

# Galactose metabolism is essential for the African sleeping sickness parasite *Trypanosoma brucei*

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The tsetse fly-transmitted protozoan parasite *Trypanosoma brucei* is the causative agent of human African sleeping sickness and the cattle disease Nagana. The bloodstream form of the parasite uses a dense cell-surface coat of variant surface glycoprotein to escape the innate and adaptive immune responses of the mammalian host and a highly glycosylated transferrin receptor to take up host transferrin, an essential growth factor. These glycoproteins, as well as other flagellar pocket, endosomal, and lysosomal glycoproteins, are known to contain galactose. The parasite is unable to take up galactose, suggesting that it may depend on the action of UDP-glucose 4'-epimerase for the conversion of UDP-Glc to UDP-Gal and subsequent incorporation of galactose into glycoconjugates via UDP-Gal-dependent galactosyltransferases. In this paper, we describe the cloning of *T. brucei galE*, encoding *T. brucei* UDP-Glc-4'-epimerase, and functional characterization by complementation of a *galE*-deficient *Escherichia coli* mutant and enzymatic assay of recombinant protein. A tetracycline-inducible conditional *galE* null mutant of *T. brucei* was created using a transgenic parasite expressing the *TETR* tetracycline repressor protein gene. Withdrawal of tetracycline led to a cessation of cell division and substantial cell death, demonstrating that galactose metabolism in *T. brucei* proceeds via UDP-Glc-4'-epimerase and is essential for parasite growth. After several days without tetracycline, cultures spontaneously recovered. These cells were shown to have undergone a genetic rearrangement that deleted the *TETR* gene. The results show that enzymes and transporters involved in galactose metabolism may be considered as potential therapeutic targets against African trypanosomiasis.

UDP-Gal | *galE* | epimerase

The tsetse fly-transmitted protozoan parasite *Trypanosoma brucei* causes human African sleeping sickness and the related cattle disease Nagana. There are 300,000–500,000 cases of the human disease per year in sub-Saharan Africa (1). The bloodstream form of the parasite divides by binary fission in the blood, lymph, and interstitial fluids of the mammalian host, and causes cachexia and anaemia in cattle and neurological disturbances in man. These conditions are fatal if not treated, and existing chemotherapies are toxic and difficult to administer. The need for new, less-toxic therapeutics is widely acknowledged (1).

The bloodstream form of *T. brucei* is rich in galactose-containing glycoproteins, most notably the variant surface glycoprotein (VSG) that, at  $5 \times 10^6$  homodimers per cell, forms a dense cell-surface coat. The VSG coat is a macromolecular diffusion barrier that protects the parasite from the innate immune system and also enables the parasite to undergo antigenic variation. Thus, although an individual parasite only expresses one VSG gene at a time, the parasite population can stay ahead of the host's specific immune response to expressed VSGs when a few parasites switch expression to one of several hundred genes encoding immunologically distinct VSGs (2).

Bloodstream-form *T. brucei* parasites use facilitated diffusion through a hexose transporter to obtain glucose from the host (3). However, galactose does not compete for this transporter and

the parasite cannot take up [<sup>14</sup>C]Gal (4). This lack of Gal transport was corroborated by unsuccessful attempts to metabolically label VSG with [<sup>3</sup>H]Gal (G. Wall and M.A.J.F., unpublished data) under conditions that were successful for [<sup>3</sup>H]Man labeling (5). Based on these observations, we considered it likely that the principal source of Gal in *T. brucei* might be via the epimerization of UDP-Glc to UDP-Gal by UDP-Glc-4'-epimerase (EC 5.1.3.2). This NADH-dependent oxidoreductase is found in most prokaryotes and eukaryotes, and interspecies sequence similarity is typically 40% or better. The precise roles of conserved residues in substrate binding and catalysis can be inferred from x-ray crystallographic studies (6).

In this study, we describe the cloning of the *galE* gene encoding the *T. brucei* UDP-Glc-4'-epimerase, the characterization of the recombinant enzyme, and the creation of a *T. brucei* conditional *galE* null mutant that shows that the enzyme, and galactose metabolism, are essential to the parasite.

## Materials and Methods

**Cloning of *T. brucei galE*.** Procyclic trypanosomes (strain 427) were grown in SDM-79 medium (7) and genomic DNA was prepared by standard methods. A 1,764-bp genomic clone was obtained by PCR amplification with a forward primer to the 5'-untranslated region (UTR) (5'-gcatacgcacatgtacatgctc-3') and a reverse primer to the 3'-UTR (5'-caagtgccttaaatagcagctcc-3'), designed from the end sequences of the 10P15 clone identified by TBLASTN search of the *T. brucei* database with the human *galE* sequence. Primers (forward, 5'-gcgatgtgttacacaagtgcg-3'; reverse, 5'-ccactctataacctcagcac-3') were used to generate a 574-bp *T. brucei galE* probe that was labeled with dUTP fluorescein by random priming (Gene Images kit, Amersham Pharmacia) for use in Southern Blotting. PCR amplification of the *T. brucei galE* ORF from *T. brucei* bloodstream-form and procyclic-form cDNA was performed with a forward primer against the minixon (5'-ggcccgtattattagaacagctttctgta-3') and a specific reverse primer to the 3' end of the gene (5'-cgatgcgtaccatt-agggtgagt-3').

**Functional Complementation.** *T. brucei galE* ORF was amplified from the genomic clone and subcloned into the *Bam*HI and *Hind*III sites of pUC18 (RFO) or with an additional one (RF + 1) or two (RF + 2) adenosine bases immediately 5' to the initiating ATG. The forward and reverse primers were 5'-cgcGGATCC(aa)atgcgcggttttggttg-3 and 5'-cccAAGCTTtatacacttagtggtcc-3', respectively, where the capital letters denote

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Abbreviations: VSG, variant surface glycoprotein; UTR, untranslated region; Tfr, *Trypanosoma brucei* transferrin receptor; TetOp, tetracycline operator.

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restriction sites and the bases in parentheses indicate the additional adenosine base(s). DC41 *galE*<sup>-</sup> *Escherichia coli* was transformed with the pUC18-*galE* plasmids by electroporation. The transformed cell lines were grown in LB medium with ampicillin and isolated clones were analyzed to ensure that they carried the correct plasmid. These clones were expanded and streaked onto McConkey agar plates (containing ampicillin and Gal as the only carbon source) pretreated with 100  $\mu$ l of 0.1 M isopropyl  $\beta$ -D-thiogalactoside (IPTG).

#### Expression and Purification of Recombinant UDP-Glc-4'-epimerase.

The *T. brucei galE* ORF was amplified from the genomic clone, silencing an *NdeI* site, and subcloned into the *NdeI/BamHI* sites of pET15b (Novagen) for expression in BL21(DE3)pLysS *E. coli* cells (Novagen). The gene was amplified in two segments by using *Pfu* (Promega). The 5' end of the ORF was amplified by using 5'-ggaattcCATATGcgcgtttgtgttg-3' and 5'-gttttgactcgccataaggacttctgggctc-3' and the 3' end was amplified using 5'-gagcccagaaagtcctatggcgagtcacaaac-3' and 5'-cgcG-GATCCttacaactagtgtcc-3' as forward and reverse primers, respectively. The two PCR products were used together in a further PCR reaction to yield a product containing the silent mutation of the *NdeI* site (italics) and 5'-*NdeI* and 3'-*BamHI* restriction sites (capital letters).

Expression was induced overnight at room temperature by adding 50  $\mu$ M IPTG to cells at a density of 0.5 OD<sub>600</sub> units. Cells were harvested, washed in PBS, and lysed by two freeze-thaw cycles and high-pressure lysis in a French press. The lysate was cleared by centrifugation (31,000  $\times$  g, 30 min, 4°C) and the supernatant was passed through a 0.45- $\mu$ m filter and loaded onto a 5-ml HiTrap Chelating Sepharose HP column (Amersham Pharmacia Biotech AB) charged with Ni<sup>2+</sup>. The column was washed with 50 mM Tris (pH 7.6)/250 mM NaCl until the A<sub>280</sub> trace was stable and then eluted with a gradient to 0.4 M imidazole in the same buffer over 100 ml. The flow rate was 5 ml/min and 4-ml fractions were collected. A<sub>280</sub> peak fractions were analyzed by 10% SDS/PAGE and those containing the 46-kDa epimerase were pooled and dialyzed against 50 mM Tris (pH 7.6). The pooled fractions were concentrated (Vivapore 7,500 MWCO concentrator, Sartorius Vivascience) and checked by SDS/PAGE and matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry. The stock solution was adjusted to 20% (wt/vol) glycerol (final concentration, 2.5 mg protein/ml) and stored in aliquots at -80°C.

**Enzyme Assay.** Assays were performed using a coupled assay according to ref. 8. Briefly, 1-cm path-length cuvettes were charged with 910  $\mu$ l of 100 mM glycine-NaOH (pH 8.7)/1 mM  $\beta$ -NAD<sup>+</sup> (Sigma)/40  $\mu$ l of 20 mM UDP-Gal (Sigma)/40  $\mu$ l of 1 unit/ml UDP-Glc dehydrogenase (Calbiochem). The reaction was started by adding 10  $\mu$ l of epimerase diluted in 50 mM Tris-HCl (pH 7.6)/1% BSA/1 mM DTT/1 mM EDTA/1 mM  $\beta$ -NAD<sup>+</sup>. Net NADH production was measured by following changes in A<sub>340</sub> at 27°C in a Shimadzu UV-1601 spectrophotometer. A linear relationship between V<sub>o</sub> and enzyme concentration was established over the range 0–1  $\mu$ g/ml, and 0.5  $\mu$ g/ml was used for determining the specific activity and K<sub>m</sub> for UDP-Gal.

**Generation of a *T. brucei* Bloodstream Form of *galE* Conditional Null Mutant.** The 309-bp 5'- and 266-bp 3'-UTR sequences immediately adjacent to the start and stop codons of the *T. brucei galE* ORF were PCR amplified from the genomic clone by using *Pfu* with 5'-ataagaatGCGGCCGCgcatacgcacatgtac-3' and 5'-gtttaaacttacggaccgtcaagcttgtaagaagggttctttcc-3' and 5'-gacggtcgtaagtttaaacggatcccttaactggcagtac-3' and 5'-ataagtaaGCGGCCGCcaagtgcttaaatagc-3' as forward and reverse primers,

respectively. The two PCR products were used together in a further PCR reaction to yield a product containing the 5'-UTR linked to the 3'-UTR by a short *HindIII*, *PmeI*, and *BamHI* cloning site (italics) and *NotI* restriction sites at each end (capital letters). The PCR product was cloned into the *NotI* site of the pGEM-5Zf(+) vector (Promega) and the *HYG* and *PAC* drug resistance genes were introduced into the targeting vector via the *HindIII* and *BamHI* cloning sites.

The *T. brucei galE* ORF was amplified from the genomic clone by using *Pfu* and 5'-cccAAGCTTatgcgctttgtgttg-3' and 5'-cgcGGATCCttacaactagtgtcc-3' as forward and reverse primers, respectively, where the capital letters indicate *HindIII* and *BamHI* restriction sites. The product was ligated into the *HindIII/BamHI* cloning site of the pLew100 tetracycline-inducible expression vector (9).

The above plasmids were prepared using Qiagen Maxi-Prep kits, digested with *NotI*, precipitated and washed twice with 70% ethanol, redissolved in sterile water, and used for electroporation of the parasites. All genetic modifications were performed using strain 427 (variant 221) bloodstream forms stably transfected to express T7 RNA polymerase and tetracycline repressor (TETR) protein under continuous G418 selection (26). For convenience, these are referred to here as "wild-type" cells. The cultivation and transfection of these cells is described in ref. 9 with minor modifications described in ref. 10. Once stable conditional *galE* null mutant clones were obtained, all selection drugs (except G418) were removed from the medium. Tet-system-approved FCS (CLONTECH) was used in experiments on the effects of tetracycline removal.

**Northern Blots.** Total RNA for Northern blotting was prepared by standard guanidinium thiocyanate methods from 1  $\times$  10<sup>8</sup> cells. Samples of RNA (5  $\mu$ g) were run on formaldehyde agarose gels and transferred to Hybond N nylon membrane (Amersham Pharmacia) for hybridization with [ $\alpha$ -<sup>32</sup>P]dCTP-labeled *T. brucei galE* probe (Stratagene, Prime-It RmT Random Primer labeling kit). The control  $\beta$ -tubulin probe was used after the *T. brucei galE* probe had decayed.

**Analysis of Tetracycline Response Elements.** The *T. brucei galE* conditional mutants contain the *TETR* gene under G418 selection (i.e., adjacent to a *NEO* gene) and the ectopic *galE* gene downstream of two tetracycline operator (TetOp) sequences. The region of DNA containing the *TETR* gene was PCR amplified from genomic DNA by using flanking primers (5'-gaagtcttgacattggg-3' and 5'-gaacgcagacgatttgcg-3'). The region of DNA containing the TetOp sequences was PCR amplified from genomic DNA by using flanking primers (5'-cgtggacacgacctc-3' and 5'-cacgtctcccacctc-3').

For Southern blot analysis, probes to the *TETR* and *NEO* genes were prepared by PCR amplification from the pLew114hyg5' plasmid (26) by using the primers 5'-cagcgcattagactgc-3' and 5'-ccgaataagaaggctgg-3' and 5'-ccaagcttgatgattg-3' and 5'-caagaaggcagatagaagg-3', respectively. The probes were labeled with dUTP-fluorescein and used to probe *NotI/SaII* digests.

#### Results

**Cloning of the *T. brucei galE* Gene.** A TBLASTN search of The Institute of Genomic Research (TIGR) database with the human UDP-Glc-4'-epimerase protein sequence identified a sheared DNA library clone (10P15) with significant homology to the C-terminal 98-aa residues followed by 298 bp of putative 3'-UTR. The other end sequence of the clone was presumed to represent 515 bp of 5'-UTR. A genomic clone was obtained using genomic DNA template and primers designed to the putative 5'- and 3'-UTRs. This 1,764-bp clone (GenBank accession no. AJ430490) contained a 1,188-bp ORF, encoding a protein with



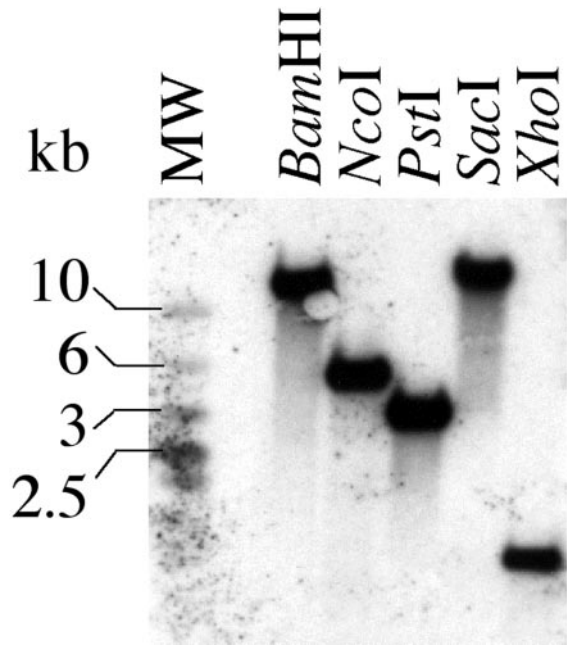


**Fig. 1.** CLUSTAL V alignment of *T. brucei* (Tb), *E. coli* (Ec), *Saccharomyces cerevisiae* (Sc), *Caenorhabditis elegans* (Ce), *Drosophila melanogaster* (Dm), and human (Hu) UDP-Glc-4-epimerase predicted amino acid sequences. The reverse-text indicates residues that match the consensus sequence.

significant homology to known UDP-Glc-4'-epimerases (Fig. 1), flanked by 309 and 267 bp of 5'- and 3'-UTR, respectively. PCR amplification of a 1.2-kb fragment generated from both bloodstream and procyclic form *T. brucei* cDNA, using primers based on the predicted C terminus of the *T. brucei* epimerase and the minixion (a 5'-spliced leader common to all *T. brucei* mRNAs), confirmed that the gene is transcribed in both life cycle stages. Southern blotting showed that the gene is single-copy per haploid genome (Fig. 2).

**Functional Complementation of *galE*-Deficient *E. coli*.** The *T. brucei galE* gene was subcloned into pUC18 in three frames, placing the gene under the control of the *lacZ* promoter. The plasmids were electroporated into a *galE*-deficient *E. coli* strain to test for functional complementation in the presence of isopropyl  $\beta$ -D-thiogalactoside (IPTG) inducer. In one frame (RF0), the *galE*<sup>-</sup> cells were rescued and grew as dark red colonies on McConkey agar (a medium with Gal as the only carbon source). By contrast, transformation with the *T. brucei* epimerase gene in the RF + 1 and RF + 2 frames failed to rescue the *galE*<sup>-</sup> growth defect and these cells grew as small, pale colonies (Fig. 3A).

**Expression and Assay of Recombinant *T. brucei* UDP-Glc-4'-epimerase.** For biochemical characterization of the *T. brucei* epimerase, we expressed recombinant protein with an N-terminal His-6-tag in *E. coli* to yield about 20 mg/l of recombinant protein (Fig. 3B). The purified recombinant protein (Fig. 3B) was assayed using a coupled assay (8) and the specific activity and *K<sub>m</sub>* for UDP-Gal were determined (Fig. 3C). The specific activity of the recombinant *T. brucei* enzyme was 5.9 units/mg, similar to that

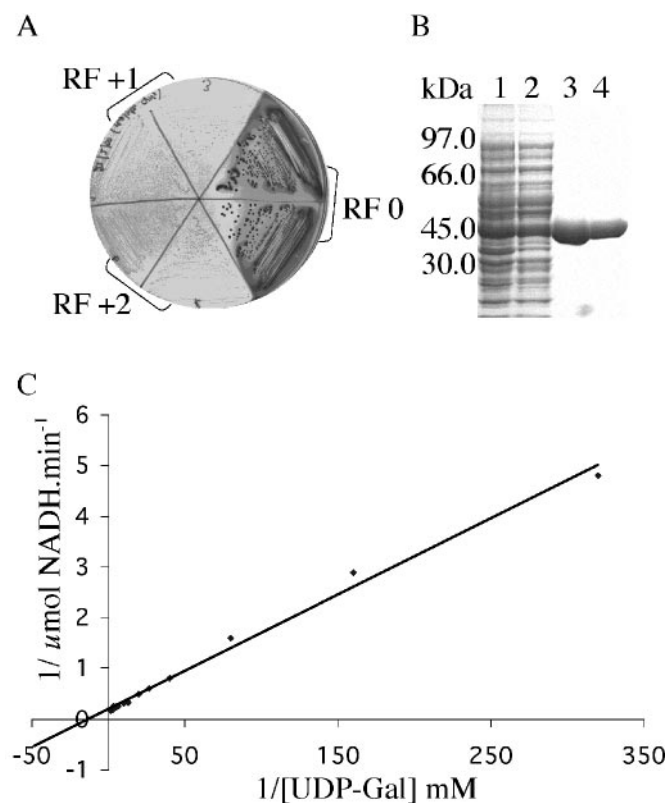


**Fig. 2.** Southern blot of *T. brucei* procyclic genomic DNA digested with a panel of restriction endonucleases and probed for *T. brucei galE*. Approximately five micrograms of DNA, digested overnight with *Bam*HI, *Nco*I, *Pst*I, *Sac*I, and *Xho*I restriction enzymes, was loaded per lane. Hybridization was at 60°C overnight. The blot was washed with 1× SSC (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7)/0.1% SDS for 20 min at 60°C, followed by 0.5× SSC/0.1% SDS for 20 min at 60°C. The single band in each lane shows that the epimerase is present as a single copy per haploid genome.

measured under identical conditions for a commercially available epimerase from *Streptococcus thermophilus* (16.7 units/mg). The apparent *K<sub>m</sub>* of the *T. brucei* epimerase for UDP-Gal was 77  $\mu$ M, similar to that measured for the *S. thermophilus* enzyme (91  $\mu$ M). These values of specific activity and *K<sub>m</sub>* are well within the normal ranges of 0.012–210 units/mg and 8–260  $\mu$ M, respectively, reported for a UDP-Glc-4'-epimerases from a variety of sources (11).

**The *T. brucei galE* Gene Is Essential to the Bloodstream Form of Trypanosomes.** Although it was possible to replace one *T. brucei galE* allele by homologous recombination with either *PAC* or *HYG*, several attempts to replace the second allele with the complementary drug-resistance gene failed, suggesting that *galE* might be an essential gene. To investigate this, a conditional null mutant was created. A trypanosome cell line that constitutively expresses T7 RNA polymerase and the tetracycline repressor (TETR) protein under G418-neomycin selection was used (9). An ectopic epimerase gene was introduced into the trypanosome rDNA locus (using the pLew100 expression vector), driven by a trypanosome (procyclic) promoter and regulated by two TetOp sequences (Fig. 4A). Thus, the addition of tetracycline relieves binding of the TETR protein to the operators and allows expression of the epimerase. Three conditional null mutant clones were obtained in this way, and Southern blots confirmed the replacement of both chromosomal *galE* alleles with antibiotic resistance genes (Fig. 4B).

To test whether the *galE* gene is essential, the conditional null mutant cells were washed three times in medium without tetracycline and cultures (with and without 1  $\mu$ g/ml of tetracycline) were inoculated at various cell densities. Cells were counted daily and cultures were split when densities approached  $3 \times 10^6$  cells per ml. In the presence of tetracycline, the cells continued to grow with normal (wild type) kinetics (Fig. 5A),

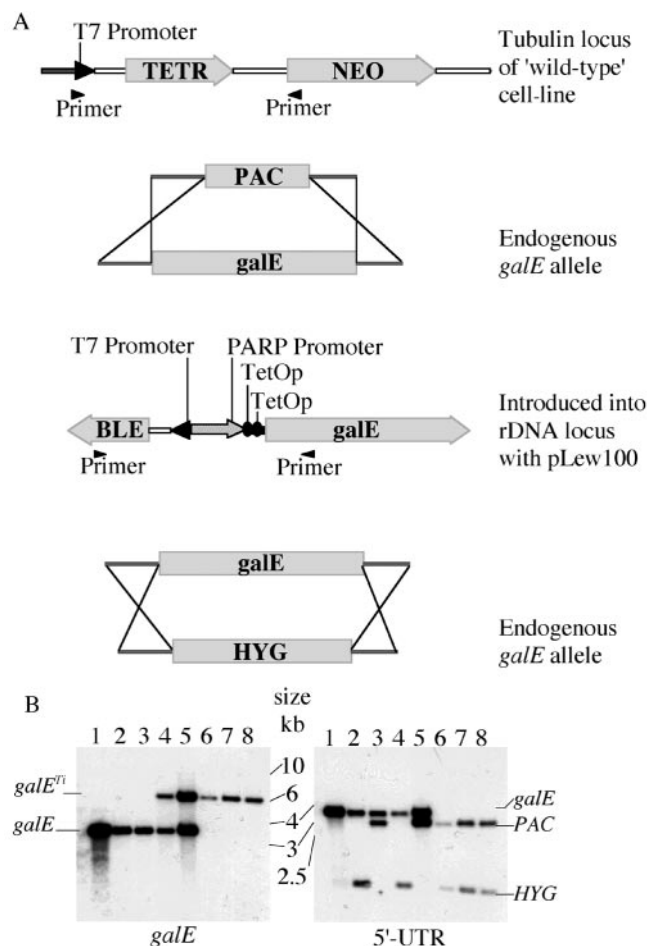


**Fig. 3.** Functionality of the *T. brucei galE* gene and recombinant protein. (A) Functional complementation of a *galE*-deficient strain of *E. coli*. Cells expressing *T. brucei galE* (RF0) from pUC18 formed dark red colonies on McConkey agar. When the epimerase is not expressed and the cells grow poorly. (B) Purification of recombinant *T. brucei* UDP-Glc-4'-epimerase; 10% SDS/PAGE gel stained with Coomassie blue of total *E. coli* lysate (lane 1), soluble supernatant (lane 2),  $\text{Ni}^{2+}$ -Sepharose purified protein (lane 3), and dialyzed purified protein (lane 4). (C) Lineweaver-Burk plot of purified recombinant *T. brucei* epimerase activity.

whereas in the absence of tetracycline the cells grew normally for 3–4 days, followed by rapid cell death (Fig. 5B). The few surviving cells failed to divide unless tetracycline was reintroduced at day 10 (Fig. 5C) or until day 16–20, when the cultures spontaneously started to grow once more at normal rates (Fig. 5B). Northern blot analysis showed that *galE* mRNA was more abundant in the conditional mutant than in wild-type cells, but that it became undetectable within 8 h of tetracycline removal (Fig. 5D). However, the cells continued to divide for 2 days, suggesting that it takes about 48 h for epimerase loss (due to dilution by cell division and protein turnover) to reach unsustainable levels. The Northern blot also showed that the cells that spontaneously recovered after 16 days in the absence of tetracycline were re-expressing *galE* mRNA.

#### The Mechanism of Re-Expression of *galE* by Conditional Null Mutants.

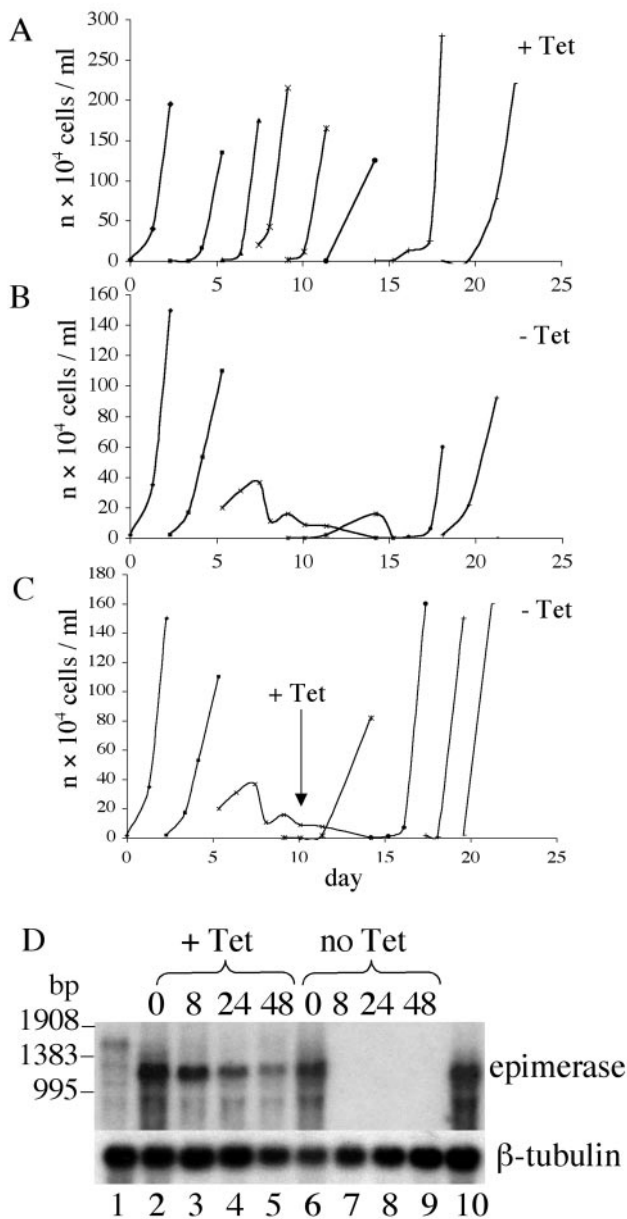
We analyzed the integrity of the genetic components of the tetracycline-inducible system in the parasites from three separate cultures that spontaneously recovered in the absence of tetracycline, as well as those of the conditional mutants grown continuously in the presence of tetracycline or starved of tetracycline for 10 days and then re-induced with the drug. Thus, DNA fragments containing the TetOp sequences upstream of the inducible *galE* gene and DNA fragments containing the tetracycline repressor protein (*TETR*) gene were amplified by PCR. The TetOp PCR products from all of the cells that had been transformed with the pLew100-*galE* construct were iden-



**Fig. 4.** Construction of *T. brucei* conditional *galE* null mutants. (A) Schematic representation showing the location of the *TETR* gene in the "wild-type" cells, the targeted replacement of the one *galE* allele with PAC, the introduction of an ectopic tetracycline-inducible copy of *galE* into the rDNA locus, and replacement of the second *galE* allele with HYG. The primers indicated are those used to amplify regions of interest in (Fig. 5). (B) Southern blot of conditional *galE* null mutants and intermediate cell lines. Aliquots ( $\approx 5 \mu\text{g}$ ) of *Pst*I-digested DNA were blotted with the *T. brucei galE* probe (Left) or the 5'-UTR probe (Right). The DNA was from wild-type *galE*<sup>+/+</sup> cells (lane 1),  $\Delta\text{galE}::\text{HYG}$  cells (lane 2),  $\Delta\text{galE}::\text{PAC}$  cells (lane 3), *galE*<sup>TI</sup>  $\Delta\text{galE}::\text{HYG}$  cells (lane 4), *galE*<sup>TI</sup>  $\Delta\text{galE}::\text{PAC}$  cells (lane 5), and conditional *galE*<sup>TI</sup>  $\Delta\text{galE}::\text{PAC}/\Delta\text{galE}::\text{HYG}$  null mutant clones derived from *galE*<sup>TI</sup>  $\Delta\text{galE}::\text{HYG}$  cells (lane 6) and from *galE*<sup>TI</sup>  $\Delta\text{galE}::\text{PAC}$  cells (lanes 7 and 8). The probe reveals allelic (*galE*) copies at 4 kb and ectopic tetracycline-inducible (*galE*<sup>TI</sup>) copies at 8 kb. The 5'-UTR probe shows differences when the allelic *galE* copies are replaced by the drug resistance genes. Note that the HYG gene contains a *Pst*I site and therefore shows a larger change.

tical (Fig. 6A). In contrast, the *TETR* gene appeared to be absent from the parasites that had spontaneously recovered from cultures without tetracycline, whereas it was present in the other cells (Fig. 6A). The absence of the *TETR* gene in the recovered cells was confirmed by Southern blot with a *TETR* probe (Fig. 6B). The deletion of the *TETR* gene was also evident from a Southern blot with a probe to the *NEO* gene (the adjacent selectable marker gene that allowed the selection of the *TETR*-expressing cell lines). The results suggests the deletion of  $\approx 4$ –5 kb of DNA, including most or all of the *TETR* gene, in all cases. The cells from the cultures rescued by reintroduction of tetracycline after 10 days appear to be a mixed population with some lacking the *TETR* gene and others retaining it.

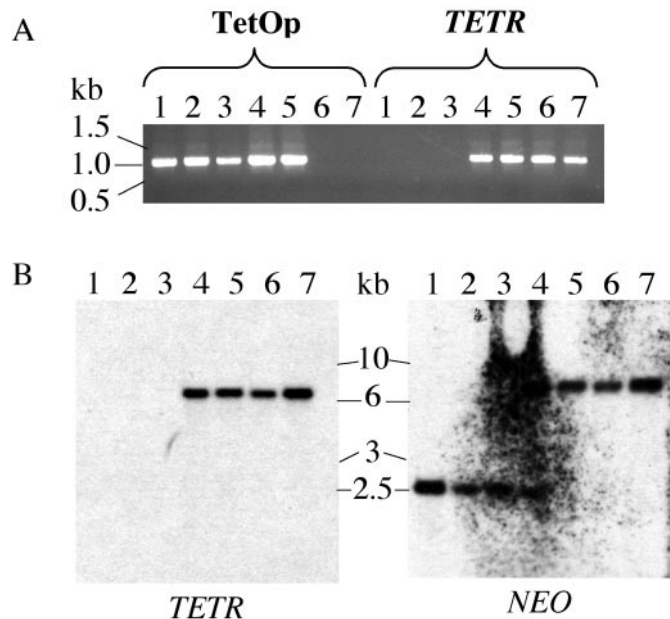




**Fig. 5.** Growth and Northern blots of the *T. brucei* conditional *galE* null mutants. Representative growth curves for one of the three conditional mutant clones are shown. Cells grown in the presence of tetracycline were washed at day 0 and reintroduced into tetracycline-containing medium (A; +Tet) or tetracycline-free medium (B and C; -Tet). Tetracycline was reintroduced into one of the cultures after 14 days (C). (D) Northern blot of total RNA prepared from "wild-type" *T. brucei* (lane 1) and conditional *galE* null mutants grown with (+Tet; lanes 2–5) and without (-Tet; lanes 6–9) tetracycline for 0, 8, 24, and 48 h and from cells that spontaneously re-emerged after 16 days without tetracycline (lane 10). The blot was probed with a *T. brucei galE* probe (Upper) and a  $\beta$ -tubulin probe (Lower).

## Discussion

Many glycoproteins of the bloodstream form of *T. brucei* contain galactose. The abundant surface coat VSG homodimers contain D-galactose ( $\beta$ -gal and  $\alpha$ -gal) in their glycosylphosphatidylinositol (GPI) anchor side chains and/or N-linked oligosaccharides (12). The *T. brucei* transferrin receptor (TfR), a VSG-like heterodimer present at about 3,000 copies in the flagellar pocket, the only site of endo- and exocytosis in the cell (13), contains one GPI anchor and seven potential N-glycosylation sites (14, 15).



**Fig. 6.** The mechanism of the recovery of the *T. brucei* conditional *galE* null mutants in the absence of tetracycline. (A) Ethidium-bromide-stained agarose gel of PCR products of the TetOp and *TETR* regions amplified from genomic DNA from conditional mutant cells from three cultures that recovered after 16 days (lanes 1–3), conditional mutant cells induced to grow after the reintroduction of tetracycline after 14 days (lane 4), conditional mutant cells grown continuously in the presence of tetracycline (lane 5), "wild-type" (*TETR*<sup>+</sup>) cells grown without tetracycline (lane 6), and "wild-type" (*TETR*<sup>+</sup>) cells grown with tetracycline (lane 7). (B) Southern blot of genomic DNA digested with *NotI* and *Sall* from the same cells described in A with *TETR* and *NEO* probes.

The TfR, like the lysosomal glycoprotein gp150/CB1-gp (16) and the invariant surface glycoprotein ISG100 (17), binds to tomato lectin, suggesting the presence of linear poly-N-acetyl-lactosamine (-3Gal $\beta$ 1-4GlcNAc $\beta$ 1-) repeats (18). It has been postulated that these  $\beta$ -Gal-containing structures may be involved in retaining the TfR, and other recycling components, in the *T. brucei* flagellar pocket and the endosomal/lysosomal membrane system (18). In addition to the aforementioned glycoproteins, five other transmembrane glycoproteins, ISG65, ISG75 (19), the ecto-phosphatase AcP115 (20), the Golgi- and lysosome-associated glycoprotein tGLP-1 (21), and the Fla-1 glycoprotein (22), the latter associated with flagellar adhesion to the cell body (23), are known to be glycosylated, but the nature of their carbohydrate chains is unknown. There are also other membrane proteins, such as Tb-29 (24), the cysteine-rich acidic membrane protein CRAM (25), the alanine-rich protein BARP (26), and the products of expression-site-associated genes (*ES-AGs*) 1, 2, 3, 4, 10, and 11 (26, 27), that are predicted to contain carbohydrate in the form of GPI anchors and/or N-linked oligosaccharides. Finally, the flagellar pocket of the parasite contains a filamentous matrix of unknown composition that binds both the  $\beta$ -Gal-specific lectin ricin (28) and tomato lectin (18). Taken together, it is clear that the bloodstream form of *T. brucei* parasites contains a significant amount and variety of glycoconjugate-associated galactose. However, the importance of galactose metabolism to parasite survival in culture or *in vivo* was unknown.

It had been noted by others that galactose does not compete with 2-deoxy-glucose for uptake through the parasite hexose transporter (3, 4), and our unsuccessful attempts to metabolically label *T. brucei* with [<sup>3</sup>H]Gal were consistent with this view. Thus, we surmised that the most likely source of galactose for glycoconjugate biosynthesis was UDP-Gal derived from UDP-Glc by

the action of UDP-Glc-4'-epimerase, and that the importance of galactose metabolism in general to the survival or infectivity of the parasite might be assessed by deletion of the *galE* gene encoding this enzyme from the parasite.

A TBLASTN search using the human UDP-Glc-4'-epimerase protein sequence revealed a fragment of a putative parasite *galE* gene that was cloned (Fig. 1) and shown to be functional by complementation of a *galE*-deficient *E. coli* mutant (Fig. 3A). Assay of the recombinant protein showed that it had a specific activity and  $K_m$  for UDP-Gal typical of UDP-Glc-4'-epimerases (ref. 11; Fig. 3C). Attempts to replace both chromosomal copies of the parasite *galE* gene were unsuccessful, even in the presence of 10 mM galactose in the medium (providing further evidence that the parasite cannot transport this sugar). However, a conditional null mutant could be created by introducing and expressing an ectopic tetracycline-inducible copy of the gene before replacement of the second chromosomal copy (Fig. 4). Withdrawal of tetracycline caused the rapid down-regulation of epimerase mRNA in these cells that was followed by cell death (Fig. 5). A few nondividing parasites survived in these cultures and could be revived by addition of tetracycline. Interestingly, these cultures spontaneously recovered after about 16 days without tetracycline.

The spontaneous recovery of *T. brucei* tetracycline-inducible conditional null mutants in the absence of tetracycline has been described previously but the mechanisms of reversion have not been described (10, 29). In this case, we analyzed the genotype of the recovered parasites and found that they had undergone a genetic rearrangement to delete the *TETR* transgene, thus becoming constitutive expressers of the ectopic *galE* gene. This escape mechanism confirms the essentiality of the *galE* gene, because parasite survival depended on a significant genetic rearrangement.

Based on current knowledge, one might speculate that a lack of UDP-Gal in the bloodstream form of *T. brucei* might be lethal because it affects the integrity of the VSG coat and/or the

uptake and/or recycling of glycoproteins like the TfR and ISG100, and/or it prevents the synthesis of the flagellar pocket matrix material of the parasite. Of these, effects on the uptake and/or recycling of the TfR may be the most acute because the receptor-mediated uptake of transferrin is known to be essential for cell growth (30). In any case, we may conclude that galactose metabolism is essential to the bloodstream form of *T. brucei*, and that we might usefully target the trypanosome UDP-Glc-4'-epimerase and/or downstream UDP-Gal transporters and/or UDP-Gal-dependent galactosyltransferases as potential drug targets. The latter group of enzymes may prove to be the most amenable to attack for three reasons. Firstly, the parasite makes several galactosyl structures that do not occur in the mammalian host (12), suggesting the presence of parasite-specific galactosyltransferases. Secondly, as a consequence, inhibitors may be designed from neutral parasite-specific acceptor substrate structures rather than from the common negatively charged UDP-Gal substrate. Thirdly, TBLASTN searches suggest that even conserved galactosyl structures (e.g., *N*-acetyl-lactosamine, Gal $\beta$ 1-4GlcNAc) are catalyzed by parasite enzymes that bare insufficient sequence similarity to their mammalian counterparts for identification and which, therefore, may be amenable to selective inhibition. Thus, the present study encourages further work to pinpoint specific galactosyl structures that are essential for parasite survival and to further investigate the therapeutic potential of affecting galactose metabolism in *T. brucei*.

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