DNA-mediated transformation of bloodstream-form *Trypanosoma brucei*

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DNA-mediated transformation of procyclic form (insect midgut stage) *Trypanosoma brucei* occurs almost exclusively by homologous recombination (1, 2, 3). Until now, DNA-mediated transformation of bloodstream-form *T. brucei* had not been accomplished because cultivation, which is necessary for selection of transformants, was generally regarded as difficult and unreproducible. Recently, we utilized a published method (4) to show that bloodstream-form *T. brucei* not only grow well in liquid medium but will also grow with high plating-efficiency as colonies on agarose plates (5). These advances in culture techniques permitted us to select transformed bloodstream-form clones soon after electroporation and measure transformation efficiency. We used a construct, polINeo (Figure 1), which can be linearized at unique sites within ribosomal RNA (rRNA) gene promoter (*Ava* I) or $\alpha\beta$ -tubulin gene intergenic region (*Mlu* I) for targeting into the rRNA or tubulin gene clusters, respectively.

polINeo, purified by cesium chloride density gradient centrifugation, was linearized by overnight digestion with a five-fold excess of either *Ava* I or *Mlu* I and the DNA was ethanol precipitated and washed twice with 70% ethanol. Residual ethanol was evaporated from the tubes in a laminar flow hood. DNA was redissolved in sterile H₂O to a concentration of 1 μ g/ μ l and aliquots of 5 and 30 μ l were distributed to sterile 1.5 ml microfuge tubes. A small amount of each DNA sample was retained for analysis by agarose gel electrophoresis to confirm complete digestion. Trypanosomes (strain 427, MiTat 1.2, clone 221) were grown in Sprague–Dawley rats to subsaturation density ($1-5 \times 10^8$ /ml blood). Blood was aseptically collected by cardiac puncture, mixed with 0.3 vol. citrate/glucose anticoagulant (0.1 M sodium citrate, 0.04 M glucose, pH 7.7), and centrifuged (1000 g, 10 min., room temperature, for this and all subsequent centrifugation steps). Buffy-coat trypanosomes were transferred to a 50 ml centrifuge tube containing 40 ml Zimmerman post-fusion medium + glucose (ZPFMG; 132 mM NaCl, 8 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 0.5 mM magnesium acetate, 0.09 mM calcium acetate, 55 mM glucose, pH adjusted to 7.0 with acetic acid). After centrifugation, cells were resuspended in 10 ml ZPFMG, counted and adjusted to a concentration of 6×10^7 /ml. 0.4 ml of the suspension was mixed with the DNA, transferred to an electroporation cuvette

(2 mm gap width; BTX Inc., San Diego, CA) and subjected to a single pulse of 1.5 kV at 24 Ohms resistance timing in a BTX electroporator. Actual voltages delivered were 0.85–0.91 kV and time constants were 0.280–0.310 msec. Immediately after electroporation, cells were transferred to 10 ml HMI-9 medium (4) in T-25 flasks. Cuvettes were rinsed once with ~0.5 ml medium from the corresponding flask. After the cells were counted they were allowed to recover for 18 hr in a humidified 37°C CO₂ (5%) incubator in the absence of drug. Cells were again counted then pelleted, resuspended in 100 μ l residual supernatant and spread on HMI-9/0.65% agarose plates (5) containing 2.5 μ g/ml geneticin (G418; Gibco BRL, Grand Island, NY). Colonies were visible after 6–8 days.

Using transient transfections with a luciferase expression construct (pHDI6, kindly provided by Christine Clayton, University of Heidelberg), a variety of conditions and parameters were tested for their ability to improve results. First, it was determined that trypanosomes grown *in vivo* transfected 3–8 fold more efficiently than cells from culture in HMI-9. The reason for the discrepancy is not clear. Chilling the cells at any point during the procedure reduced the transfection efficiency, therefore, all steps were invariably performed at room temperature. Blood cells contaminating the buffy-coat-derived trypanosomes had no effect. Sheared salmon sperm DNA was tested as a non-specific carrier (6) but reduced the transfection

Figure 1. Scheme of the polINeo vector. The neomycin phosphotransferase gene (Neo, from Tn5) is flanked at its 5' side by a procyclic acidic repetitive protein- α splice acceptor site (PARP- α SAS) and at its 3' site by the intergenic region between the *T. brucei* α - and β -tubulin genes. The Neo gene is transcribed from a *T. brucei* rRNA gene promoter (flag). These elements were inserted in the multiple cloning region of the bacterial plasmid pUC18 (9).

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Table 1. Efficiency of bloodstream-form *T.brucei* transformation with polINeo

Linearization Site	Target Locus	Amount of DNA	Experiment	Number of Colonies ¹	Transformation Efficiency ²
Ava I	rRNA	5 µg	A	10	1.3×10^{-7}
			B	14	1.4×10^{-7}
Ava I	rRNA	30 µg	A	8	1×10^{-7}
			B	16	1.8×10^{-7}
Mlu I	$\alpha\beta$ -tubulin	5 µg	A	0	—
			B	0	—
Mlu I	$\alpha\beta$ -tubulin	30 µg	A	3	3×10^{-8}
			B	0	—

¹Average of duplicate plates.²Calculated as [average number of colonies divided by ($2^n \times$ plating efficiency)] divided by number cells/transfection; where n=number of doublings during recovery period (2.8–3.0). Plating efficiencies were 0.40 and 0.45 for experiments A and B, respectively, and were determined by mixing 10^2 Neo^R trypanosomes (from a previous transfection with polINeo) with 10^6 Neo^S trypanosomes and spreading the cells on two G418-containing agarose plates.

efficiency. Finally, when HEPES buffered saline solution (7; 20 mM HEPES pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 6 mM dextrose), culture medium (HMI-9) and ZPFMG were tested with a range of electroporator settings (1.3–1.9 kV and 24–72 Ohms), ZPFMG gave the best results, specifically at 1.5 kV and 24 Ohms (data not shown).

Results from two experiments using the polINeo vector to transform bloodstream-form trypanosomes are shown in Table 1. Targeting of the vector to the rRNA genes was approximately ten-fold more efficient than for targeting to the $\alpha\beta$ -tubulin clusters, even though the homologous regions for each targeting event are approximately of equal length. This discrepancy could be due to a difference in target number if each cell contains more rRNA genes than its 26–34 $\alpha\beta$ -tubulin genes (8). Transformation efficiency did not depend on the amount of DNA used, but a greater range of DNA quantities will have to be tested in order to determine whether this is a general feature of DNA-mediated transformation of bloodstream-form trypanosomes. The observed transformation efficiency (10^{-8} to 10^{-7}) for bloodstream-form trypanosomes is significantly lower than that of procyclic-form trypanosomes, which is 10^{-6} to 10^{-5} (2; V.B.C, unpublished observations). At least part of this may be due to an observed difference in survival rates; 2–5% of the bloodstream-form trypanosomes survive electroporation compared to 30–40% of the procyclic-form trypanosomes. On the other hand, bloodstream-form transformants grow approximately twice as fast as procyclic-form transformants, thus selection and expansion for analysis can be accomplished in 8–12 days versus 17–23 days. Southern blot analysis of several transformants showed that, in the majority of cases, the polINeo vector integrated at the correct target locus (data not shown).

We anticipate that recent developments in bloodstream-form culture (4, 5) combined with our demonstration of bloodstream-form transformation will stimulate further experimentation to address some long-standing questions concerning African trypanosomes.

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