells, which still contain two procyclin genes. By using a combination of Southern blot analysis and PCR, it was established that Nour 6C trypanosomes had retained the first gene in the *Pro C* locus, which encodes the GPEET form of procyclin, and that recombination most probably occurred via a conserved stretch of 70 bp that spans the splice acceptor site and 5′ UTR of all procyclin genes (Fig. 3 *C*). To confirm these results, pKOS was used to transfect a second hygromycin-resistant clone, Hyg 12C (Fig. 2 *B*). Once again, the resulting clones (SAT 1-5) had retained the same gene (data not shown).

The fact that the last procyclin gene could not be deleted would suggest that trypanosomes need at least one of the eight genes to survive in culture, but is the type of procyclin important? To answer this question, hygromycin-resistant trypanosomes were transfected with the plasmid pKOSα, which was designed to eliminate the GPEET gene while leaving the EP gene intact (Fig. 3 *C*). Several nourseothricin-resistant clones were analyzed (Fig. 2 *B*, *KOSα1-6*), but in all cases, they showed aberrant integration of the construct and still expressed GPEET (see below). These results indicate that the two types of procyclin are not equivalent: the EP form is dispensable when trypanosomes are maintained in culture, while at least one GPEET gene seems to be required.

Coexpression of EP and GPEET Procyclins

GPEET procyclins have not been localized previously since no antibodies were available. To study whether GPEET was also expressed on the surface of procyclic forms, we first generated specific antibodies by immunizing rabbits with a synthetic peptide (see Materials and Methods). A well-characterized mAb, TBRP1/247, reacts with the dipeptide repeat of EP procyclins (35). When trypanosomes were labeled simultaneously with anti-GPEET and anti-EP antibodies, it could be demonstrated that all wild-type cells coexpressed both forms of procyclin on their surfaces (Fig. 4 *A*). In contrast, only the GPEET form was detectable on Nour 6C cells. Despite the fact that the deletion mutants no longer expressed EP, they were morphologically indistinguishable from the wild-type cells. To examine these trypanosomes in more detail, transmission electron microscopy was performed on ultrathin sections (Fig. 4 *B*). Once again, there were no sig-
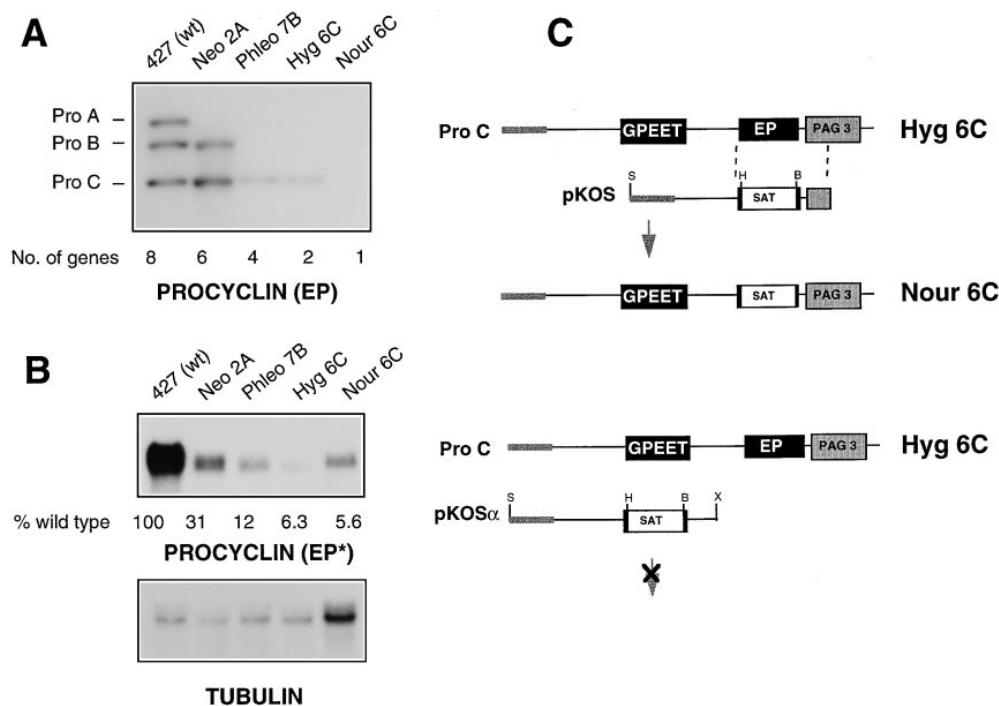
Figure 3. (*A*) Southern blot analysis of sequential deletion mutants. Genomic DNA was digested with PstI, which separates the *Pro A* and *Pro B* loci from the two copies of *Pro C*. The blot was hybridized with a probe (*EP*) corresponding to the coding region of EP1, and was washed under stringent conditions (0.1 × SSC, 0.05% SDS at 65°C). (*B*) Northern blot analysis of deletion mutants. Total RNA was isolated from individual clones and hybridized with a longer probe of 460 bp, EP* (see Materials and Methods). This probe was used because it is 75% identical to the corresponding region of the GPEET transcript and includes two regions, a stretch of 110 bases at the 5′ end and 180 bases at the 3′ end, which are >93% identical. Posthybrization washes were performed under moderately stringent conditions (1 × SSC, 0.05% SDS, 65°C) to maximize the signal obtained with GPEET. The blot was normalized by hybridization with a probe containing tandemly linked α- and β-tubulin genes (42), and was quantified on a PhosphorImager. (*C*) Schematic depiction of the integration of selectable markers into the last *Pro C* locus. A construct designed to delete both procyclin genes (*pKOS*) replaced only the EP gene. A second construct, designed to delete the GPEET gene (*pKOSα*), gave rise to stable transformants that were antibiotic resistant but had retained both procyclin genes.
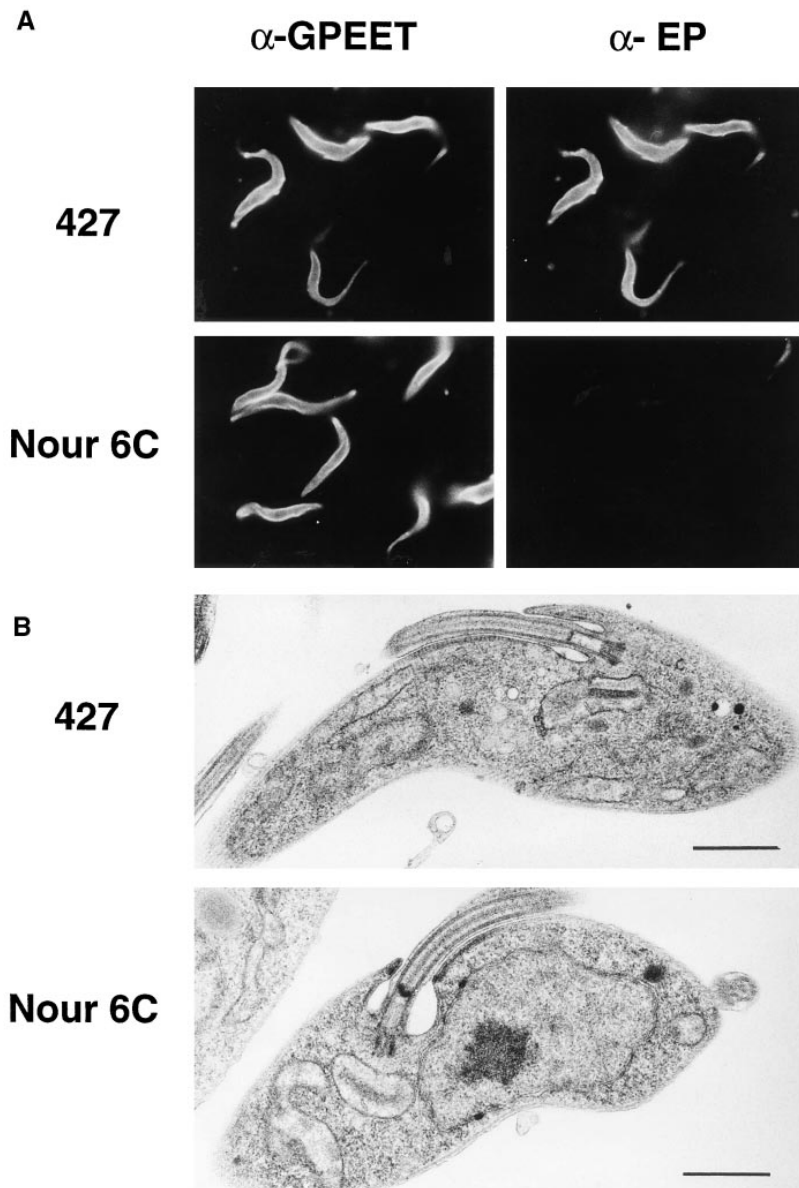
A

α-GPEET   α- EP

427

Nour 6C

B

427

Nour 6C

Figure 4. (*A*) Colocalization of EP and GPEET on the surface of trypanosomes. *T. brucei* 427 and Nour 6C procyclic forms were simultaneously labeled with the anti-EP mAb TBRP1/ 247 (34, 35) and anti-GPEET (K1) antibodies. Both forms of procyclin are expressed on the surface of all 427 cells, whereas Nour 6C cells express only GPEET. Anti-GPEET antibodies also bind to the surface of living trypanosomes, even in the presence of EP (data not shown). (*B*) Transmission electron micrographs of 427 and Nour 6C. Despite the absence of EP in the latter, there are no discernible differences in the plasma membranes or flagellar pockets. Bars, 500 nm.

nificant differences between the cell surfaces of wild-type and Nour 6C cells. Furthermore, the 427 and Nour 6C trypanosomes grew at virtually the same rate in culture (average population doubling times 9.5 and 9 h, respectively). We could also find no alterations in their susceptibility to various proteases (trypsin, chymotrypsin, and Pronase) or to lysis by complement (data not shown).

Reexpression of EP Procyclins

The deletion mutants were the end-product of several rounds of transfection and cloning, so we might have unwittingly selected cells with altered properties, such as changes in transmissibility, that were unlinked to the presence or absence of procyclins. Before we embarked on a set of experiments to assess the role of procyclins in the tsetse fly, Nour 6C trypanosomes were retransformed with a construct containing an EP gene. Since Northern blot

analysis indicated that ~70% of the transcripts in wild-type cells were derived from the two genes in the *Pro A* locus (compare 427 and Neo 2A in Fig. 3 *B*), we constructed a bicistronic plasmid containing an EP 1 gene (Fig. 1) and the puromycin-resistance gene (Fig. 5 *A*, *pEPΔ164-PUR*) and targeted it to the *Pro A* locus by a combination of flanking sequences and drug selection. The procyclin coding sequence in this construct is followed by a truncated 3′ UTR that increases expression twofold over the wild-type 3′ UTR (16a, 18), so it was anticipated that the single EP gene in the retransformed cells would give rise to between 70 and 100% of the amount of procyclin found in wild-type cells. When two clones were examined, however, it was found that they overexpressed the RNA fivefold (N6-EPα 1A) or threefold (N6-EPα 2A) relative to the wild-type cells (Fig. 5 *B*), and this was also reflected by the amount of EP detected by Western blot analysis (data not shown). The retransformants grew slightly more slowly

N6-EPα 2A
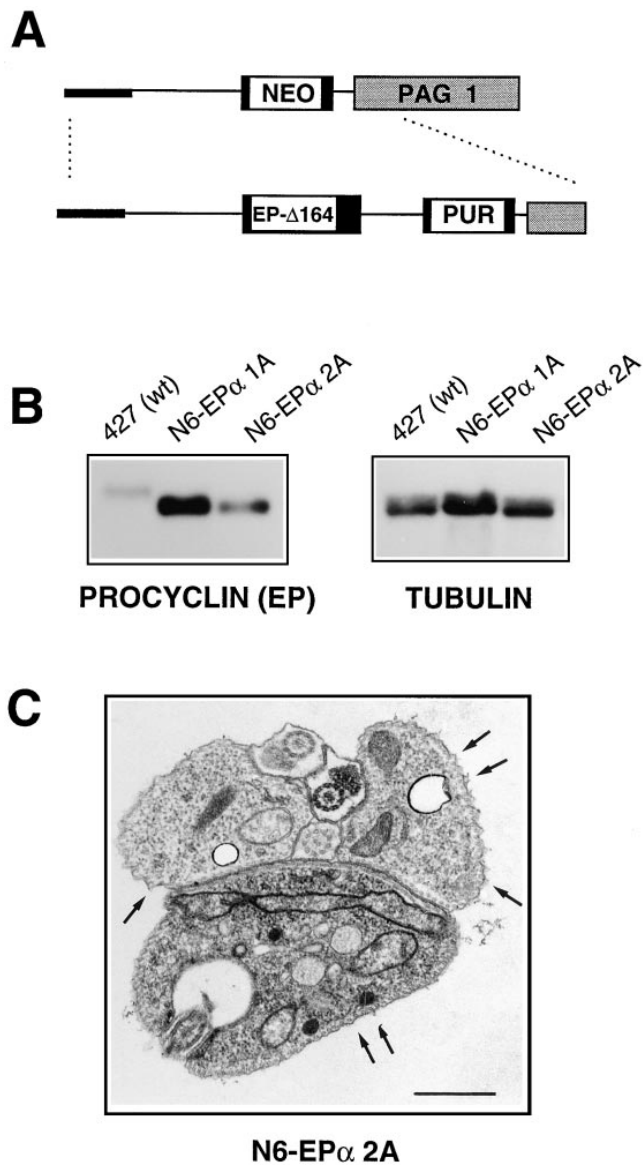
Figure 5. Overexpression of EP in Nour 6C retransformants. (*A*) Schematic drawing of the replacement of the Neo gene in the *Pro A* locus of Nour 6C cells by integration of the bicistronic construct pEPΔ164-PUR. Puromycin-resistant clones were isolated and analyzed for correct integration (data not shown). The deletion of the first 164 bp of the procyclin α 3′ UTR increases expression of a reporter gene approximately twofold compared to the wild-type 3′ UTR (16a, 18). (*B*) Northern blot analysis of procyclin expression. The blots were hybridized sequentially with probes for EP procyclin and tubulin, and were normalized as described in the legend to Fig. 3. N6-EPα 1A has five times more steady-state procyclin mRNA and N6-EPα 2A has three times more than the wild-type 427. (*C*) Transmission electron microscopy reveals irregularities (*arrows*) in the surface of cells that overexpress EP. Bar, 500 nm.

than either 427 or Nour 6C (average population doubling time 10.3 h), and although no changes in morphology could be detected when the trypanosomes were examined by light microscopy, transmission electron microscopy revealed slight distortions of the surface membranes (Fig. 5 *C*). We also observed that when these cells were passaged

for several months, there was tendency for a proportion of the population to stop expressing the gene, and that this was exacerbated in the absence of puromycin.

Ectopic Expression of GPEET

Since EP procyclins could be overexpressed, it was expected that this would also hold true for GPEET when a similar construct was integrated into the *Pro A* locus. The EP coding region in the construct shown in Fig. 5 *A* was replaced with GPEET, and the new construct, pGPEETΔ164-PUR, was used to transfect Nour 6C cells. Trypanosome clones were analyzed for correct integration of the construct (data not shown). These cells now contained two GPEET genes, the endogenous gene in the *Pro C* locus, and a second copy in the *Pro A* locus, whose transcripts could be distinguished on the basis of their size (Fig. 6 *A*, *N6-GPEET 1A*). Unlike the EP retransformants, however, these cells did not show increased levels of steady-state RNA. On the contrary, quantitation of the Northern blots revealed a 25% reduction in the relative amount of RNA from the gene in the *Pro C* locus that was compensated for by transcription of the gene in the *Pro A* locus.

Recombinational hot and cold spots have been described in other lower eukaryotes, such as yeast, and an alternative explanation for the retention of the last GPEET copy was that it was inaccessible for integration. To test this possibility, the N6-GPEET 1A cell line was transfected with a plasmid (Fig. 2 *A*, *pKOCN*) that was designed to delete the GPEET gene and the flanking SAT gene. Several stable transformants were examined and shown to have the predicted integration of the Neo gene (data not shown) and to express only the truncated form of the GPEET mRNA (Fig. 6 *A*, *2CKO 7*). Interestingly, the steady-state level of this mRNA had now increased to the same level as that of the endogenous mRNA in Nour 6C cells. In addition, Western blot analysis confirmed that there were similar amounts of the protein in the different cell lines (Fig. 6 *B*). These results confirm that the gene in the *Pro C* locus can be deleted provided a second copy is present, and they suggest that GPEET expression is much more tightly regulated than that of EP procyclins.

EP Procyclins Enhance Infections in the Tsetse fly Midgut

When procyclic form trypanosomes are mixed with RBCs and fed to tsetse flies through an artificial membrane, they are capable of establishing an infection in the gut with the same efficiency as bloodstream forms. To assess the role of procyclins on survival in vivo, tsetse flies were infected with either wild-type trypanosomes, EP null mutants (Nour 6C), or EP overexpressors (N6-EPα 2A and N6-EPβ 1A). The flies were dissected 12–14 d later and examined for the presence of parasites. Infections in midgut-positive flies could be clearly classified into three categories: weak, intermediate, and heavy. Five separate experiments are shown in Fig. 7. More than 1,100 flies were examined in total, but since these experiments were performed with different batches over a period of 12 mo, direct comparisons can only be made within an experiment. Despite the inherent variability of the system, it is striking that wild-type trypanosomes gave rise to heavy infections in 16.7–26.8%
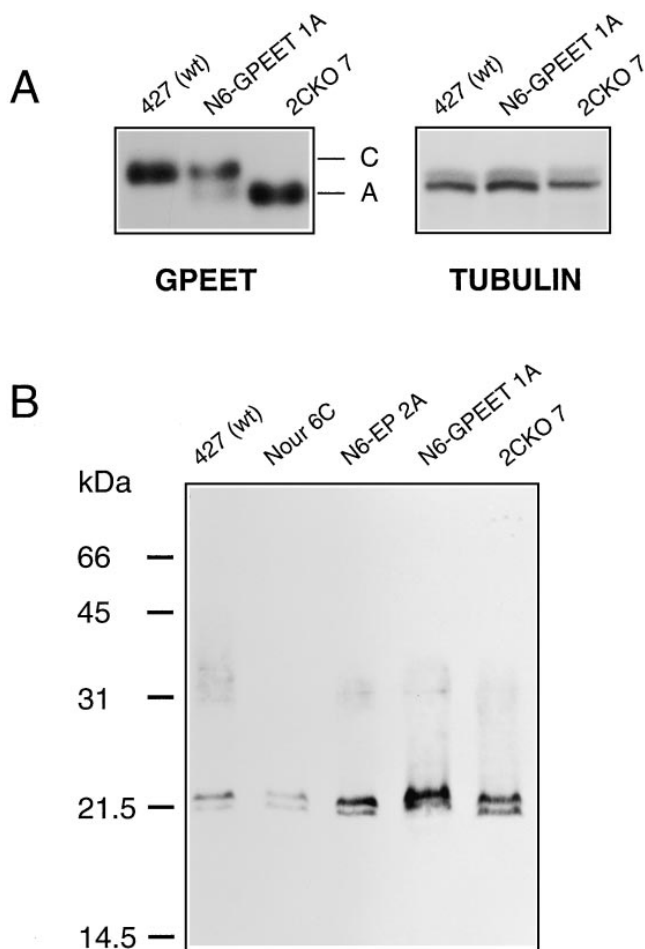
GPEET **TUBULIN**

Figure 6. Ectopic integration of GPEET does not result in overexpression. The construct pGPEETΔ164-PUR was used to integrate a tagged GPEET gene into the *Pro A* locus of N6-GPEET 1A trypanosomes, and the expression of GPEET mRNA in this cell line was compared to that in 427 and Nour 6C. (*A*) Blots were hybridized with probes corresponding to the GPEET coding region and tubulin. *A*, the truncated GPEET transcript from the *Pro A* locus; *C*, the endogenous transcript from the *Pro C* locus. Once the second endogenous copy of GPEET was deleted (clone 2CKO 7), only the smaller transcript could be detected. (*B*) Western blot analysis: total cell lysates (2×10^6 cell equivalents per lane) were separated by SDS-PAGE and transferred to nitrocellulose. The membrane was incubated with anti-GPEET (K1) polyclonal serum, and bound antibody was detected by enhanced chemiluminescence. K1 antiserum reacts primarily with two polypeptides of 20 and 21 kD. At longer exposures, variable amounts of several larger polypeptides can also be detected. All can be specifically inhibited by coincubation with 1 μg · ml^{-1} of the synthetic peptide (GPEET)$_3$C (data not shown). It is possible that the higher molecular weight species represent O-glycosylated and/or phosphorylated forms of GPEET, or forms with additional modifications to the GPI-anchor. In contrast to GPEET, EP procyclins migrate as a diffuse band of 40–45 kD (35).

of the flies (mean $21.0 \pm 4.2\%$), whereas the Nour 6C deletion mutants caused heavy infections in only 2.4–5.4% of flies (mean $3.8 \pm 1.2\%$). In general, there were also more flies that were negative or only weakly infected with Nour 6C. N6-EPα 2A cells, which overexpress the EP1 form of procyclin (Fig. 1), were considerably more successful at es-

tablishing heavy infections (10.4 and 14.7%, Fig. 7, experiments III and IV) than their EP-negative parent, although they were not as efficient as the wild type. N6-EPα 2A trypanosomes express a glycosylated form of EP, corresponding to EP1 in Fig. 1 (7a, 35). To assess the importance of N-linked carbohydrates, we also tested a trypanosome clone (N6-EPβ 1A) that overexpressed a form of EP without a glycosylation site (Fig. 1, *EP2*). These cells were equally effective at promoting strong infections as the N6-EPα 2A cells were (11.1%, Fig. 7, experiment V), suggesting that N-linked sugars do not play a crucial role.

There were significant differences in the number of heavy infections produced by EP-positive cells (wild-type 427 and both forms of N6-EP) and EP-negative cells, as well as in the number of total infections (Fig. 7). In contrast, although there was a small, but significant difference in the number of heavy infections produced by 427 and N6-EP cells, the total infection rate fell within the same range. In conclusion, these results demonstrate that the expression of EP procyclins correlates with improved survival and growth within the fly, but other determinants must also be involved, since even Nour 6C cells are capable of establishing heavy infections in a small percentage of flies.

Discussion

The analysis of deletion mutants has provided new insights into the expression and function of the different forms of procyclin. While it has been known for some time that the procyclin messenger RNAs that can be detected in a cloned line of trypanosomes must stem from at least two of the four expression sites (24, 30), it could not be ruled out that individual procyclic forms used only a single expression site, as is normally the case for the VSG expression site in bloodstream forms. In the course of deleting the procyclin genes, we inserted different selectable markers into each of the four loci and obtained parasites that were resistant to all four antibiotics, demonstrating that it was possible for all the loci to be transcribed simultaneously. Despite the fact that the promoters are virtually identical in sequence (9, 40), the contributions of the expression sites are not equal, since ∼70% of the procyclin transcripts could be attributed to the *Pro A* locus, 18% to *Pro B,* and 12% to the two copies of *Pro C*. It remains to be established whether the *Pro A* locus is dominant in all isolates, or whether procyclic forms have the capacity to switch between loci.

The *Pro A* and *Pro B* loci both contain two genes for EP procyclins, while each of the two *Pro C* loci contains a GPEET gene followed by an EP gene. Three pairs of genes were sequentially deleted by homologous recombination, but all attempts to knock out the last pair, from a *Pro C* locus, invariably left one GPEET gene intact. Constructs that were designed to remove this gene, while leaving the neighboring copy of EP, resulted in aberrant integrations and the retention of GPEET. Other attempts to delete both GPEET genes from the *Pro C* loci were also unsuccessful, even when the four EP genes from the other two loci were still present (Ruepp, S., and A. Furger, unpublished observation). Once a tagged copy of GPEET was integrated into the *Pro A* locus, however, it was possi-
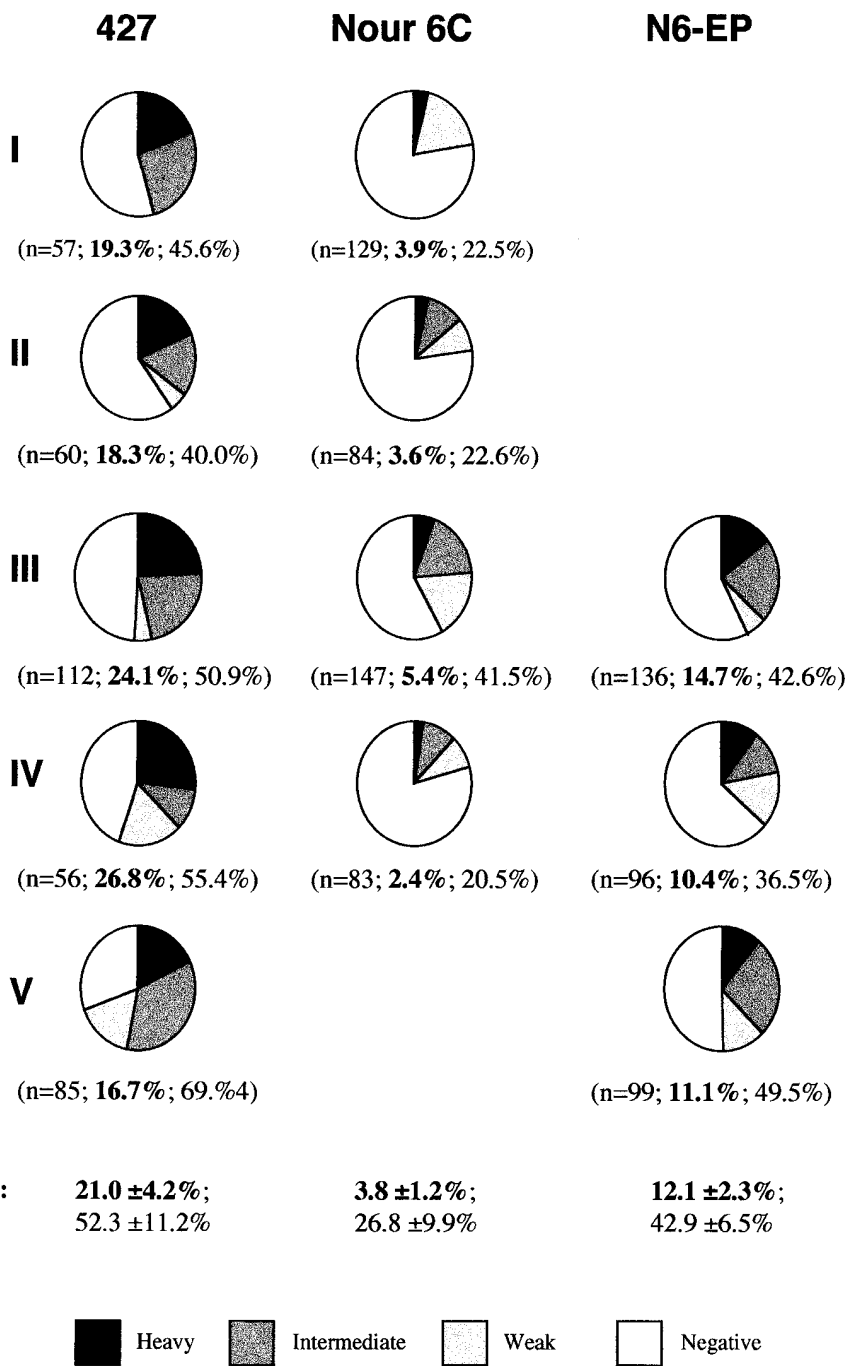
427 **Nour 6C** **N6-EP**

I
(n=57; **19.3%**; 45.6%) (n=129; **3.9%**; 22.5%)

II
(n=60; **18.3%**; 40.0%) (n=84; **3.6%**; 22.6%)

III
(n=112; **24.1%**; 50.9%) (n=147; **5.4%**; 41.5%) (n=136; **14.7%**; 42.6%)

IV
(n=56; **26.8%**; 55.4%) (n=83; **2.4%**; 20.5%) (n=96; **10.4%**; 36.5%)

V
(n=85; **16.7%**; 69.%4) (n=99; **11.1%**; 49.5%)

Heavy: **21.0 ±4.2%**;   **3.8 ±1.2%**;   **12.1 ±2.3%**;
Total: 52.3 ±11.2%   26.8 ±9.9%   42.9 ±6.5%

■ Heavy ▨ Intermediate ▥ Weak □ Negative

Figure 7. Effect of EP deletion or overexpression on the survival and proliferation of trypanosomes in the tsetse fly midgut. Flies were infected with procyclic forms, and then dissected 12–14 d later. Infections in midgut-positive flies were divided into three categories: weak, intermediate, and heavy. A description of the three categories is given in Materials and Methods. The numbers in brackets refer to the number of flies that were dissected in each group followed by the percentage of heavy infections (*boldface type*) and total infections. N6-EP: Experiments III and IV were performed with N6-EPα 2A cells that overexpress the glycosylated EP1 form (see Fig. 1). Experiment V was performed with N6-EPβ 1A trypanosomes that overexpress a nonglycosylated form corresponding to EP2.

ble to delete the last endogenous gene, confirming that it was not intrinsically inaccessible to recombination. These results indicate that the two forms of procyclin are not functionally equivalent, since it is possible to generate null mutants for EP (e.g., Nour 6C), but not for GPEET.

The two forms of procyclin also do not appear to be subject to the same control mechanisms. The integration of a single EP gene (with a truncated 3′ UTR that increased expression) into the *Pro A* locus of Nour 6C trypanosomes was sufficient to produce up to five times more mRNA and protein than wild-type cells. In contrast, when a GPEET gene with the identical 3′ UTR was integrated into the same locus, the amount of steady-state RNA was only one quarter of that of *Pro C*–derived transcript. Once the GPEET gene was deleted from the *Pro C* locus, however, there was an increase in the amount of the transcript from the *Pro A* locus. These data suggest first that these cells can only tolerate a narrow range of GPEET expression, and secondly that the level of steady-state RNA might be determined by a regulatory element in the coding region.

The evidence for GPEET expression was formerly restricted to the detection of the mRNA (31), since there were no antibodies that were specific for this form. By using antibodies raised against a synthetic pentapeptide repeat, we have shown that GPEET is coexpressed with EP

on the surface of *T. brucei* 427. Western blot analysis with the same antibodies demonstrated that the two forms of procyclin have markedly different electrophoretic mobilities. EP migrates as a broad band in the range 40–45 kD (35), whereas GPEET is predominantly detected as a doublet of 20/21 kD (Fig. 6 *B*). It is interesting to note that acidic proteins of the same size were also detected when cells were labeled with proline and separated on two-dimensional gels (8). Furthermore, procyclic form trypanosomes labeled with tritiated myristic acid incorporated a small proportion of the label into proteins of 20/21 kD (15), which is consistent with the fact that GPEET, like EP, is GPI anchored (7a, 46a).

The first requirement for successful transmission of *T. brucei* is the establishment of an infection in the fly midgut. To study the role of EP, the properties of four trypanosome clones were compared: wild-type cells with the full complement of genes, the EP null mutant Nour 6C, and two EP overexpressors derived from Nour 6C (N6-EPα 2A and N6-EPβ 1A), which both produced about three times more EP than wild-type cells. Removal of the EP coat had no significant effect on the growth characteristics or morphology of Nour 6C procyclic forms in culture, nor could we detect an alteration in their sensitivity to several proteases or complement compared to wild-type cells. In contrast, N6-EPa 2A trypanosomes grew more erratically than either 427 or Nour 6C, and transmission electron microscopy revealed that they had a more ruffled surface (Fig. 5 *C*). Furthermore, EP expression in these cells was not completely stable: EP-negative cells could be detected in cultures that had been passaged for several months in the presence of the appropriate antibiotic, and these tended to overgrow the EP-positive cells when the selective pressure was removed. Although 427 and Nour 6C trypanosomes were virtually indistinguishable in culture, striking differences emerged when we compared their infectivity for tsetse flies. Wild-type cells were 5 to 10 times more likely to give rise to heavy midgut infections than Nour 6C cells; the correlation between EP expression and the degree of infection was confirmed by the finding that both of the EP overexpressors were also capable of producing heavy infections three to four times more often than Nour 6C trypanosomes. In light of our results, it would seem that some of the functions that have previously been proposed for procyclins may be oversimplifications. For example, it has been suggested that procyclins might be a target for a trypanocidal factor—possibly a lectin—in the fly midgut (28). If this were the case for EP, Nour 6C cells should survive better than the wild-type strain, but the converse is true. In addition, cells that overexpress either EP 1 or EP 2 behave very similarly, which would argue against a role for N-linked carbohydrates in the interaction.

Our results clearly demonstrate the importance of one class of procyclins in the first stage of transmission. Procyclins may also play a role in the later stages of differentiation, but this cannot be tested with derivatives of strain 427; these parasites establish normal midgut infections, but do not migrate to the salivary glands. Experiments to determine the effects on parasite maturation using transmissible strains may not be trivial, however. When procyclic forms are maintained in culture for more than a few months, they lose the ability to complete the cycle, so that repeated rounds of transfection and selection to remove several genes may result in changes in transmissibility that are unlinked to procyclin expression. There might be alternative ways to determine whether procyclins are involved in parasite tropism. In contrast to *T. brucei*, the epimastigote and metacyclic forms of *T. congolense* develop in the proboscis. It should be possible to investigate the role of surface molecules by expressing heterologous procyclins in the two species (19) and studying their route through the fly.

The procyclin coat has previously been regarded as both homogeneous and invariant. We now know that although it is composed of closely related surface glycoproteins, these appear to have distinct functions in different contexts. The requirement for GPEET by procyclic culture forms suggests that it might either play a role in parasite–parasite interactions or function as a receptor for an unknown soluble ligand. The finding that EP is not required in culture but is important for survival in vivo implies that its prime importance lies in parasite–tsetse interactions, which may involve the recognition of soluble, matrix- or cell-associated ligands. At the present time, we can only speculate on the biological relevance of the three variants of EP that are encoded in the genome (see Fig. 1). Our data show that both glycosylated and unglycosylated forms can function equally well in enhancing midgut infections in *Glossina morsitans centralis*, but this does not exclude specific functions for either form at a later stage in the life cycle. It should also be borne in mind that *T. brucei* can infect different species of tsetse flies, although not necessarily with equal efficiency (28). It is possible that the survival in a given species of tsetse fly is favored by a certain form of EP, and that the expression of different variants has enabled the parasite to increase its host range. Finally, changes in the balance between EP and GPEET might also explain why some stocks of trypanosomes can be more readily transmitted than others.

Note Added in Proof. The relative amounts of EP and GPEET expressed by procyclic forms have now been determined independently by Bütikofer et al. and Treumann et al. The ratio of two forms of procyclin can vary markedly between strains (Bütikofer et al., 1997) or between different passages of the same culture (Treumann et al., 1997), with a shift toward increased GPEET expression with time. The clone of *T. brucei* 427 used in these experiments stably expresses about onefold more GPEET than EP, as measured by the incorporation of labeled precursors into the GPI anchors of the two forms. The requirement for GPEET is not simply due to the high levels of expression, however, because we have been unable to delete the last copy in derivatives of *T. brucei* 427 in which the ratios are reversed.

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