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Transposon Mutagenesis of *Trypanosoma brucei* Identifies Glycosylation Mutants Resistant to Concanavalin A*

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We have engineered *Trypanosoma brucei* with a novel *mariner* transposition system that allows large populations of mutant cells to be generated and screened. As a proof of principle, we isolated and characterized two independent clones that were resistant to the cytotoxic action of concanavalin A. In both clones, the transposon had integrated into the locus encoding a homologue of human *ALG12*, which encodes a dolichyl-P-Man: $Man_7GlcNAc_2$-PP-dolichyl-α6-mannosyltransferase. Conventional knock-out of *ALG12* in a wild-type background gave an identical phenotype to the *mariner* mutants, and biochemical analysis confirmed that they have the same defect in the *N*-linked oligosaccharide synthesis pathway. To our surprise, both *mariner* mutants were homozygous; the second allele appeared to have undergone gene conversion by the *mariner*-targeted allele. Subsequent experiments showed that the frequency of gene conversion at the *ALG12* locus, in the absence of selection, was 0.25%. As we approach the completion of the trypanosome genome project, transposon mutagenesis provides an important addition to the repertoire of genetic tools for *T. brucei*.

The medical and economic relevance of *Trypanosoma brucei* and the many peculiarities of this differently evolved parasite (1–3) make it an interesting model eukaryote. Among the many important adaptations that occur during the life cycle of *T. brucei*, the change of its surface coat is crucial. In the infectious bloodstream and metacyclic forms, the cell surface is covered by the variant surface glycoprotein. Upon differentiation in the tsetse (*Glossina*), variant surface glycoprotein is shed and replaced by members of the procyclin family of GPI[1]-anchored glycoproteins. There are two major classes of procyclins, dubbed EP- and GPEET-procyclins, according to the sequence of amino acid repeats that comprise about 50% of the protein (4). EP-procyclins contain up to 30 Glu-Pro repeats, whereas GPEET-procyclins have five or six Gly-Pro-Glu-Glu-Thr repeats followed by the sequence $(Glu\text{-}Pro)_3$-Gly (5–7). In *T. brucei* Lister 427, the EP-procyclin isoforms are encoded by the *EP1*, *EP2*, and *EP3* genes (4, 8). Only the products of *EP1* and *EP3* contain a glycosylation site (Asn^{29}), which is modified exclusively by a $Man_5GlcNAc_2$ glycan (7, 9). Procyclins are important for survival in the tsetse (10–12) but can be dispensed with in culture, after a period of adaptation that involves increased expression of free GPIs on the parasite surface (13).

Despite the major advances in reverse genetics approaches to study *T. brucei* (14, 15), tools that would allow large scale functional analysis of its genome are urgently needed. Even though African trypanosomes undergo some so far uncharacterized forms of genetic exchange in the tsetse salivary glands (16), just the fact that this only happens in the fly severely restricts its application to genetic analysis. On the other hand, transposon mutagenesis could potentially provide a valuable tool for forward genetics in *T. brucei*. Members of the *mariner*/Tc1 superfamily are probably the most widespread DNA transposons in nature (17, 18). Transposition occurs via a nonreplicative cut-and-paste mechanism, in which a staggered double-stranded break at each end of the transposable element releases it from the donor DNA molecule, freeing it to be ligated into a staggered cut at the target site (19). Transposition is independent of host-specific factors (20), which has allowed *mariner* to be used for gene disruption in a variety of organisms, including *Leishmania* (21), nematodes (22), and vertebrates (23, 24).

We have used the MosI mariner transposon from *Drosophila mauritiana* (25). This 1.3-kb transposon is flanked by 28-bp inverted repeats and encodes a single protein of 345 amino acids, the transposase. MosI integrates at a TA dinucleotide, which is duplicated upon insertion (reviewed in Ref. 17). By expressing MosI transposase constitutively, from a chromosomally integrated gene, and supplying a transposable donor cassette on a single-copy autonomously replicating plasmid (26), we were able to build a system that gives a high transposition efficiency in cell populations of unlimited size, which would not be possible if the system depended upon transient transfection of the *mariner* components.

As a test of the system, we decided to select mutants that resisted the cytotoxic effects of concanavalin A (ConA), which binds to the *N*-glycans on the EP1 and EP3 procyclins and kills

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[1] The abbreviations used are: GPI, glycosylphosphatidylinositol; ALG, asparagine-linked glycosylation; ConA, concanavalin A; IR, inverted repeat; LOH, loss of heterozygosity; OSL, oligosaccharide-lipid; ORF, open reading frame; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS, mass spectrometry; PNGase F, N^4(*N*-acetyl-β-glucosaminyl) asparagine amidase F; RNAi, RNA interference; PP, pyrophosphoryl; HF, hydrogen fluoride.

procyclic *T. brucei* via an unknown mechanism that resembles programmed cell death in metazoa (27, 28). ConA has been used to isolate glycosylation mutants of *T. brucei* after chemical mutagenesis (29), but the lack of methods for genetic mapping prevented the mutated genes from being identified.

From a *T. brucei* population selected for *mariner* transposition events, we isolated and characterized two independent mutants, in which the transposon had disrupted a previously uncharacterized ORF at different sites. The ORF encodes a probable orthologue of the ALG12 mannosyltransferase. Mutations at the asparagine-linked glycosylation (ALG) pathway are the origin of a number of severe inherited human diseases, known collectively as congenital disorders of glycosylation (30). Cloning of human *ALG12* showed that mutation in this locus is the underlying cause of a new subtype of congenital disorders of glycosylation, type I (31). Targeted deletion of both *ALG12* alleles in wild-type *T. brucei* produced the same phenotype as the *mariner* mutants. The *N*-glycan precursor in wild-type *T. brucei* is $Man_9GlcNAc_2$-PP-dolichol, whereas *ALG12* mutants make a smaller precursor, $Man_7GlcNAc_2$-PP-dolichol.

EXPERIMENTAL PROCEDURES

Constructs and Cell Lines—One copy of the MosI transposase coding sequence from pBluescribe M13+/MosI (25) was targeted to the *TUBULIN* locus on chromosome I of wild-type procyclic *T. brucei* of the Lister 427 strain by transfection with NotI-linearized pSgl29, which was derived by several cloning steps from a version of the *TUBULIN*-targeting construct pHD309 (32) containing a puromycin resistance gene in place of hygromycin phosphotransferase. The donor transposon cassette, pSgl33 (see Fig. 1*A*), is a derivative of the single-copy autonomously replicating *T. brucei* plasmid pT13-11 (26). As an intermediate step, pBSMos1Hyg was made by cloning the hygromycin phosphotransferase gene, flanked by 5′ and 3′ *ACTIN*-derived splicing and polyadenylation signals (the SmaI-StuI fragment of pHD309), into the SalI site in the transposase ORF of pBluescribe M13+/MosI. The XhoI-HindIII fragment from pBSMos1Hyg was blunt end-cloned into the EcoNI site of pT13-11 to create pSgl33. *ALG12* knock-out constructs were made by PCR amplifying 300 bp of genomic sequences flanking the *ALG12* ORF and inserting selectable marker cassettes between them. The neomycin (pSgl39) and hygromycin (pSgl41) cassettes, both containing *ACTIN*-derived splicing and polyadenylation signals, were obtained by SmaI-StuI digestions of pLew114 and pHD309 (33), respectively. Bloodstream and procyclic *ALG12* null mutants were obtained by sequentially transfecting wild-type *T. brucei* with NotI-linearized pSgl39 and pSgl41. Elimination of the *ALG12* gene was confirmed by Southern hybridization. Trypanosomes were cultivated and transfected as previously described (32).

DNA Analysis—Southern analysis was carried out according to standard protocols (34). Approximately 2 μg of genomic DNA was run on an 0.8% agarose gel and transferred to Hybond N membranes (Amersham Biosciences) by standard procedures. The probes were generated using a Prime-It II kit (Stratagene) with gel-purified DNA. Hybridization and washing of membranes were carried out at 65 °C in a hybridization oven (Hybaid). Post-hybridization washes were for 30 min at 65 °C in 0.1× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate) and 0.1% SDS. The hybridization signals were detected by autoradiography. For pulsed field gel electrophoresis of chromosomal DNA, agarose blocks of the different cell lines were prepared as described previously for mammalian cells (34) at a final cell concentration of 2×10^8 ml^{-1}. The parasites were resuspended in LB (0.10 M EDTA, pH 8, 0.010 M Tris-HCl, 0.020 M NaCl) and mixed with 1 volume of 1.6% low melting point agarose (Sigma) in LB. Lysis was carried out by two sequential treatments with 1% lauroyl sarcosine and 1 mg ml^{-1} proteinase K in LB for 48 h at 50 °C. Electrophoresis was performed in a contour clamped homogeneous electric field apparatus (CHEF DRII; Bio-Rad). *Saccharomyces cerevisiae* (New England Biolabs), *Schizosaccharomyces pombe* (Bio-Rad), and *Hansenula wingei* (Bio-Rad) chromosomes were electrophoresed concurrently. The electrophoresis parameters were 1% agarose, 1400–700-s linear ramped pulse times, 144 h, 2.5 V cm^{-1}, 0.5× TBE (0.045 M Tris borate, pH 8, 1 mM EDTA) with buffer recirculation at 15 °C. The chromosome bands were visualized by staining with 0.5 mg ml^{-1} ethidium bromide. The gels were blotted onto Hybond N membranes (Amersham Biosciences) by standard procedures. Transfer, hybridization, and washing of membranes were carried out as described above.

Identification of Mariner Genomic Insertion Sites—Genomic DNA from hygromycin^R clones was isolated, digested with HindIII, self-ligated under dilute conditions, and then used as template in PCRs, using primers SG3 (GGCAAATACTTTGAATAA, beginning 67 bp from the *mariner* 3′ inverted repeat (IR)) and SG4 (TTTATGACAATCGATAAA, beginning 49 bp from the *mariner* 5′ IR) with the Expand Long Template PCR system (Roche Applied Science). The resulting PCR products were purified, sequenced, and analyzed by a BLAST search of *T. brucei* sequence data bases.

ConA Screening—6×10^7 transposase-expressing procyclic trypanosomes were transfected with the *mariner* donor plasmid (pSgl33) and amplified for 9 days in the presence of 50 μg ml^{-1} G418. Approximately 4×10^8 G418^R cells were washed and resuspended in SDM-79 containing 25 μg ml^{-1} of hygromycin. The cells were grown for 10 days to allow expression of mutant phenotypes. The culture (100 ml) was treated with 2 μg ml^{-1} of ConA (Sigma) overnight and then centrifuged ($50 \times g$ for 5 min) to eliminate large clumps of cells. One round of ConA addition, centrifugation, and recovery for a few days in the absence of ConA is considered a "treatment." We performed one more treatment with 2 μg ml^{-1} of ConA, five with 5 μg ml^{-1} of ConA, and one with 10 μg ml^{-1} of ConA. The entire procedure took approximately 1 month.

Identification of TbALG12 Gene—The hygromycin and ConA-resistant cells were cloned, and the *mariner* integration sites were determined as described above, except that a different pair of primers was used: SG16 (CGGCACGAAACTCGACATGTTGACTGCACT, beginning 161 bp from the *mariner* 5′ IR) and SG17 (CGAAGACGCCGAACTGCAAGCATTATTGGA, beginning 872 bp from the *mariner* 3′ IR). The inverse PCR product was cloned into pGEM-T Easy vector (Promega) after A-tailing (performed according to the manufacturer's directions). The cloned DNA was sequenced and analyzed as described above.

Preparation of Cell Lysates and Radiolabeling of Oligosaccharide-Lipids—Hypotonic lysates of wild-type, *ALG12*$^{+/-}$, *ALG12*$^{-/-}$, and *mariner* ConA^R trypanosomes were prepared as described (35), except that tunicamycin was omitted during preparation of the membranes. For labeling oligosaccharide-lipid (OSL) and other glycolipids, we used a protocol designed for GPI synthesis (35). Thawed cell lysates (1 ml) were washed twice with 10 ml of HKML buffer (50 mM HEPES, pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, 1.25 μg ml^{-1} leupeptin) by centrifugation ($4{,}550 \times g$ for 10 min at 4 °C). The membranes (1×10^8 cell equivalents) were suspended in HKML buffer and incubated for 1–2 min at 27 °C with 5 mM $MnCl_2$ and 1 mM dithiothreitol. Mannose-containing glycolipids were pulse-labeled by transferring the membranes (5×10^7 cell equivalents) into another tube containing GDP[3,4-^{3}H]Man (0.5 μCi ml^{-1}, 18.3 Ci mmol^{-1}; PerkinElmer Life Sciences) and 2 mM UDP-GlcNAc for 5 min and chased with 1 mM nonradioactive GDP-Man for 20 min at 27 °C. The reactions were terminated by adding $CHCl_3$/CH_3OH (1:1, v/v) to give a final $CHCl_3$/CH_3OH/H_2O (10:10:3, v/v/v), and the lipids were extracted for 10 min in a bath sonicator. After centrifugation for 5 min at $14{,}000 \times g$, the insoluble debris was re-extracted with 200 μl of $CHCl_3$/CH_3OH/H_2O (10:10:3, v/v/v) under sonication and recentrifuged. The pool of organic supernatants was dried under a stream of nitrogen, and the lipids were extracted by adding 100 μl of *n*-butanol and 100 μl of water. After a quick centrifugation, the organic upper phase was saved, and the lower aqueous phase was re-extracted twice with 100 μl of water-saturated *n*-butanol. The pooled organic phases were then dried in a SpeedVac concentrator and resuspended in 10 μl of $CHCl_3$/CH_3OH/H_2O (10:10:3, v/v/v) for thin layer chromatography on silica gel 60 TLC plates (Merck), where lipids were resolved using the same solvent. For glycan analysis, the samples were fractionated on a predried TLC silica gel 60 (Merck) by running three times with *n*-butanol/acetone/water (6:5:4, v/v/v). For autoradiography, the plates were sprayed with En^{3}Hance (DuPont) and exposed to x-ray film (Amersham Biosciences) at −80 °C.

Mild Acid Hydrolysis of OSL—Dried [^{3}H]Man-labeled lipids were resuspended in 100 μl of 0.1 M HCl and heated at 100 °C. After 10 min, the tubes were cooled to 0 °C, and the released glycans were separated from glycolipids. *n*-Butanol (100 μl) was added, and the sample was equilibrated twice with 100 μl of water-saturated *n*-butanol. Glycans, in the aqueous phase, were dried in a SpeedVac and dissolved in 40% 1-propanol for TLC analysis using *n*-butanol/acetone/water (6:5:4, v/v/v).

MALDI-TOF-MS Analysis of Procyclins—For mass spectrometry, procyclins were purified from 2×10^8 freeze-dried parasites by sequential extraction with organic solvents (7, 9). To remove GPI anchors from the procyclins, the dry butanolic extracts were treated with 25 μl of 48% aqueous hydrofluoric acid for 14 h at 0 °C. After treatment, the samples were immediately frozen in dry ice ethanol, dried in a SpeedVac, and resuspended in 20 μl of 0.1% trifluoroacetic acid. An aliquot (~0.5 μl; ~5×10^6 parasite equivalents) was co-crystallized with α-cyano-4-

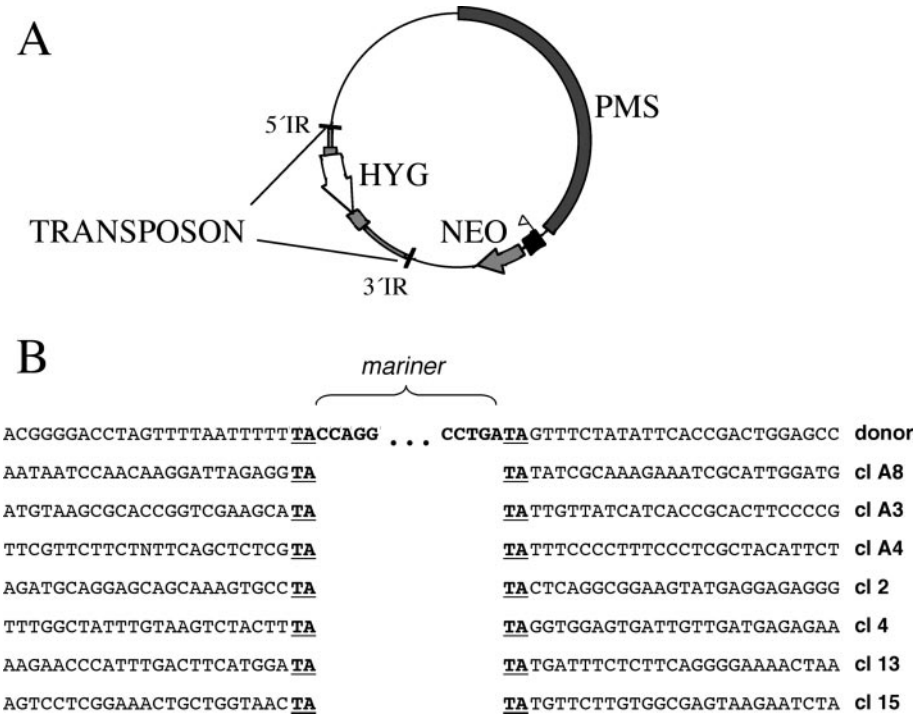
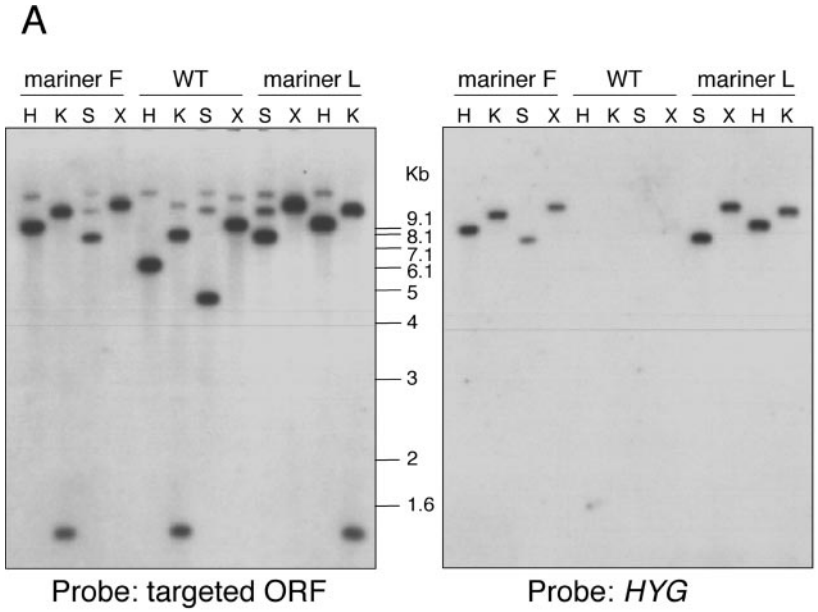


FIG. 1. **Transposition of *mariner* in *T. brucei*.** *A*, donor transposon vector (pSgl33) contains the plasmid maintenance sequence (*PMS*) (26), the procyclin *GPEET* promoter (represented by a *flag*) driving a *NEO* cassette flanked by *GPEET*-derived untranslated regions, and the modified MosI donor element, which contains a transposable cassette in which *HYG* is flanked by *T. brucei ACTIN*-derived untranslated regions and *mariner* IRs. *B*, DNA sequence of *mariner* insertions in representative hygromycin-resistant clones. The TA dinucleotides at the boundaries of the element are in *bold type*. The sequences flanking the transposon cassette in the donor plasmid are shown for comparison with the sequences at the insertion sites.



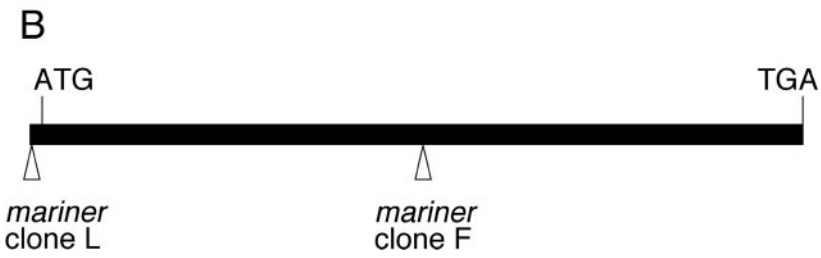


FIG. 2. **Mariner disrupted the same ORF in both ConA^R clones.** *A*, Southern analysis of *mariner* clones. Approximately 2 μg of genomic DNA was digested with HindIII, KpnI, SmaI, or Xho (*H*, *K*, *S*, or *X*, respectively). The blot was probed with the target ORF (*left panel*), then stripped, and reprobed with the *HYG* ORF (*right panel*). The faint more slowly migrating bands in some lanes are due to incomplete digestion. *B*, schematic representation of *mariner* insertions in ConA^R clones F and L. *WT*, wild type.

hydroxycinnamic acid as the matrix and analyzed in a PerSeptive Biosystems Voyager Elite mass spectrometer (7). To confirm assignments, HF-treated samples (2 μl, $\sim 2 \times 10^7$ parasite equivalents) were submitted to mild acid hydrolysis with 40 mM trifluoroacetic acid for 15 min at 100 °C, and an aliquot was analyzed by MALDI-TOF-MS as described above (7).

Enzymatic Deglycosylation of Procyclin Polypeptides—Aqueous HF-treated ($\sim 5 \times 10^7$ parasite equivalents) procyclins from wild-type, *ALG12*$^{+/-}$, *ALG12*$^{-/-}$, and *mariner* ConA mutants were deglycosylated with 250 units of peptide N^4(*N*-acetyl-β-glucosaminyl) asparagine amidase F (PNGase F; New England Biolabs) in 10 μl of 25 mM sodium phosphate, pH 7.5, at 37 °C for 2 h. To confirm the presence of a terminal *N*-acetyllactosamine group in procyclins from *ALG12*$^{-/-}$ and *mariner* Con-A mutants, aqueous HF-treated proteins ($\sim 5 \times 10^7$ parasite equivalents) were incubated with a mixture of 1 unit ml^{-1} of bovine testis β-galactosidase (GLYKO) and 10 units ml^{-1} of jack bean β-hexosaminidase (GLYKO) in 100 mM of sodium citrate, pH 4.5, at 37 °C for 18 h. After digestion, the samples were desalted using a ZipTip containing C18 silica

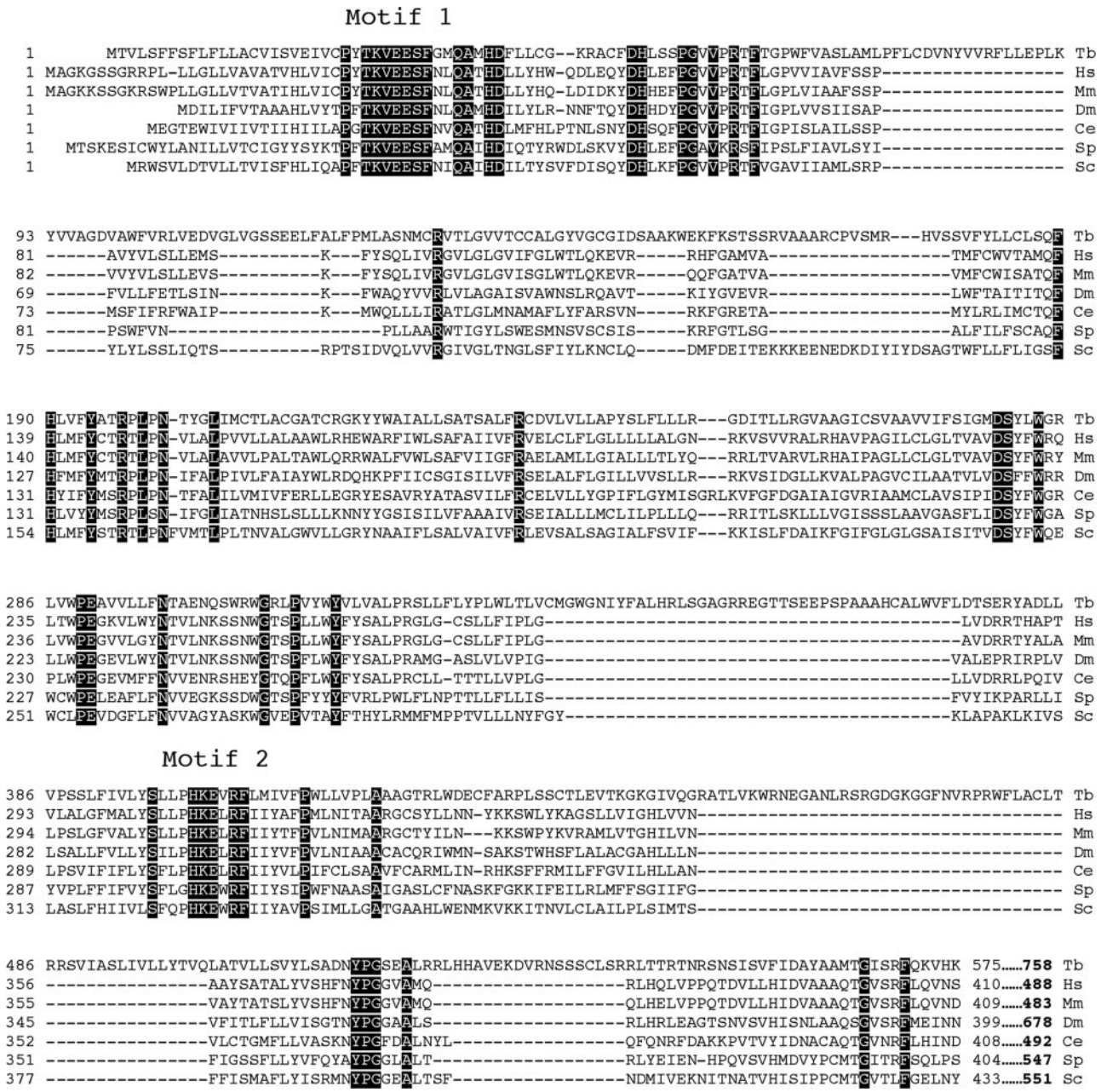


FIG. 3. **ALG12 sequence alignment.** *T. brucei* ALG12 (www.genedb.org, ORF Tb927.2.4720) aligned with its human (GenBank™ accession number AJ303120), *Mus musculus* (AJ429133), *D. melanogaster* (AE003684), *C. elegans* (U53155), *S. pombe* (AL031856), and *S. cerevisiae* (Z71645) homologues. The sequences were aligned using the Clustal W algorithm implemented in MegAlign (Lasergene/DNASTAR). Invariant residues are highlighted on a *black background*. The alignments are truncated. Beyond the indicated positions, there are no amino acids that are identical in all species. The *final number* indicates the total length of each protein.

(Millipore Corp.), as described by the manufacturer, and an aliquot was analyzed by MALDI-TOF-MS as described above.

RESULTS

Transposition in T. brucei—To circumvent the low transfection efficiency of *T. brucei*, we started with the objective of establishing permanent cell lines that could be amplified prior to selecting for transposition and screening or selecting for specific phenotypes. The approach that we used was to split the two components of *mariner*, creating a donor transposon containing a selectable marker that could only be expressed after transposition into a transcribed region of a chromosome and providing tranposase activity *in trans*. Some early experiments that failed to result in transposition included attempts to mobilize a chromosomally integrated transposon cassette by placing a chromosomally integrated copy of transposase under the control of an inducible promoter. On the other hand, co-transfection of separate plasmids encoding the transposase and a donor cassette resulted in transposition into the genome, so it was clear that, in principal, *mariner* could function in *T. brucei*.

The approach that achieved our objectives was to create a cell line in which the *mariner* transposase gene was integrated into the *TUBULIN* locus on chromosome I, where it is constitutively expressed by read-through transcription, and cloning the donor element into pT13-11, an autonomously replicating single-copy episome (26), creating pSgl33 (Fig. 1*A*). pSgl33 is stable under G418 selection but does not confer hygromycin resistance; *HYG* is in the opposite orientation to the direction of transcription, which originates from the single EP-1 procyclin promoter that drives *NEO* expression. Transposase-expressing trypanosomes were transfected with pSgl33 and amplified in the presence of

G418, which was subsequently replaced by hygromycin, and the cells were distributed into multi-well plates. Hygromycin-resistant clones were observed at a frequency of ~5% (240 cells were distributed per 24 wells in a typical experiment, yielding ~12 clones/plate). Southern analysis of these clones suggested that the donor element had transposed to different regions of the genome (data not shown). The *mariner* integration sites from several clones were recovered by inverse PCR. Sequence analysis (Fig. 1*B*) showed that they all contained the *mariner* cassette flanked by a TA dinucleotide and sequences that were absent from the donor plasmid DNA but were represented in the *T. brucei* genome data base.

ConA Screening and Identification of the TbALG12 Gene—To test the potential for isolating specific mutants with this system, we chose to screen procyclic *T. brucei* for resistance to ConA, which was expected to identify genes involved in *N*-glycosylation. *T. brucei* mutants with reduced ConA binding had been isolated after chemical mutagenesis (29, 36) but, although these mutants were biochemically characterized, the genetic defect responsible for the altered phenotype could not be identified.

The transposase-expressing cell line was transfected with donor plasmid pSgl33, and the population was amplified under G418 selection. The G418-resistant population was then washed, split, and grown either in the absence or presence of 25 μg ml^{-1} of hygromycin for 10 days to allow full expression of any mutant surface glycoproteins before adding ConA. After the sixth cycle of ConA treatment, only the cells growing in the presence of hygromycin no longer agglutinated. After two more cycles of ConA treatment, an analysis of ConA binding by flow cytometry confirmed the low affinity of the surviving cells for ConA. Preliminary DNA analysis (data not shown) suggested that we had a mixed population of mutants that were resistant to both hygromycin and ConA. After cloning by limiting dilution, Southern blot analysis of individual clones showed two patterns (data not shown). One clone (F and L) of each type was selected for further characterization. Once the integration site in clone F was amplified, cloned, and sequenced, primers were made to amplify the flanking sequences of the targeted ORF and the ORF itself. DNA analysis (Fig. 2*A*) showed that the plasmid-derived transposable cassette had integrated into the same gene in both clones. More surprising was the absence of bands characteristic of the wild-type allele in both mutants (see below). The insertion site in clone F was a TA dinucleotide in the middle of the gene, whereas the insertion in clone L had occurred in a TA located 7 bp upstream of the translation initiation codon (Fig. 2*B*). BLAST searching identified the target as an unannotated ORF on chromosome II.

The disrupted ORF appeared to be an orthologue of the human *ALG12* gene, which encodes dolichyl-P-Man: $Man_7GlcNAc_2$-PP-dolichyl α6-mannosyltransferase (31). The predicted amino acid sequences of *T. brucei*, *Caenorhabditis elegans*, *Drosophila melanogaster*, budding yeast, fission yeast, mouse, and human ALG12 are aligned in Fig. 3. TbALG12 has 38 and 33% similarity with *S. cerevisiae* and human ALG12, respectively. Motif 1 (TKVEESF) is conserved only in the ALG12 family of α_6-mannosyltransferases, whereas motif 2 (the sequence HKE*X*RF flanked by hydrophobic regions) also occurs in the α_2-mannosyltransferases (37). *ALG12* is a single-copy gene on chromosome II, and Northern analysis showed that it is expressed in both procyclic and bloodstream *T. brucei* (data not shown).

ConA Resistance Phenotype Is Due to Gene Conversion—*T. brucei* is diploid, so transposon-mediated insertional mutagenesis was expected to result in the isolation of heterozygous cells. However, restriction mapping (Fig. 2*A*) and pulsed field gel electrophoresis separation of chromosomal DNA (Fig. 4) suggested that both *mariner* clones F and L had undergone a gene conversion-mediated loss of heterozygosity (LOH). Conventional targeted deletion of the *ALG12* ORF from wild-type cells showed that the wild-type and *ALG12*$^{+/-}$ heterozygotes were equally sensitive to ConA; deletion of both alleles was necessary to confer resistance (data not shown).

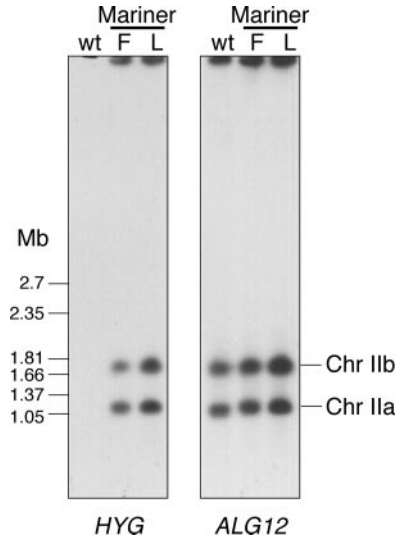


FIG. 4. **Chromosomal DNA analysis as evidence for *mariner*/*ALG12* gene conversion.** Agarose blocks of wild-type (*wt*) cells and ConA^R clones F and L were separated by pulsed field gel electrophoresis, blotted, and probed with the *HYG* ORF. The blot was stripped and reprobed with *ALG12*. *Mb*, mega base pairs of markers.

Mariner ConA^R Mutants Express Procyclins with Altered Glycosylation—To biochemically characterize the changes in the surface glycans of ConA^R clones, we extracted procyclins and analyzed their polypeptides by negative ion MALDI-TOF-MS, after removal of their GPI anchors. MS analysis of aqueous HF-treated wild-type procyclin revealed only three major [M-H]$^-$ pseudomolecular ions at *m/z* 11,531, 10,430, and 9,723 (Fig. 5*A* and Table I), which match the expected masses of the glycosylated products of the EP-procyclin genes *EP1-1*, *EP1-2*, and *EP3*, respectively (7, 8). *EP1-1* and *EP1-2* are allelic genes; their products differ only in the number of EP repeats. The assignments suggest that each procyclin polypeptide carries a $Hex_5HexNAc_2$ glycan, which corresponds to the previously characterized $Man_5GlcNAc_2$ (9). No traces of the nonglycosylated EP2 and GPEET procyclins (*m/z* 8,344 and 6,142, respectively) were detected. In contrast, analysis of aqueous HF-treated procyclins from *mariner* ConA^R, clone F (Fig. 5*B*), and clone L (not shown) showed a different profile of [M-H]$^-$ pseudomolecular ions in the same *m/z* range (9,000–12,000). The assignments are consistent with each EP procyclin species (EP1-1, EP1-2, and EP3) having been modified by a glycan of composition $Hex_4HexNAc_2$ (species A) or $Hex_5HexNAc_3$ (species B) (see Table I for mass assignments). There is also a small amount of unglycosylated EP1-2, and small amounts of unglycosylated EP3 and EP1-1 are probably also present but obscured by other peaks. PNGase F treatment of aqueous HF-treated procyclins yielded peaks of identical mass (*m/z* 8,506, 9,213, and 10,314), corresponding to the unglycosylated polypeptides from wild-type and *mariner* ConA^R clones (Fig. 6). Finally, analysis of the C-terminal fragments after mild trifluoroacetic acid hydrolysis, which cleaves the EP procyclins at their mild acid-labile Asp-Pro bonds (7), showed the same characteristic C-terminal ions from EP1-1, EP1-2, and EP3 (*m/z* 7,001, 5,870, and 5,191) in all samples (data not shown). Taken together, these analyses suggest that, in contrast to wild-type

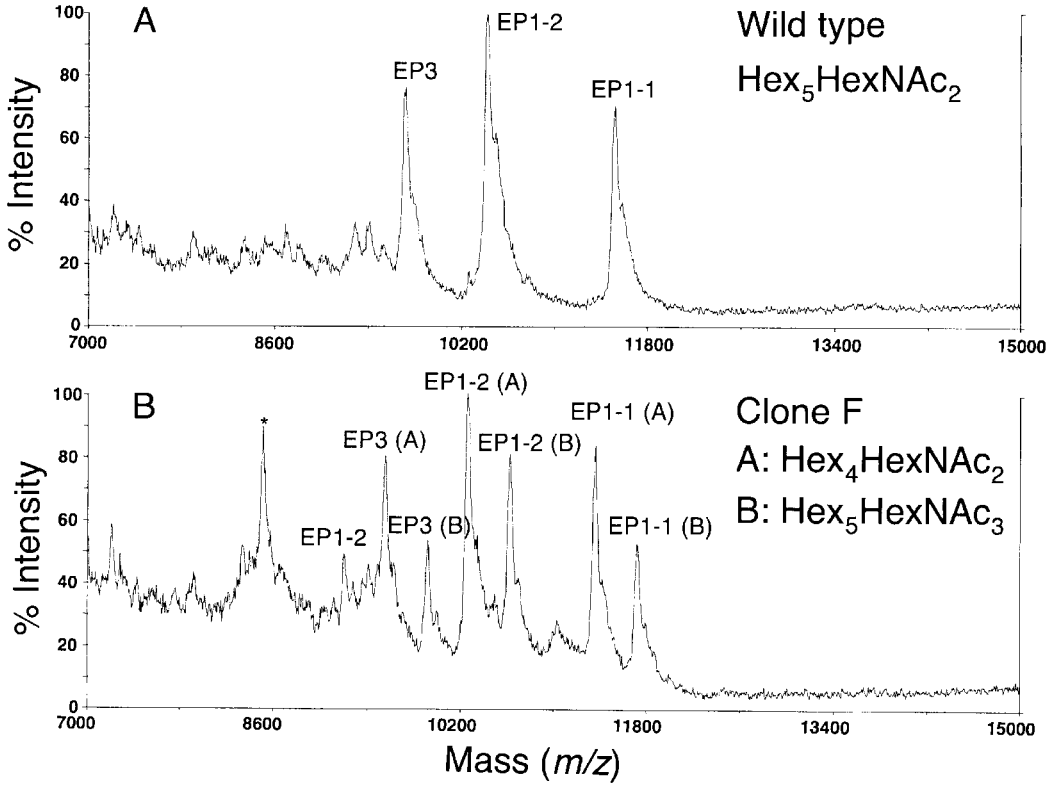


FIG. 5. **Negative ion MALDI-TOF mass spectra of wild-type and *mariner* ConA^R clone F procyclins.** *A*, analysis of wild-type procyclin polypeptides after removal of GPI anchors by aqueous HF. See text and Table I for assignment of ions. *B*, analysis of the ConA^R clone F procyclin polypeptides after removal of GPI anchors by aqueous HF. Species A and B contain $Hex_4HexNAc_2$ and $Hex_5HexNAc_3$ glycans, respectively. The *asterisk* indicates a contaminant.

TABLE I
Procyclin species observed in the mass spectrum of wild-type, ALG12$^{+/-}$, ALG12$^{-/-}$, and mariner ConA^R clones by negative ion MALDI-TOF-MS (Figs. 5–7)

Ion no.	Mass[a]	Number of EP repeats[b]	Type of glycan	Assignment
1	11,531	30	$Hex_5HexNAc_2$	EP1-1[c]
2	9,213	25	None	EP1-2[d]
2	10,430	25	$Hex_5HexNAc_2$	EP1-2[c]
3	9,723	22	$Hex_5HexNAc_2$	EP3[c]
4	11,369	30	$Hex_4HexNAc_2$	EP1-1(A)[e]
5	11,734	30	$Hex_5HexNAc_3$	EP1-1(B)[e]
6	10,268	25	$Hex_4HexNAc_2$	EP1-2(A)[e]
7	10,633	25	$Hex_5HexNAc_3$	EP1-2(B)[e]
8	9,561	22	$Hex_4HexNAc_2$	EP3(A)[e]
9	9,926	22	$Hex_5HexNAc_3$	EP3(B)[e]

[a] Determined as $[M-H]^-$.

[b] The number of EP repeats was also confirmed by analysis of the C-terminal fragments after trifluoroacetic acid mild acid hydrolysis of the Asp-Pro bonds (not shown).

[c] Form containing a $Hex_5HexNAc_2$ glycan and ethanolamine linked to the C-terminal glycine. This species was observed only in procyclins from wild-type and *ALG12$^{+/-}$* cells (Fig. 5*A*).

[d] Form with ethanolamine linked to the C-terminal glycine but missing the *N*-glycan chain. This species was observed only in procyclins from ALG12$^{-/-}$ and *mariner* clones (Figs. 5*B* and 7*A*).

[e] Glycosylated polypeptide with altered *N*-glycan chain and an ethanolamine linked to the C-terminal glycine. This species was observed only in procyclins from *ALG12$^{-/-}$* and *mariner* clones (Figs. 5*B* and 7*A*).

cells, which express glycosylated EP procyclins bearing a $Hex_5HexNAc_2$, both *mariner* ConA^R clones express the same proteins with altered glycosylation, predominantly modified by a shorter high mannose glycan ($Hex_4HexNAc_2$) or a hybrid-type glycan with an extra HexHexNAc sequence that is probably a terminal *N*-acetyllactosamine (see below).

Analysis of Procyclins from ALG12$^{-/-}$ Cells—To confirm that the altered glycosylation of procyclins from the *mariner* ConA^R clones is a direct consequence of *ALG12* disruption, we analyzed the glycosylation of EP procyclins from *ALG12$^{-/-}$* cells made by targeted deletion. Although disruption of one allele did not detectably affect glycosylation, as determined by MS analysis (data not shown), procyclins from *ALG12$^{-/-}$* cells (Fig. 7*A*) revealed the same $[M-H]^-$ pseudomolecular ions detected in both *mariner* clones, including a small amount of nonglycosylated EP1-2. Inspection of the ions produced after mild acid hydrolysis of the Asp-Pro bonds (data not shown) and PNGase F (Fig. 7*C*) confirmed that the *ALG12$^{-/-}$* cells only express procyclins EP1-1, EP1-2, and EP3. Thus, *ALG12$^{-/-}$* cells also express procyclin proteins modified with a $Hex_4HexNAc_2$ and $Hex_5HexNAc_3$ (species A and B, respectively).

The presence of peptides carrying a $Hex_5HexNAc_3$ glycan (denoted as species B) suggests that one of the terminal αMan residues is capped with an *N*-acetyllactosamine (βGal-βGlcNAc) repeat that is similar to the modification found in the *T. brucei* ConA^R clone ConA 1-1 (29, 38). To confirm this interpretation, we incubated aqueous HF-treated *ALG12$^{-/-}$* procyclins with a mixture of bovine testis β-galactosidase and jack bean β-hexosaminidase. Simultaneous incubation with these enzymes removes *N*-acetyllactosamine units (9). As shown in Fig. 7*B*, this treatment eliminated species B but species A remained intact, confirming that only the former glycopeptides contain a terminal *N*-acetyllactosamine. Identical results were obtained after treatment of procyclins from both *mariner* ConA^R clones (data not shown).

Analysis of the OSL Precursor from Mariner ConA^R Mutants and ALG12$^{-/-}$ Cells—A defect in *ALG12* implies that *mariner* mutants should make OSL precursor with a shorter glycan core, which, in turn, will account for the altered glycan structure on their procyclins. To characterize the OSL precursor,

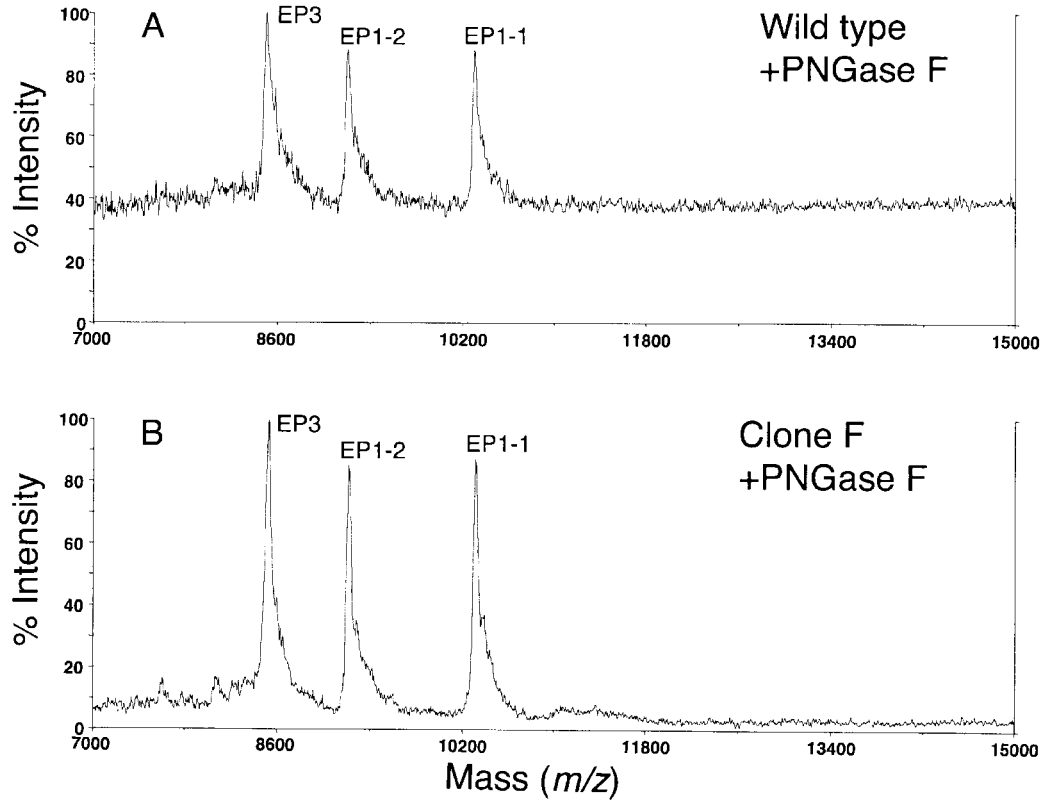


FIG. 6. **Negative ion MALDI-TOF mass spectra of aqueous HF-treated procyclins after PNGase F deglycosylation.** *A*, wild type. *B*, *mariner* ConA^R clone F.

glycolipids were synthesized in a cell-free system consisting of washed trypanosome membranes. Under these conditions, incubation with UDP-GlcNAc and GDP-[^{3}H]Man results in labeling of both OSL and GPI precursors and their biosynthetic intermediates (35). Although the mature OSL ($Man_9GlcNAc_2$-PP-lipid) was synthesized in the wild-type cell-free system, as expected (39), and by *ALG12*$^{+/-}$ membranes (Fig. 8*A*, *first* and *second lanes*), it was absent from the *ALG12*$^{-/-}$ and the two *mariner* mutant samples (*lanes 3–5*), where less polar species, designated "mutant OSL," were observed, migrating slightly faster than PP1. Other components were identified as GPI/OSL intermediates and PP1 and P3, the well characterized GPI anchor precursors of procyclic trypanosomes (40, 41). Mutant OSL components were sensitive to mild acid hydrolysis, and their synthesis was inhibited by tunicamycin (data not shown), consistent with their proposed designation as *N*-glycan precursors. Based on this experiment, we concluded that the *mariner* and *ALG12*$^{-/-}$ mutants make a less polar oligosaccharide-PP-dolichol, whose glycan structure must be shorter than that from wild-type cells.

To prove that mutant OSL has a smaller glycan, total [^{3}H]Man-labeled glycolipids from *ALG12*$^{-/-}$ and from *ALG12*$^{+/-}$, which produces large amounts of native OSL (Fig. 8), were subjected to mild acid hydrolysis under conditions that do not affect GPIs. The released glycans were recovered from the aqueous phase after butanol-water partitioning, and the products were analyzed by TLC. After mild acid hydrolysis, a ladder of glycans ranging from $Man_3GlcNAc_2$ up to $Man_9GlcNAc_2$ were released from *ALG12*$^{+/-}$ OSLs (Fig. 8*B*, *left lane*). The same ladder of [^{3}H]Man-labeled glycans was observed from *ALG12*$^{-/-}$ OSLs, except that the most hydrophilic component was smaller and co-migrated with the $Man_7GlcNAc_2$ standard. Thus, as predicted, disruption of *ALG12* in procyclic *T. brucei* produces a mature OSL that has a shorter glycan, containing a $Man_7GlcNAc_2$ instead of $Man_9GlcNAc_2$, as in wild-type cells (39).

DISCUSSION

Because of their ancient divergence from the lines of evolution that have provided most of the model organisms used for the study of basic biological processes, trypanosomes differ from other eukaryotes in a wide range of pathways: from gene transcription to glycolysis. Trypanosomes are also important pathogens that exert their virulence through novel mechanisms such as antigenic variation, which gives us two important motives to study them. Although trypanosomes represent the most intensively studied group of differently evolved eukaryotes, their novelty results in a greatly reduced ability to identify the functions of important genes by bioinformatics methods. To facilitate the elucidation of important processes in trypanosomes, it is vital to develop forward genetics tools to supplement the available reverse genetics approaches.

Experiments in *Leishmania major* provided the first example of trans-kingdom transposition of the *Drosophila mariner* element (21) and prompted us to evaluate its potential as a genetic tool for *T. brucei*. Our first attempts to establish the *mariner* system in *T. brucei* were based on the construction of a cell line in which we could regulate expression of the *mariner* transposase, using the tet repressor system (33, 42), and a separately integrated copy of a donor transposon that allowed for the selection of transposition events. This approach did not work, even when the donor was provided on a transiently transfected plasmid. Fortunately, the constitutive expression of transposase that we subsequently adopted did not lead to unstable insertions that we could detect within the time frame of our experiments. When hygromycin-resistant cell lines were cultivated for more than 2 months, in the absence of selection, the Southern blot pattern of the *HYG* gene was unchanged

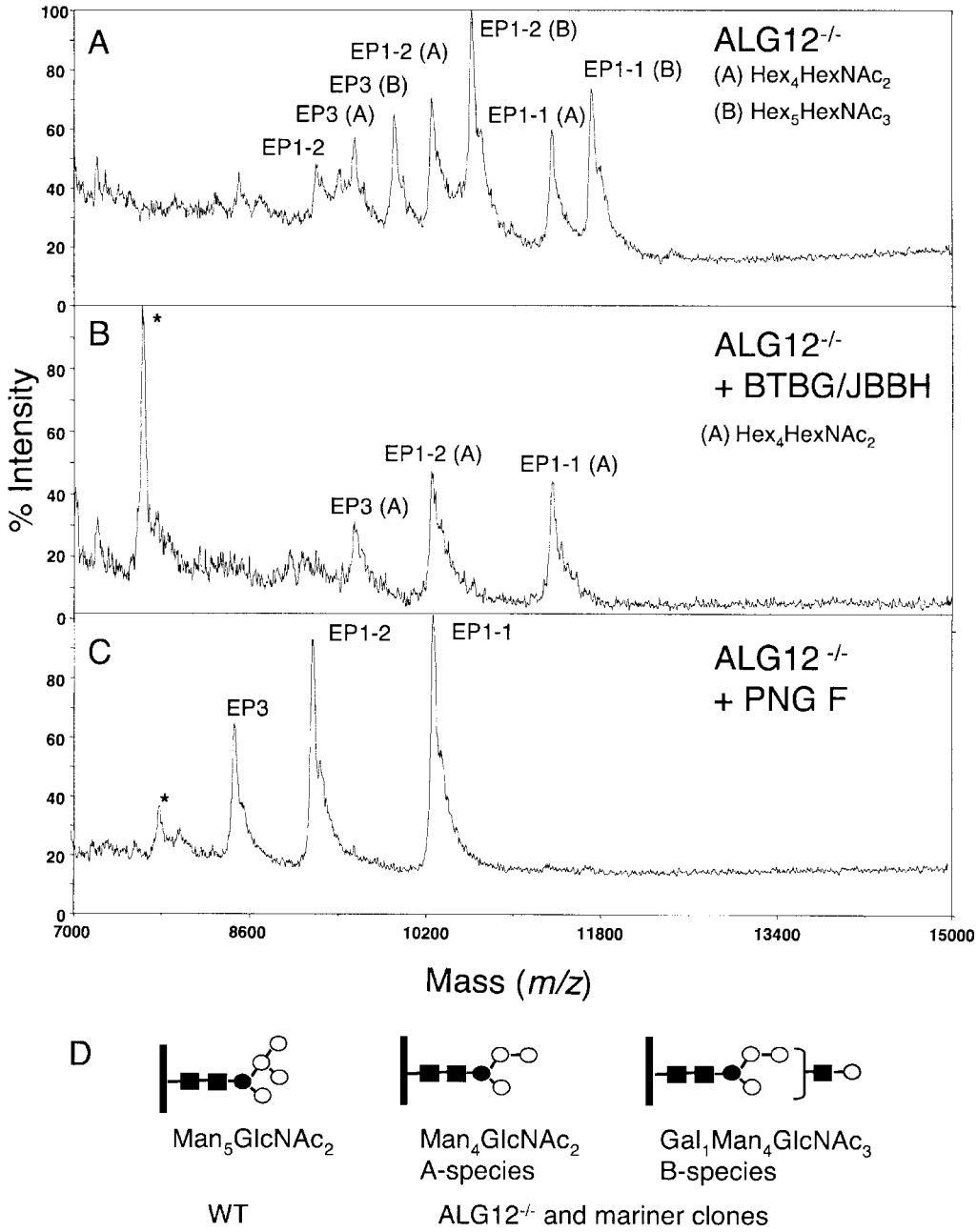


FIG. 7. **Negative ion MALDI-TOF mass spectra of *ALG12*$^{-/-}$ procyclins.** *A*, analysis of procyclin polypeptides after removal of GPI anchors by aqueous HF. See Table I for assignment of ions. *B*, aqueous HF-treated procyclin after incubation with a mixture of bovine testis β-galactosidase and jack bean β-hexosaminidase to remove the terminal *N*-acetyllactosamine unit. *C*, aqueous HF-treated procyclin after deglycosylation with PNGase F. The *asterisks* in *B* and *C* indicate contaminant peptides after digestion. *D*, proposed structures of the *N*-glycans linked to procyclins from WT and *ALG12*$^{-/-}$ and *mariner* ConA^{R} clones. *Open circles*, αMan; *filled circles*, βMan; *filled squares*, βGlcNAc; *dotted circle*, βGal.

(data not shown). In *D. melanogaster*, MosI-dependent mobilization of mariner transposons containing various inserts, including green fluorescent protein at the SalI site, is very inefficient: no mobilization was detected in 831 samples (43).

We based our decision to use ConA resistance as a real life test of *mariner* mutagenesis on the foreknowledge that ConA-resistant mutants could be generated by chemical mutagenesis and on the preliminary characterization of one such mutant (ConA 1-1).[2] The initial biochemically identified defect in ConA 1-1 was a reduction in the conversion of polyprenol to dolichol that was tentatively ascribed to mutation of one allele of an unidentified polyprenol reductase gene. Thus, a *mariner* hit in one allele of the same unidentified gene was expected to confer resistance to ConA. Subsequent work, however, showed that the dolichol-linked glycan precursor in ConA 1-1 also contained a modified glycan that would be consistent with a concomitant mutation in *ALG12*, although this has not been confirmed. The possibility remains that the ConA 1-1 phenotype is due to disruption of one polyprenol reductase allele and that the consequent dolichol deficiency causes an alteration in the structure of the OSL (39).

The *mariner*-targeted gene in the two clones that we characterized in detail is *ALG12*, based initially on sequence homology and confirmed by structural analysis of the mutant glycans. The *T. brucei* ALG12 protein is considerably larger than it is in other organisms. It contains several insertions

[2] P. T. Englund, personal communication.

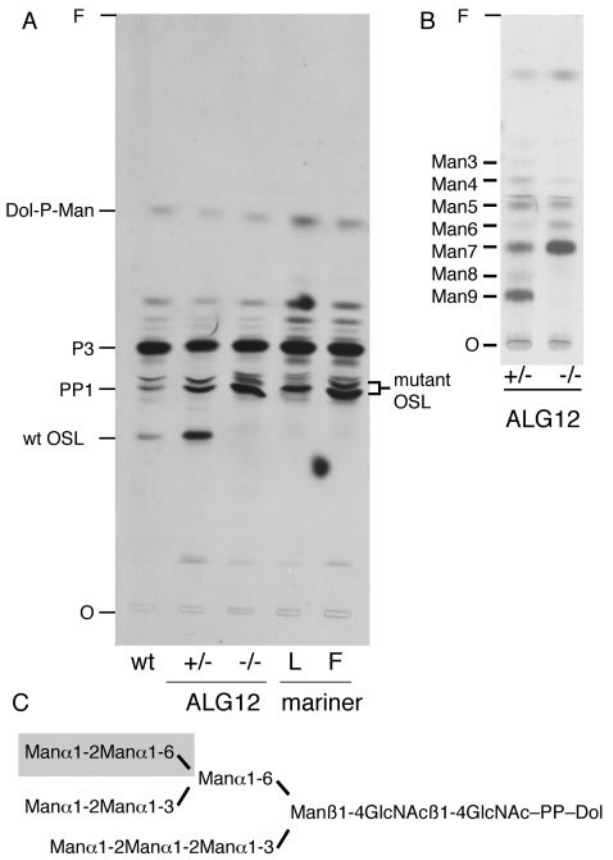


FIG. 8. *A*, cell-free synthesis of lipid-linked oligosaccharides. Washed trypanosome membranes were incubated with GDP-[^{3}H]Man and UDP-GlcNAc and chased with nonradioactive GDP-Man. The glycolipids were extracted, fractionated by TLC, and detected by autoradiography. *Dol-P-Man*, dolichol phosphoryl Man; *PP1* and *P3*, GPI precursors; *O*, origin; *wt*, wild type. *B*, characterization of released glycans. [^{3}H]Man-labeled glycans, released by mild acid hydrolysis, were analyzed by TLC using *n*-butanol/acetone/water (6:5:4, v/v/v) as solvent system and detected by autoradiography. The positions of glycan standards (3 μg Dextran), visualized by orcinol-H_2SO_4 staining, are indicated on the *left*. *C*, proposed structure of the *N*-glycan precursor in wild-type cells. The residues in the *shaded box* are those predicted to be absent from both *ALG12*$^{-/-}$ and *mariner* ConA^R mutants.

within the region aligned in Fig. 3 and a C-terminal extension. There is no structural information that can be used to interpret these differences at present.

Our structural data on the procyclin *N*-glycans from the ALG12$^{-/-}$ and *mariner* ConA^R clones are consistent with the lack of transfer of the α1–6-linked Man residue (the enzymatic product of ALG12) to the Manα1–6βManβ1 arm of the trimannosyl core (Fig. 8*C*). This is also consistent with the formation of an OSL carrying a smaller glycan ($Man_7GlcNAc_2$) by these mutant cells (Fig. 8, *A* and *B*). The implications of such a defect, with regard to the structure of mature glycans linked to surface glycoproteins, have been discussed extensively (29, 36, 38, 39). One issue that has been clarified, from our study of ALG12$^{-/-}$ cells, is that upon transfer of the truncated ($Man_7GlcNAc_2$) OSL onto nascent proteins, the $Man_4GlcNAc_2$ glycan that results from the trimming of all α1–2Man residues can also be modified by the addition of a terminal *N*-acetyllactosamine repeat. The hybrid-type glycans are responsible for ConA resistance, because they are poorly bound by ConA. It also suggests that the trypanosome UDP-GlcNAc:glycoprotein GlcNAc transferase type I, GnTI, prefers $Man_4GlcNAc_2$ instead of $Man_5GlcNAc_2$ (as in other organisms) as a substrate. It also may explain why procyclic trypanosomes, which are known for making exclusively high Man oligosaccharides, never modify the $Man_5GlcNAc_2$ glycan linked to procyclin molecules. Further research on the expression and substrate specificity of the trypanosome GnTI is necessary to fully clarify this issue.

RNA interference (RNAi) is another potentially powerful approach that has recently been used for large scale genetic screening in *T. brucei*. Procyclic *T. brucei* were stably transfected with an inducible RNAi-generating vector containing a library of random genomic sequences. The selection of ConA-resistant mutants was also used as one test of this approach but did not result in the identification of glycosylation mutants. Instead, ConA selection led to the isolation of cells that had switched to the expression of one of the nonglycosylated procyclin variants. The change was shown to be due to the RNAi-induced silencing of the hexokinase gene, a totally unanticipated result (45). A screen for tubercidin resistance also produced an unexpected result that led directly to the demonstration of phosphoglycerate kinase as a target for tubercidin in *T. brucei* (46). Both of these reports emphasize the importance of applying forward genetics to *T. brucei*. The fact that RNAi and *mariner* mutagenesis identified different genes that mediated ConA resistance demonstrates the value of using alternative approaches.

Transposon mutagenesis and RNAi libraries could provide parallel and complementary approaches to forward genetics in *T. brucei*. RNAi is not limited by the ploidy of the organism, but it has limitations that do not apply to the *mariner* system. Most importantly, it is limited by transfection and chromosome integration efficiency, which precludes its use in the bloodstream forms, and it is limited by the leakiness of the currently available vector systems, which prevent its use for genes that are very sensitive targets for RNAi. Insertional mutagenesis by electroporation of preformed transposon complexes into *T. brucei* procyclic forms has also been reported (47), but the efficiency of this approach precludes its general application, especially to the pathogenic stage. Transposon mutagenesis in general, however, was expected to be limited to identifying mutations where disruption of only one allele would cause a discernable phenotype.

Unexpectedly, we found that ConA selection resulted in the isolation of mutants in which both alleles of *ALG12* had been disrupted, by *mariner* insertion at one allele followed by gene conversion-mediated LOH. That the loss of the second *ALG12* allele in both cases was indeed due to gene conversion and not a second *mariner* hit was inferred from the fact that the insertion was present at the same TA site in both alleles in both mutants (data not shown). In any case, a second *mariner* transposition should not have been possible; the plasmid supplying the selectable transposon is single-copy and rapidly lost after removal of G418. It was possible that LOH was a very low frequency event that we detected only because of the strong selection by ConA over a significant period of time. If the intrinsic LOH frequency were low, it could be difficult, because of the size of the populations that would have to be screened, to use *mariner* for mutant screens when strong selection was not an option. We therefore measured the rate of LOH at the *ALG12* locus in the absence of selection. To do this, we took an *ALG12*$^{+/-}$ heterozygote clone, generated by targeted gene disruption, and analyzed the proportion of cells that spontaneously lost their ability to bind fluorescein-conjugated ConA. These negative cells were sorted and immediately cloned, and PCR was used to verify that, in most of the ConA-negative clones, the second *ALG12* allele had been lost. No ConA-negative clones were obtained from populations of wild-type cells. The proportion of *ALG12*$^{-/-}$ cells arising from spontaneous LOH was 0.24 and 0.25% in two independent experiments, which was far higher than anticipated. These experiments will

be reported in detail elsewhere, after they have been extended to additional loci. LOH has also been measured in *L. major* (21, 48), where the rate ranged from 10^{-4} to 10^{-6} at the *DHFR-TS* locus. The authors were unable to account for the large variation in frequency between experiments but were able to demonstrate that the LOH frequency could be increased by γ-irradiation. As previously noted (48), an LOH rate at the upper end of this range could have important implications for genetic variability within this group of organisms.

Our experiments provide the first example of the use of transposon insertion to isolate specific mutations in trypanosomatids and provide an approach that can be easily scaled up to select or screen for mutations in other pathways and processes of interest. *Mariner* mutagenesis will be most useful if it can identify genes involved in trypanosome infectivity and virulence. We are currently extending the system to the pathogenic bloodstream stage of *T. brucei.* The transposon donor cassette has been cloned into pT11-bs, a version of the pT13-11 plasmid that can be propagated in the bloodstream stage, to create pSgl35. Although the original description of these plasmids noted that pT13-11 propagated poorly in bloodstream *T. brucei*, in contrast to pT13-11 in procyclic forms (26), other investigators did not find this to be a problem (11), although an initial lag in growth of G418-resistant plasmid-containing cells has been observed by us and by others.

With a detectable transposition efficiency in the range of 5% (another 5% of insertions will be in the wrong orientation, with respect to the direction of transcription, and perhaps 5% will be in transcriptionally silent loci, indicating an overall transposition rate closer to 15%, comparable with the value of 23% calculated for *L. major* (21)), we have an efficient new tool to manipulate the *T. brucei* genome. We estimate the chance of *mariner* disrupting a single allele to be $>10^{-5}$/cell division, based on the assumption that *T. brucei* contains about 5,000 genes and knowing that genes are closely packed on the chromosomes. This rate would be at least 10-fold higher than the rate of spontaneous mutation calculated for the Herpes thymidine kinase gene inserted into a variant surface glycoprotein expression site (44) and slightly higher than the measured frequency of *mariner* insertion into the *DHFR-TS* locus in *L. major* (21), where the spontaneous mutation frequency was in the range of 10^{-6} to 10^{-7}. With the end of the *T. brucei* genome project in sight, the challenge now lies in devising good screening techniques to help unravel the many complexities of this parasite.

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